

Prospero-related homeobox 1 gene (*Prox1*) is regulated by canonical Wnt signaling and has a stage-specific role in adult hippocampal neurogenesis

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Neural stem cells (NSCs) generate new granule cells throughout life in the mammalian hippocampus. Canonical Wnt signaling regulates the differentiation of NSCs towards the neuronal lineage. Here we identified the prospero-related homeodomain transcription factor *Prox1* as a target of β -catenin–TCF/LEF signaling in vitro and in vivo. *Prox1* overexpression enhanced neuronal differentiation whereas shRNA-mediated knockdown of *Prox1* impaired the generation of neurons in vitro and within the hippocampal niche. In contrast, *Prox1* was not required for survival of adult-generated granule cells after they had matured, suggesting a role for *Prox1* in initial granule cell differentiation but not in the maintenance of mature granule cells. The data presented here characterize a molecular pathway from Wnt signaling to a transcriptional target leading to granule cell differentiation within the adult brain and identify a stage-specific function for *Prox1* in the process of adult neurogenesis.

New neurons are born throughout adulthood in two distinct areas of the adult brain, the subventricular zone (SVZ) of the lateral ventricles and the hippocampal dentate gyrus (DG) (1). Adult hippocampal neurogenesis is important for hippocampus-dependent learning and memory and has been implicated in a number of neuropsychiatric diseases such as major depression, epilepsy, and age-related cognitive decline (2, 3). Although neural stem cells (NSCs) that show neurogenic potential in vitro can be isolated from various adult brain regions, only the SVZ and the DG produce substantial numbers of new neurons throughout life (4). Previous reports suggested that the niche provides important cues to exploit the neurogenic potential of adult NSCs (5). Within the hippocampal niche, several signaling pathways have been identified that are critical for stem cell maintenance and neuronal differentiation, such as sonic hedgehog (Shh) and Wnt signaling (6, 7). Wnt3 is produced by local hippocampal astrocytes, regulating the generation of newborn granule cells (7, 8). Upon binding of Wnt to its receptors of the Frizzled and Lrp families, β -catenin becomes stabilized during canonical Wnt signaling, leading to its nuclear accumulation. There, β -catenin acts as a transcriptional coactivator of TCF/LEF transcription factors, resulting in the expression of Wnt target genes. Recently, it has been shown that NeuroD1, a basic helix-loop-helix transcription factor required for granule cell genesis in the embryonic and adult DG, is regulated by β -catenin–dependent signaling (9–12). However, NeuroD1 is not only important for hippocampal neurogenesis but is also involved in the generation of SVZ-derived olfactory neurons (11, 13, 14). Thus, the network of genes regulated by the Wnt pathway to selectively generate DG granule cells in the adult hippocampus remains unknown. Here we show that *Prox1* is regulated with β -catenin–TCF/LEF signaling and controls the generation of newborn granule cells in the adult hippocampus.

Results

Prox1 Promoter Contains Functional TCF/LEF-Binding Sites. To identify potential targets of Wnt signaling within the hippocampal niche, we focused on genes expressed in the adult DG. *Prox1* is a prospero-related homeodomain transcription factor, which is highly expressed in DG granule cells (10, 15, 16). *Prox1* has previously been shown to be important for lens development, lymphangiogenesis, differentiation of certain spinal cord interneurons, and, very recently, in hippocampal granule cell genesis (17–20). We analyzed the murine *Prox1* promoter/enhancer regions for TCF/LEF sites and found two TCF/LEF consensus sites 49 kb and 43 kb upstream of the *Prox1* start codon (Fig. 1A and Fig. S1), in accordance with a previous report showing TCF/LEF sites at conserved positions in the human *Prox1* enhancer sequence that appear to mediate *Prox1* regulation in intestinal cancers (–43 kb, CTTCAAAG; –49 kb, CTTTGAAG) (21). First, we performed chromatin immunoprecipitations (ChIP) to analyze a potential association of β -catenin with the *Prox1* enhancer region in adult NSCs. Chromatin from NSCs was precipitated with antibodies against β -catenin followed by quantitative RT-PCR (qPCR) using site-specific primers directed against the putative TCF/LEF sites within the *Prox1* enhancer region. We found an enrichment of β -catenin on both the –49 kb and –43 kb sites compared with control ChIP using IgG antibody, suggesting that β -catenin is associated with these genomic regions of the *Prox1* enhancer (Fig. 1B). To test the functionality of the two predicted TCF/LEF sites within the *Prox1* enhancer, we cloned the –49 kb and –43 kb sites with approximately 1 kb surrounding genomic DNA into a luciferase reporter and analyzed the effect of β -catenin expression on luciferase activity. β -Catenin enhanced luciferase activity in adult hippocampal NSCs (Fig. 1C), demonstrating a functional relevance of β -catenin–mediated activity on the *Prox1* enhancer in hippocampal NSCs. This finding was confirmed by using a heterologous cell system (293T cells; Fig. S1). The specificity of β -catenin–mediated induction of luciferase activity on *Prox1* enhancer constructs was confirmed using site-directed mutagenesis against the consensus TCF/LEF sites: mutation of the TCF/LEF sites reduced the β -catenin–induced increase of lu-

