Supporting Information

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SI Materials and Methods

ChIP and Luciferase Assays. For ChIP experiments, the primers designed for qPCR amplification of the samples were as follows: for the −43 kb site, forward, CTTTCCAGTTCTTTCCCTTCA-GTC; reverse, ACAATATCAGACATTCCCTCTTCC; and for the −49 kb site, forward, ATCTCGACATCACTAAATGACT-GG; reverse, TGCTGGGGAAATATCCAAATACT, targeting an approximately 150 bp region around the TCF/LEF sites in the murine Prox1 enhancer region. For luciferase assay, the primers used to clone the respective sites were as follows: for the −49 kb site, forward, GAGCTCTTACTGCCACAGCAACTGTATCTT; reverse, CTCGAGCTGCCTCTCA-GGGTCTTTCAAA; and for the −43 kb site, forward, GAGCTCCTTTGAAACGGGAGAG-AAGAATCT; and reverse, CTCGAGATACGTGTCTGTCC-TCTCTTTAAAC 3′. To introduce mutations into the TCF/LEF binding sites, we deleted 5 bp from the core region (TTCAA for −43 kb and TTGAA for −49 kb) of the binding sites using the following sense primers for −49 kb and −43 kb sites, respectively: TCTCTTACAAATGCCTGTCTCCCACC-GCCTAC and CC-AGCAGAGAGCCGTTCAGGGGACTTCT TCAG. All mutations were carried out according to the QuikChange Site-Directed Mutagenesis Kit (Stratagene). As positive control, we also electroporated Super8xTOPFLASH (with WT TCF/LEF binding sites) or Super8xFOPFLASH (mutant TCF/LEF binding sites) together with a β-catenin construct to assess the capability of β-catenin to activate TCF/LEF sites. Assay results were also confirmed by using 293T cells.

Cell Culture. For gain- and loss-of-function experiments of Prox1 in vitro, we used NSCs isolated from the DG of adult rats. Cells were grown and maintained as described previously (1). Cells were transduced with the respective viruses and kept for 24 h under FGF-2 (20 ng/mL; PeproTech) conditions. For proliferation experiments, cells were pulsed for 1 h with 10 μM BrdU (Sigma) and then fixed. For differentiation experiments, cells were transferred into medium containing retinoic acid (1 μM; Sigma) and forskolin (5 μM; Sigma). For Wnt3 overexpression, we transduced NSCs isolated from the adult, murine DG with a lentivirus expressing Wnt3 or control virus (2). RNA was collected 3 d after viral transduction. Cells were harvested (for RNA extraction, we used TRIzol; Invitrogen) or immunocytochemically analyzed at the times indicated. To test the efficiency of shRNAs to knockdown Prox1 protein levels, 293T cells were collected in RIPA+ 48 h after transfection with respective Prox1-overexpressing and shRNA constructs using Lipofectamine 2000 (Invitrogen). Knockdown of Prox1 protein was analyzed by Western blots (as detailed in the next section).

Immunostaining, Western Blotting, and in Situ Hybridization. Primary antibodies used were rat α-BrdU (1:500; Abcam), mouse α-BrdU (1:200; BD Sciences), mouse α-MAP2ab (1:250; Sigma), chicken α-GFP (1:500; Aves Lab), rabbit α-Prox-1 (1:2,000; Chemicon), mouse α-Calbindin (1:250; Swant), mouse α-NeuN (1:100; Millipore), goat α-SOX2 (1:500; Santa Cruz), mouse α-GFAP (1:500; Sigma), mouse α-PCNA (1:200; Santa Cruz), goat α-NeuroD1 (1:250; Santa Cruz), chicken α-β-Gal (1:500; Prosciences), and goat α-DCX (1:250; Santa Cruz). All secondary antibodies were obtained from Jackson Laboratory.

For in vitro experiments, NSCs transduced by retroviruses were analyzed by using a Zeiss Axiovert Observer-D1 inverted microscope. ImageJ 1.42q was used to quantify colocalization of GFP-labeled cells with MAP2ab for differentiation experiments and with BrdU for proliferation assays in the cells infected by Prox1-overexpressing/shRNA or their respective control viruses. For all images, only general contrast enhancements and color level adjustments were performed. All counting was performed by an investigator blind to the experimental conditions.

