

Supporting Information

Pozniak et al. 10.1073/pnas.1016485107

SI Methods

Retroviral Cloning and Production. The retroviral transfer vector pLNCX2 (Clontech) was modified in the following manner: A new multiple cloning site (5'-BglII-PacI-XhoI-HindIII-AvrII-HpaI-NotI-ClaI-3') was introduced downstream of the immediate early CMV promoter, and the neomycin resistance gene was replaced with histone H2B fused to either enhanced green fluorescent protein (pLH2BeGFP) or monomeric red fluorescent protein (pLH2BmRFP), so that transduced cells are identifiable by a fluorescent nucleus. Mouse Sox8, Sox9, Sox10, Olig1, and Olig2 cDNAs were obtained from the Mammalian Gene Collection and, with the exception of Olig2, were PCR-amplified to flank the ORFs with a 5' XhoI and a 3' ClaI site to facilitate their subcloning into the above described retroviral vectors. The Olig2 ORF was excised as a TaqI-AvrII fragment that was inserted into the XhoI and AvrII sites of pLH2BmRFP. The N-terminal HA epitope-tagged mouse Sufu ORF was excised as a HindIII-NotI fragment from pCMV5-HASufu (a kind gift from Pao-Tien Chuang, University of California, San Francisco) that was inserted into the HindIII and NotI sites of pLH2BeGFP. Sox10-3×HA was created by PCR amplification of Sox10 without stop codon to flank the ORF with a 5' XhoI and a 3' NotI site that were used for subcloning into pLH2BmRFP. Subsequently, a 111-bp NotI fragment containing three copies of the HA epitope, a stop codon and a BglII site was inserted. pSIREN-HygEGFP was generated in the following manner: The EcoRI site in the dual-function marker of pHygEGFP (Clontech) was mutated, and the CMV_{ie}-HygEGFP cassette from this plasmid was used to replace the PGK-Puro cassette in pSIREN-RetroQ (Clontech). Subsequently, a luciferase control siRNA (Clontech) and two previously described Sufu siRNAs [ref. 1; first nucleotides of the 21-bp target sequences correspond to position 740 (siRNA1) and position 583 (siRNA2) of Sufu mRNA with GenBank accession nos. AF134893] were inserted into the BamHI and EcoRI sites of this self-inactivating retroviral siRNA expression vector. All constructs were sequenced to confirm the absence of undesirable mutations. Cloning details, vector maps, and sequence files are available upon request.

For virus production, GP2-293 cells (Clontech) were plated on 10-cm dishes at $5\text{--}6 \times 10^6$ cells per plate in DME/H-21 with 10% FBS, 1% penicillin-streptomycin, and 1% glutamax 24 h before transfection. The culture medium was changed 6 h before transfection, and a total of 24 μg of plasmid DNA was used for calcium phosphate-mediated transfection (www.flemingtonlab.com) of each 10-cm dish. Retroviral supernatants were prepared by cotransfection of GP2-293 cells, using 12 μg of transfer vector, 4 μg of envelope vector (pVSV-G; Clontech), 2 μg of packaging vector (pVpack-G+P; Stratagene) and 6 μg of carrier DNA (pBKSII⁺; Stratagene). The culture medium was replaced 12 h after transfection, and viral supernatant was collected 24 and 48 h later. The viral supernatants were cleared by low-speed centrifugation, filtered through a 0.45- μm PES syringe filter (Nalgene), and stored at 4 °C until further processing. Viral supernatants were concentrated 185-fold by ultracentrifugation at $100,000 \times g$ (Beckman) for 1 h 45 min. Viral pellets were resuspended in sterile PBS and stored at -80 °C.

Infection of Neurospheres. Low-passage neurospheres (passage 1–2) were dissociated with a 0.05% trypsin-EDTA solution for 8 min at 37 °C and then gently triturated with a P1000 pipetman 25 times to generate a single-cell suspension. The cells were washed with sterile PBS, pelleted for 2 min at $170 \times g$ and re-

suspended in 500 μL of complete media containing EGF, FGF, B27, and 5 $\mu\text{g}/\text{mL}$ polybrene (Sigma). Concentrated virus (50–100 μL ; approx 1×10^5 transducing units) was added to $\approx 300,000$ cells, which were spin infected for 90–120 min at $170 \times g$. The viral supernatant was removed after infection, and the cells were plated in fresh complete media containing growth factors in a 25-cm² flask for 24 h to allow integration and expression. Cells were collected the next day, resuspended in the appropriate media, and plated on laminin-coated (1 mg/mL; Invitrogen) eight-well chamber slides (Nunc) at a density of $\approx 37,500$ cells per well. Differentiation media contained complete media with 2% heat-inactivated FBS or 3, 3', 5-triiodo-L-thyronine sodium salt (T3) and thyroxine (T4) (Sigma; 30 ng/mL and 40 ng/mL, respectively).