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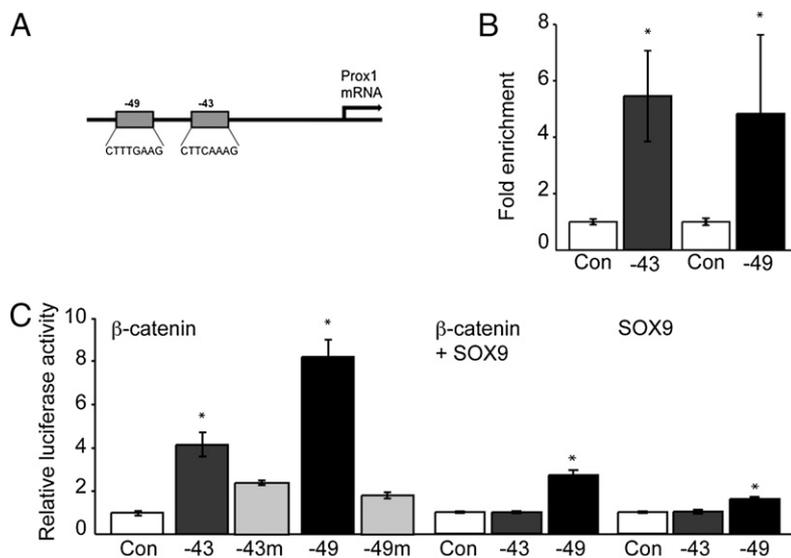


Fig. 1. *Prox1* enhancer contains functional TCF/LEF sites. (A) Two TCF/LEF sites are present in the *Prox1* enhancer region –49 kb and –43 kb upstream of the *Prox1* start codon. (B) ChIP analyses indicate that β -catenin is associated with the two TCF/LEF sites in the *Prox1* enhancer region as shown by qPCR using primers surrounding the TCF/LEF sites, showing an enrichment of these sites with β -catenin precipitation compared with IgG precipitated chromatin. (C) β -Catenin expression enhanced luciferase activity of the two TCF/LEF sites containing regions cloned from the *Prox1* promoter in reporter assays (dark gray bars, Left). This effect of β -catenin was blocked by site-directed mutagenesis deleting base pairs in the respective TCF/LEF sites (light gray bars, Left). SOX9 coexpression represses the effect of β -catenin at the –43 kb and, to a lesser extent, at the –49 kb site (middle bars), whereas SOX9 expression alone had no or only a small effect on luciferase activity (right bars); * $P < 0.05$.

ciferase activity (Fig. 1C). Furthermore, we found that lentiviral expression of Wnt3 led to an increase in *Prox1* mRNA in NSCs (Fig. S1) using qPCR. We next analyzed if neuronal differentiation of adult NSCs upon exposure to retinoic acid/forskolin, which is associated with an increase in TOPFLASH reporter activity (Fig. S1), enhances *Prox1* expression in hippocampal NSCs. Again supporting the association of TCF/LEF signaling and *Prox1*, we found a significant time-dependent increase of *Prox1* mRNA in hippocampal NSCs upon neuronal differentiation (Fig. S1).