For Western blots, proteins were separated by 4% to 12% polyacrylamide gels, transferred to nitrocellulose membranes, and probed overnight at 4 °C with rabbit α-Prox1 (Chemicon) and mouse α-GAPDH (HyTest) primary antibodies. Membranes were incubated with 1:5,000 dilution α-rabbit HRP–conjugated (Promega) and 1:10,000 dilution α-mouse alkaline phosphatase–conjugated secondary antibodies (Promega) for 2 h at room temperature. Bands were detected by using enhanced chemiluminescence and quantified by using ImageJ 1.42q.

For immunohistochemistry of embryonic day 18 to postnatal day 0 brains, the tissue was embedded in paraffin and cut as described previously (3). Six representative sections throughout the rostrocaudal extent of the brain from Emx1^{IRESCre}/β-catenin^{exo3} mutant mice and their respective controls (n = 3 per genotype) were chosen and stained using Prox1-specific antibodies. Images were acquired using an epifluorescence a Zeiss Axiovert Observer-D1 inverted microscope (10× objective, 0.3 N.A.). Areas in the dorsal cortex were traced for DAPI and Prox1 signal. Prox1-expressing areas were quantified by using ImageJ 1.42q.

For in situ hybridization, brains were cut in 20-μm (adult) thick sections using a cryostat (Microm). A digoxigenin-labeled RNA probe was generated from a pCMV-Sport6 *Prox1* clone (Imagene) using T7 RNA polymerase, followed by alkaline hydrolysis. Sections were fixed in 4% PFA at room temperature for 20 min, washed in PBS solution, and treated with triethanolamine-HCL/acetic anhydride to reduce nonspecific binding of the probe. Sections were subjected to 4 h of prehybridization followed by 12 h of hybridization at 65 °C. After a series of highly stringent wash steps and 1 h of blocking with 20% lamb serum, cryosections were incubated with an anti-DIG antibody conjugated with alkaline phosphatase (1:2,000; Roche) for 2 h at room temperature. Endogenous alkaline phosphatase activity was inhibited by the addition of levamisole (Sigma) to the subsequent washing buffer. Antibody visualization was done by incubation with the chromogenic substrates NBT/BCIP (Sigma) at 37 °C in the dark for several hours.

Quantitative RT-PCR. RNA was extracted using TRIzol (Invitrogen), and genomic DNA contamination was subsequently removed using Turbo DNase treatment (Ambion). cDNA was generated with SuperScript II (Invitrogen) according to manufacturer's recommendations. The PCR reactions were performed by using TaqMan Master Mix and target-specific TaqMan primer/probe assays (Applied Biosystems) against rat *Prox1*. A primer/probe assay against rat GAPDH was used for normalization. Data acquisition was done on an ABI Prism 7000 RT-PCR machine, followed by data visualization/analysis with Sequence Detection System software (Applied Biosystems). The detection thresholds automatically calculated by the Sequence Detection System software were used to determine Ct values. Fold changes of mRNA expression levels were calculated according to the ΔΔCt method, and statistical analysis was done on individual sample ΔCt levels.

1. Jessberger S, Toni N, Clemenson GD, Jr., Ray J, Gage FH (2008) Directed differentiation of hippocampal stem/progenitor cells in the adult brain. *Nat Neurosci* 11:888–893.
2. Lie DC, et al. (2005) Wnt signalling regulates adult hippocampal neurogenesis. *Nature* 437:1370–1375.
3. Miquelajauregui A, et al. (2007) Smad-interacting protein-1 (Zfhx1b) acts upstream of Wnt signaling in the mouse hippocampus and controls its formation. *Proc Natl Acad Sci USA* 104:12919–12924.