Data Analysis. The microarray analysis performed by Nimblegen used a 60-mer catalog array of mouse build 5 from the University of California, Santa Cruz (mm5, May 2004 genome assembly) of 34,062 genes at a minimum of 11 probes per gene and also included 5,000 random probes. The probe intensities were quantile-normalized, and 11+ probes per gene were summarized by using the robust multiarray average method (RMA), developed originally for Affymetrix arrays. To normalize the data for each array, the raw intensities were extracted from NimbleGen PAIR files to calculate M, the log₂ ratios of IP-enriched DNA (Cy5) and input total DNA (Cy3), for each probe. M was then loess-normalized to remove the strong signal-dependent dye imbalance. Qualitative diagnostic plots and several measures of signal to noise strength were used collectively for quality check. The normalized M (referred as M hereafter) was subject to a two-step sliding window approach to identify sites with significant IP enrichment: (i) Sliding window statistics. Calculate statistics over probes within a sliding window and apply cutoffs to call significant windows (see details below); (ii) Site formation. The significant windows were then merged to form nonredundant sites if they overlap for at least one probe. For each probe location, the following three statistics were calculated over probes that are located within the [–500 bp, +500 bp] window of that probe: (i) medM: median M of probes in window, (ii) pM: *P* value of *t* test that $M = 0$ vs. $M \neq 0$, (iii) pA: *P* value of one-sided *t* test that the average Cy5 and Cy3 signals of the probe in window =, vs. >, random probe signals. A window is called significant by using the following criteria: medM > log₂(1.5) and pM < 10^{-4} and pA < 10^{-2} . A significant site should have good signal (small pA), and good enrichment (large medM, small pM). Note that medM > 0.58 corresponds to a 1.5 \times enrichment in IP-enriched DNA relative to input DNA. The window size (1 kb) and cutoffs are somewhat ad hoc but arguably reasonable and fairly typical in the ChIP-chip literature. The DNA fragments used in the protocol are 1,000 bp, and the resolution of these arrays yield ≈ 10 probes per 1,000-bp window for statistical inference. We do not attempt to adjust the *P* values for multiple testing because of the sophisticated dependency between overlapping sliding windows, but instead chose a more stringent *P* value cutoff.

Western Blotting. Sox10 or Empty retrovirus-infected neurospheres plated on laminin for 24 or 48 h in EGF + FGF were collected in 1 mL of PBS and spun down for 15 s at $16,100 \times g$. Brain tissue was removed and immediately snap frozen on dry ice and stored at -80 °C until further analysis. Tissue was homogenized and cells resuspended in RIPA lysis buffer (Invitrogen) containing protease inhibitors (Roche), rocked for 30 min at 4 °C, and centrifuged for 30 min at $16,100 \times g$ at 4 °C. The supernatants were collected, and a BCA protein assay (Pierce) was performed to

determine sample concentration. Samples were separated on a 10% Tris-SDS polyacrylamide gel (Bio-Rad) and transferred to a nitrocellulose membrane, which was blocked for 1 h in Western blocking reagent (Roche) and subsequently probed with anti-Sufu antibody (rabbit 1:200; Santa Cruz) in blocking solution for 1 h at 25 °C or overnight at 4 °C. The membrane was washed three times in Tris-Buffered Saline with 0.3% Tween-20 (TBST) before a 1-h incubation with a secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (1:5,000, Amersham or Jackson Immuno Research) at 25 °C. The immunoreactive proteins were visualized by using an ECL detection reagent (Amersham) after three TBST washes. To ensure equal loading of proteins the membrane was stripped (Tris at pH 6.8, 20% SDS, β -mercaptoethanol), washed extensively in TBST, reblocked, incubated with an anti- β -actin antibody (mouse, Sigma; 1:5,000) or anti-tubulin antibody (rabbit, Covance; 1:1,000) for 1 h at 25 °C or overnight at 4 °C, and detected with a secondary goat anti-mouse IgG HRP antibody (Amersham; 1:10,000) or goat anti-rabbit IgG (Jackson ImmunoResearch; 1:5,000) as described above.

Immunohistochemistry. For histological analysis brains from embryos (E15.5) or postnatal day 2 (P2) pups were rinsed briefly in ice-cold 1 \times PBS and drop fixed in 4% paraformaldehyde overnight (E15.5) or perfused with 4% paraformaldehyde (P2). During the next day, brains were cryoprotected overnight in 10% sucrose in PBS followed by 30% sucrose in PBS and then serially cryosectioned at 12- μ m thickness onto precoated glass slides (Fisher Scientific). Sections were blocked and permeabilized for 30 min with PBS containing in 0.3% Triton X-100 and 10% lamb serum before incubation with primary antibodies at 4 °C overnight. The next day, cells or tissue were washed three times with PBS and incubated with fluorescent secondary antibodies (1:200; Invitrogen) and DAPI (1:10,000, Sigma) for 2 h at 25 °C. Three to four matched sections from three to four brains were analyzed from wild-type and Sufu heterozygous mice. Total numbers of PDGFR α -positive cells were quantified for each section and averaged to generate a mean number of PDGFR α cells per brain.

1. Varjosalo M, Li SP, Taipale J (2006) Divergence of hedgehog signal transduction mechanism between *Drosophila* and mammals. *Dev Cell* 10:177–186.

Other Supporting Information Files

[Table S1 \(DOC\)](#)