Taken together, we identified two functional TCF/LEF sites in the murine *Prox1* enhancer that become activated upon β -catenin expression in hippocampal NSCs. Interestingly, it has been recently shown that TCF/LEF sites and binding sites for the *Sry*-related HMG box (SOX) transcription factors often overlap, forming a SOX/LEF binding site (12), with the –43 kb site representing one of them. Thus, we analyzed if SOX proteins may repress the effects of β -catenin on the *Prox1* enhancer region as it has been shown at comparable sites in the *NeuroD1* promoter (12). We found a strong repression of β -catenin-induced luciferase activity on the –43 kb enhancer region when we coexpressed SOX9 (Fig. 1C), a SOX family member that is highly expressed in adult NSCs and that may function to repress neuronal differentiation (22, 23). Similarly, SOX9 repressed luciferase activity at the –49 kb site, albeit to a lesser extent compared with the –43 kb site (Fig. 1C). Given this finding, it appears that a switch on the *Prox1* enhancer region from a suppressive state mediated by SOX proteins to an activated state mediated by β -catenin may be critical for *Prox1* expression.

Hippocampal *Prox1* Expression Is Associated with TCF/LEF Signaling in Vivo. We next aimed to characterize the relationship between TCF/LEF signaling and *Prox1* expression in vivo. First, we analyzed *Prox1* mRNA and protein expression within the adult brain. By using in situ hybridization, we found that *Prox1* mRNA expression was largely restricted to the DG granule cell layer, a finding confirmed by qPCR, wherein *Prox1* mRNA levels among different brain regions were compared (Fig. 2A and Fig. S2). This is in contrast to earlier stages of neural development during which *Prox1* is also expressed in the SVZ (16). Reflecting the mRNA expression pattern, we found *Prox1* protein expression to be restricted to the DG in the adult brain (Fig. 2B–E), confirming previous reports (16). By using single BrdU injections and previously characterized marker proteins for NSCs and their progeny, we found *Prox1* to be expressed as early as in the nonradial stage of SOX2-expressing cells in the DG (24) (Fig. 2B and Fig. S2). *Prox1* expression was also

high in doublecortin (DCX)- and *NeuroD1*-expressing cells, two markers for newborn neuronal cells in the adult DG (25, 26). In contrast to other transcription factors associated with hippocampal neurogenesis, such as *NeuroD1* (12), *Prox1* remained to be expressed at high levels also in mature, calbindin-expressing hippocampal granule cells (Fig. 2C–E) and was exclusively expressed in the DG but not in the SVZ, the second neurogenic region of the adult brain (Fig. S2).

We then analyzed *Prox1* expression in a transgenic mouse line (BAT-gal) expressing the *lacZ* gene coding for β -gal under the control of multiple TCF/LEF consensus sites (27). We observed a strong overlap of *Prox1* and β -gal activity in the dentate area of BAT-gal mice (Fig. 3A), suggesting an association between TCF/LEF signaling and *Prox1* expression in the adult hippocampus. To analyze a functional relationship between TCF/LEF signaling and *Prox1* expression in the adult DG, we used a retrovirus, selectively targeting dividing cells and their progeny, to overexpress a dominant-negative LEF (dnLEF), thus inhibiting TCF/LEF-dependent transcription in vivo (Fig. S3). Given the importance of canonical Wnt signaling for dentate neurogenesis, inhibition of TCF/LEF-dependent transcription may lead to a block of hippocampal neurogenesis, making the interpretation of such loss-of-function experiments difficult. Nevertheless, we found 4 d after retroviral injection that the number of dnLEF-expressing cells colabeling with *Prox1* was reduced compared with newborn cells labeled with a control virus (Fig. 3B and C). These findings support the data showing the in vitro activity of β -catenin on the *Prox1* promoter.

Overexpression of Active β -Catenin Induces Ectopic *Prox1* Expression. We next aimed to analyze if β -catenin is sufficient to induce *Prox1* expression. As the vast majority of newborn cells in the adult hippocampus, under physiological conditions, express *Prox1* (Fig. S2), we switched to the developing forebrain. We decided to address whether elevated levels of active β -catenin induce *Prox1* expression. To enhance active β -catenin levels, we used a mouse line in which exon 3 of the *β -catenin* gene is flanked by loxP sites (28). Exon 3 encodes all amino acids essential for phosphorylation of β -catenin and its subsequent degradation. Deletion of exon 3 results in the generation of a truncated but stabilized form of β -catenin, which is not targeted for degradation and retains its transcriptional activity (28). We used *Emx1*^{IRESCre} mice to express Cre recombinase under the *Emx1* promoter, which is active in cortical progenitors and excitatory neurons in the embryonic