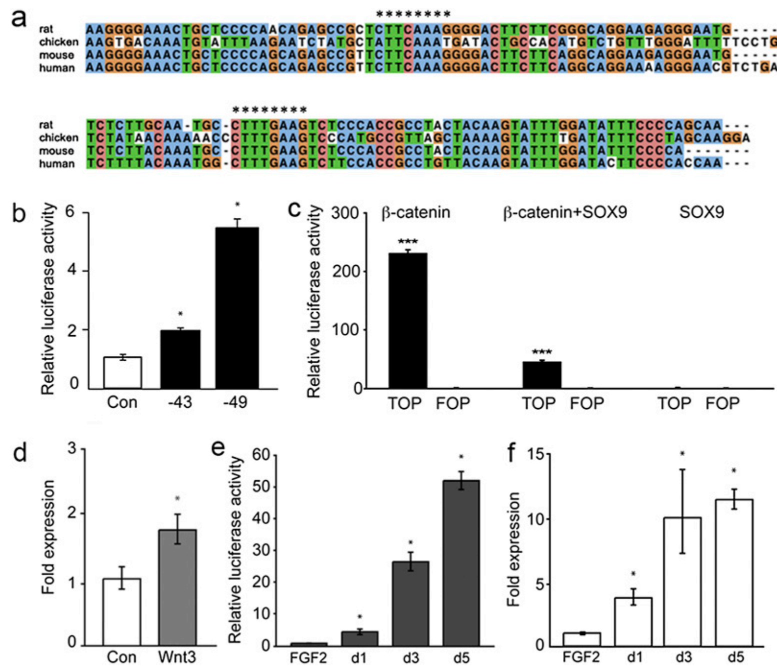

Fig. S1. Regulation of Prox1 with β-catenin and neuronal differentiation. (*A*) The *Prox1* enhancer region contains conserved TCF/LEF sites (stars) at −49 kb (*Upper*) and −43 kb (*Lower*) upstream of the *Prox1* start codon. (*B*) β-Catenin expression enhanced luciferase activity of the two TCF/LEF sites containing regions (−43 kb and −49 kb upstream of the *Prox1* start codon) cloned from the *Prox1* promoter in reporter assays performed in 293T cells. (*C*) β-Catenin expression strongly enhanced luciferase activity of a TOPFLASH reporter construct in rodent NSCs, whereas mutation of TCF/LEF binding sites (FOPFLASH reporter construct) abolished increased luciferase activity. TOPFLASH reporter activity was largely blocked by coexpression of SOX9. (*D*) Overexpression of Wnt3 enhances Prox1 expression in NSCs. Shown is the fold increase of *Prox1* mRNA as measured with qPCR 3 d after transduction of NSCs with a lentivirus expressing Wnt3 compared with control virus. (*E*) Neuronal differentiation of hippocampal NSCs stimulates TOPFLASH reporter activity indicative of β-catenin–TCF/LEF–dependent transcriptional activity. Shown is a time course 1, 3, and 5 d after induction of neuronal differentiation using retinoic acid/forskolin compared with proliferating conditions (FGF-2). (*F*) The increase of TOPFLASH reporter activity during neuronal differentiation of hippocampal NSCs is associated with strong up-regulation of *Prox1* mRNA as measured by qPCR (*$P < 0.05$ and **$P < 0.01$).

Fig. S2. Prox1 expression is highly enriched in the DG and expressed in the course of adult neurogenesis (*A*) qPCR analysis showed high levels of *Prox1* mRNA expression in the DG compared with the second neurogenic region of the adult brain, the SVZ, to area CA3 of the hippocampus and to the cortex, confirming the in situ data. (*B*) Occasionally, we observed Prox1-expressing cells (red) that colabeled with the cell cycle-associated protein proliferating cell nuclear antigen (PCNA, green, arrowheads), suggesting that a fraction of Prox1-expressing cells is proliferating within the dentate neurogenic niche. (*C*) BrdU time course experiments show that Prox1 colabels with BrdU as early as 24 h after a single BrdU injection, and relative numbers of BrdU/Prox1-colabeled cells remain constant at least until 7 wk after BrdU injection, supporting the expression pattern observed with endogenous markers of neurogenesis and granule cells shown in Fig. 3. (*D*) Prox1 protein (green) is abundant in the adult DG (*Upper*) but absent in the SVZ (*Lower*), the second neurogenic area. Note that both areas contain a high number of newborn neurons expressing DCX (red). *Right*: Single channel for Prox1 (gray). Nuclei were counterstained with DAPI (gray, *Left*). GCL, granule cell layer; LV, lateral ventricle; CA3, cornu ammonis area 3; Cx, cortex. (Scale bars: 100 μm.)

Fig. S3. Expression of dnLEF reduces transcriptional activity of the TOPFLASH reporter upon β-catenin stimulation and knockdown of Prox1 protein using shRNA targeting Prox1 mRNA. (*A*) Luciferase assays shows that cotransfection of a dnLEF in 293T cells reduces luciferase activity of the TOPFLASH reporter after β-catenin expression. (*B*) Two of the tested shRNA target sequences resulted in a robust decrease in Prox1 protein as measured with cotransfection experiments in 293T cells. Shown are representative Western blots indicating the decrease of Prox1 protein at the expected size with target sequence 1 (L1) and target sequence 3 (L3), which were used for all subsequent experiments. Note that target sequence 2 (L2) had little effect on Prox1 protein expression and was not used for further experiments. (*C*) Cells transduced with a control lentivirus expressing GFP (green) and nontargeting shRNA showed high levels of Prox1 (red) expression (arrowheads, *Upper*). In striking contrast, lentivirus expressing GFP (green) and shRNA targeting *Prox1* mRNA showed Prox1 expression below the detection level (red, *Lower*, arrows), confirming the in vitro effectiveness of shRNAs used against Prox1. *Left*: Overlays of Prox1 and GFP signals. *Right*: Single GFP (*Upper*) and Prox1 signal (*Lower*). GCL, granule cell layer. $^{*}P < 0.05$ and $^{***}P < 0.001$. (Scale bar: 20 μm.)