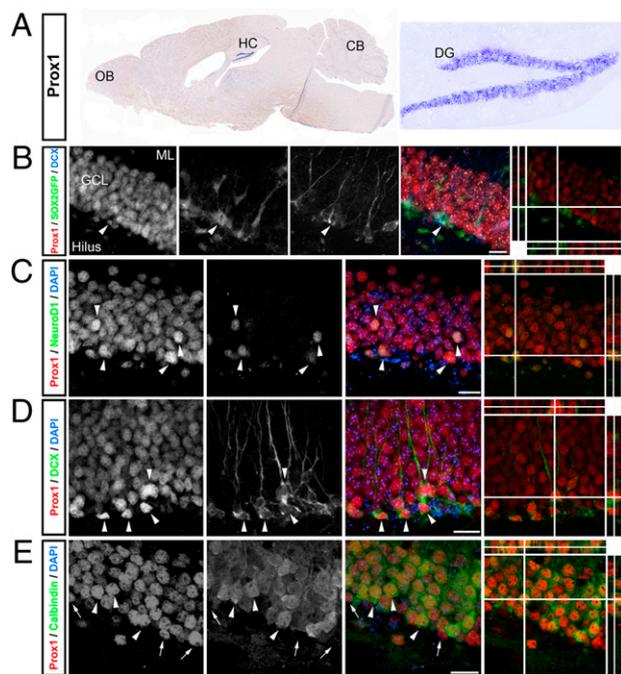


Fig. 2. Prox1 is expressed in the course of adult hippocampal neurogenesis. (A) In situ hybridization with a riboprobe against *Prox1* shows the selective expression of *Prox1* mRNA in the DG of adult mice. Shown are an overview of a sagittal brain section (Left) and a high-power view of the DG (Right). (B) Prox1 (red) is expressed in nonradial SOX2GFP-expressing cells (green) that are also weakly positive for DCX (blue, arrowheads). Grayscale images show single channels. Right: Collapsed z-stack overlay of single channels (nuclei were counterstained with DAPI, gray) and a 3D reconstruction of a triple-positive cell. (C–E) Prox1 (red) is expressed in early neuronal progeny expressing NeuroD1 (blue, C) and DCX (blue, D) but is also expressed at high levels in mature, calbindin-expressing granule cells (blue, E). Right: Collapsed z-stack overlay of single channels (nuclei were counterstained with DAPI, gray) and a 3D reconstruction of a triple-positive cell. OB, olfactory bulb; CB, cerebellum; HC, hippocampus; ML, molecular layer; GCL, granule cell layer. (Scale bars: B–E, 30 μ m.)

forebrain (29), and crossed heterozygous mice to produce animals with the genotype $Emx1^{IRESCre}/\beta\text{-catenin}^{exo3}$.

In mice overexpressing the dominant active form of β -catenin, the Prox1 expression domain was dramatically increased in the $Emx1^{IRESCre}/\beta\text{-catenin}^{exo3}$ mutants compared with WT littermates. At embryonic day 18 to postnatal day 0, we found robust ectopic expression of Prox1 in dorsal cortical areas of $Emx1^{IRESCre}/\beta\text{-catenin}^{exo3}$ mutants whereas Prox1 expression in controls was largely restricted to the developing DG (Fig. 4). Taken together, these experiments show that β -catenin is sufficient to induce ectopic Prox1 expression in the developing forebrain, supporting a regulation of *Prox1* by β -catenin–TCF/LEF signaling.

Prox1 Has a Stage-Specific Role in the Course of Adult Hippocampal Neurogenesis. It has been recently shown that Prox1 is required for granule cell genesis during embryonic and adult neurogenesis in the hippocampus (17). Given the expression pattern of Prox1 in newborn but also fully mature granule cells, we aimed to analyze the functional role of Prox1 in early and late steps of adult neurogenesis. Thus, we used shRNA-expressing lentiviruses—transducing dividing but also postmitotic cells in the DG to target Prox1 expression levels in the adult hippocampus (Fig. S3)—in combination with endogenous markers and thymidine analogue labeling of cells in S-phase and their progeny.

By using DCX to analyze the levels of neurogenesis (26), we found a substantial reduction of newborn neurons after shRNA-

mediated Prox1 knockdown compared with animals injected with nontargeting shRNA-expressing control virus (Fig. 5). As a complementary strategy, we injected the thymidine analogue chlorodeoxyuridine (CldU) 2 wk after stereotactic injections of Prox1-targeting or nontargeting shRNA-expressing lentiviruses. Animals were killed 3 wk after CldU injection. We found that Prox1 knockdown led to impaired differentiation and reduced numbers of CldU-DCX-labeled cells (Fig. S4).

We next aimed to analyze the effects of Prox1 knockdown on the NSC compartment. Notably, the number of hippocampal, radial glia-like NSCs expressing SOX2 and GFAP was not affected by Prox1 knockdown (Fig. S4), suggesting that Prox1 knockdown affects the level of newborn neurons but not the proliferative activity and/or maintenance of hippocampal NSCs at this time point after Prox1 knockdown. However, this may change in a situation in which Prox1 is chronically depleted: Lavado and colleagues found defective maintenance of radial glia-like NSCs 2 mo after Prox1 KO (17).

We then asked if Prox1 overexpression selectively in newborn cells of the adult DG would cell-autonomously enhance neuronal differentiation. Animals were injected with retroviruses expressing Prox1 or control retroviruses and killed 1 wk later. We found that Prox1 overexpression enhanced neuronal differentiation as the number of GFP-labeled cells coexpressing DCX was enhanced compared with cells transduced with a control retrovirus (Fig. S5).

To analyze if Prox1 is required for granule cell maintenance after cells have fully matured, we again used a lentivirus-based strategy. Adult-born granule cells were birthdated with BrdU labeling and injected 16 wk later with shRNA-expressing virus targeting *Prox1* mRNA or control lentiviruses, at a time point when newborn granule cells are stably integrated into the hippocampal circuitry and many of them express calbindin (15, 30, 31). In contrast to the obvious loss of differentiating granule cells, we found no differences in lentivirus-infected BrdU-labeled cells expressing shRNAs against Prox1 compared with control cells (Fig. 6). There were also no differences in the total number of BrdU-labeled cells expressing NeuN or calbindin, suggesting that Prox1 is not required for the survival of granule cells after they have matured (Fig. 6).

Supporting these *in vivo* findings, we found that Prox1 overexpression enhanced neuronal differentiation of cultured NSCs, whereas shRNA-mediated knockdown of Prox1 impaired neuronal differentiation (Figs. S6 and S7). Taken together, our data suggest that Prox1 is (i) required for proper neuronal differentiation of granule cells, (ii) sufficient to enhance neuronal differentiation of newborn cells in the adult DG, and (iii) not required for granule cell survival after they have fully matured. Thus, our findings confirm and extend the results recently obtained by using a transgenesis-based approach (17).

Discussion

Here we show that the homeodomain transcription factor Prox1 is regulated with β -catenin–TCF/LEF activation downstream of Wnt signaling and that Prox1 has a stage-specific function in the course of adult hippocampal neurogenesis. Thus, we have identified a molecular mechanism of how niche-derived signals are translated into the production of new granule cells within the adult hippocampus.

The hippocampus is one of the two regions within the brain that generates new neurons throughout adulthood (1). Despite recent advances in understanding the molecular mechanisms underlying lifelong neurogenesis (2), it remains largely unknown how local signals become translated into the production of new neurons. One of the key molecules expressed by local astrocytes within the neurogenic niche of the adult hippocampus is Wnt3, which is required for the generation of new granule cells (7, 32). Supporting the critical role of Wnt signaling in adult neurogenesis, it has been recently shown that NeuroD1, a basic helix-