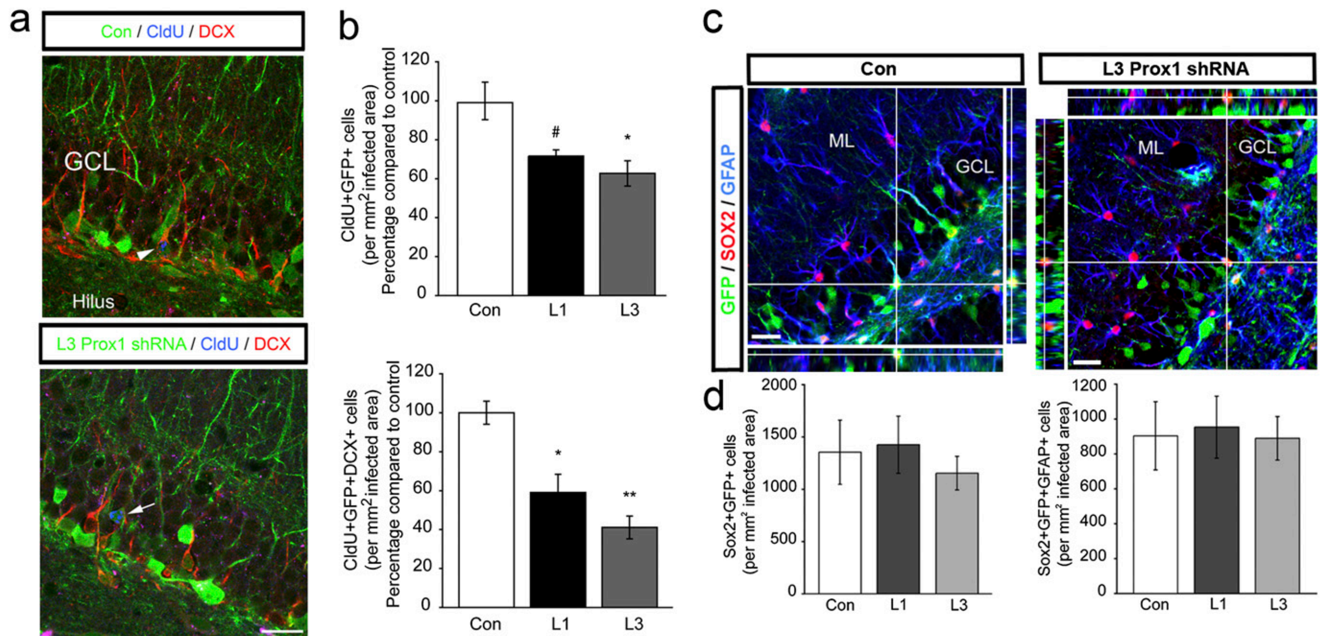

Fig. S4. Reduced survival and differentiation of CldU-labeled newborn cells after Prox1 knockdown but no effect on the number of hippocampal NSCs. (*A*) In contrast to animals injected with a nontargeting shRNA-expressing lentivirus (green, *Upper*) in which we found many GFP/CldU-labeled cells (blue) expressing DCX (red, arrowhead), knockdown of Prox1 using shRNAs directed against *Prox1* mRNA (green, *Lower*) decreased the total number of GFP/CldU-labeled cells (blue, arrow) and GFP/CldU/DCX colabeled cells. (*B*) Two weeks after injection of lentiviruses expressing nontargeting control shRNA (Con) or two shRNAs directed against *Prox1* mRNA (L1 and L3), dividing cells and their progeny were labeled by a single CldU injection. Animals were killed 3 wk later. We found a significant reduction of GFP/CldU-labeled cells using L3 and a trend toward decreased GFP/CldU-labeled cells using the L1 shRNA. Lentivirus-mediated expression of both shRNAs (L1 and L3) targeting *Prox1* mRNA reduced the number of GFP/CldU/DCX-colabeled cells, confirming the results obtained by DCX analyses. (*C*) The number of NSCs expressing SOX2 (red) and GFAP (blue) was not different between areas transduced comparing control lentiviruses (green, *Left*) and viruses expressing shRNAs directed against Prox1 mRNA (green, *Right*). Shown are 3D reconstructions of SOX2/GFP/GFAP colabeled cells. (*D*) Quantification of SOX2/GFP and SOX2/GFAP/GFP colabeled cells. ML, molecular layer; GCL, granule cell layer. $^{\#}P = 0.06$, $^{*}P < 0.05$, and $^{**}P < 0.01$. (Scale bars: *A*, 30 μm; *C*, 50 μm.)

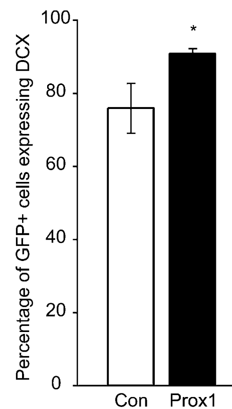

Fig. S5. Enhanced neuronal differentiation after retrovirus-mediated Prox1 overexpression. Retroviruses expressing GFP-only (Con) or Prox1-GFP (Prox1) were injected into the hippocampus of adult mice. Seven days after virus injection, animals were killed and the phenotype of cells was analyzed. Prox1 overexpression increased the percentage of newborn cells expressing DCX, suggesting that Prox1 overexpression enhances neuronal differentiation of newborn cells in the adult DG ($^{*}P < 0.05$).

Fig. S6. Prox1 overexpression enhances neuronal differentiation of NSCs in vitro. (*A* and *B*) BrdU pulse-labeling 24 h after viral infection reveals decreased numbers of BrdU-labeled cells (red) in Prox1-expressing, GFP-labeled cells (green, *Left*) compared with cells transduced with a control virus (green, *Right*). Nuclei were counterstained with DAPI (blue). (*C* and *D*) Enhanced neuronal differentiation of Prox1-overexpressing, GFP-labeled cells (green, *Right*, arrows) compared with cells transduced with a control virus (green, *Left*), as indicated by MAP2ab (red) expression. Nuclei were counterstained with DAPI (blue). *$P < 0.05$. (Scale bars: 50 μm.)

Fig. S7. Prox1 knockdown impairs neuronal differentiation of NSCs in vitro. (*A* and *B*). Shown are examples of hippocampal NSCs transduced with viruses expressing nontargeting shRNAs (Con, green, *Left*) or shRNAs targeting *Prox1* (L3, green, *Right*). Prox1 knockdown did not affect the number BrdU-labeled cells (red). (*C* and *D*) Prox1 knockdown using shRNAs targeting *Prox1* (L3, green, *Right*) impairs neuronal differentiation of NSCs into MAP2ab-positive neuronal cells (red, arrowhead) compared with control cells transduced with lentivirus expressing nontargeting shRNA (green, *Left*). Nuclei were counterstained with DAPI (**$P < 0.01$). (Scale bars: 50 μm.)