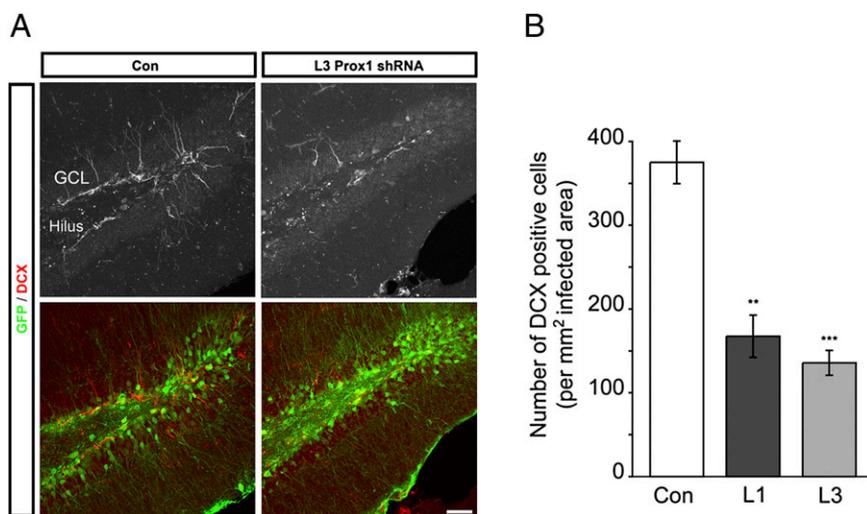


Fig. 5. Prox1 has a stage-specific role for neurogenesis in the adult hippocampus. (A) Intra-hippocampal injections of lentiviruses expressing control, nontargeting shRNA and GFP (green, *Left*), or shRNA against Prox1 mRNA (green, *Right*) show a decrease in newborn DCX-expressing neurons (red) after Prox1 knockdown. *Upper*: DCX. *Lower*: Overlay of DCX and GFP. (B) The density of DCX-expressing cells is reduced after Prox1 knockdown by using two independent shRNAs. GCL, granule cell layer. ** $P < 0.01$ and *** $P < 0.001$. (Scale bar: 100 μm .)

regulating adult hippocampal neurogenesis by showing that β -catenin–TCF/LEF–dependent transcription selectively up-regulates Prox1 expression in the adult hippocampus. Future studies will have to characterize if Wnt-regulated Prox1 and NeuroD1 may function together or independently in the formation of new granule cells in the adult hippocampus.

What could be the downstream targets of Prox1 that are important for neuronal differentiation in vitro and in vivo? Several transcriptional targets of Prox1 have been identified, among them a number of genes that could be also important for hippocampal neurogenesis, such as VEGF receptor 3, FGF receptor

3 (35), and $\alpha 9$ -integrin (36). Furthermore, a recent study suggested that Prox1 deletion in NSCs and their progeny results in impaired Notch signaling in NSCs, leading to the depletion of the stem cell pool over time (17). However, significant targets of Prox1 in the context of adult neurogenesis are still unknown.

The levels of Prox1 remain high in fully mature granule cells, which is also reflected by high TCF/LEF-dependent transcriptional activity in mature granule cells as seen in the BAT-gal reporter mouse (7). Thus, two models could explain the functions of Prox1: (i) Prox1 regulates the expression of differentiation/survival factors that are required for early and late steps of hippocampal neurogenesis or (ii) downstream targets of Prox1 are distinct between early and late steps of neurogenesis (e.g., in immature vs. mature granule cells). At this time, neither of the two possibilities can be formally ruled out. However, the finding that the survival of fully mature granule cells was not affected by Prox1 knockdown suggests that the function of Prox1 in mature granule cell is distinct to its role during initial neuronal differentiation of adult-born granule cells.

The data presented here identify Prox1 as a critical stage-specific regulator of hippocampal neurogenesis in the adult brain. Understanding the transcriptional code governing the neuronal differentiation of newborn granule cells is a prerequisite for the development of new therapeutic strategies to target adult neurogenesis in the context of neuropsychiatric diseases.

Materials and Methods

Plasmids and Viruses. For Prox1 overexpression, full-length mouse Prox1 cDNA (ImaGenes) was subcloned into a retroviral backbone containing a chicken β -actin promoter followed by an internal ribosome entry site GFP as described previously (37). To block β -catenin signaling, we cloned a dnLEF (7) into the same retroviral backbone. As control, a chicken β -actin–driven GFP vector was used. For knockdown experiments, two different shRNAs directed against Prox1 rat and mouse mRNA were used (target sequence L1, GGGCAAT-GAAAACGAAAGA; L3, CGGCAAACCAAGAGGAGAA). As control shRNA we used a target sequence not matching to any rodent mRNA sequence (CCTA-AGGTTAAGTCGCCCT). For retroviral experiments, shRNAs were cloned into a vector backbone that contains an U6-driven cloning site and a chicken β -actin–driven GFP as described previously (37). For lentiviral experiments, the same shRNA oligos were cloned into the LentiLox 3.7 vector (Addgene) that contains a U6-driven cloning site and a CMV-driven GFP. For overexpression of Wnt3 we used lentiviruses described previously (7). Viruses were produced as described previously (38).

Luciferase Assays and CHIP. For CHIP, we used murine NSCs as described previously (39). Chromatin was isolated 5 d after growth factor withdrawal to induce neuronal differentiation. Samples were prepared according to the manufacturers recommendations using the EZ CHIP Kit (Millipore). For im-

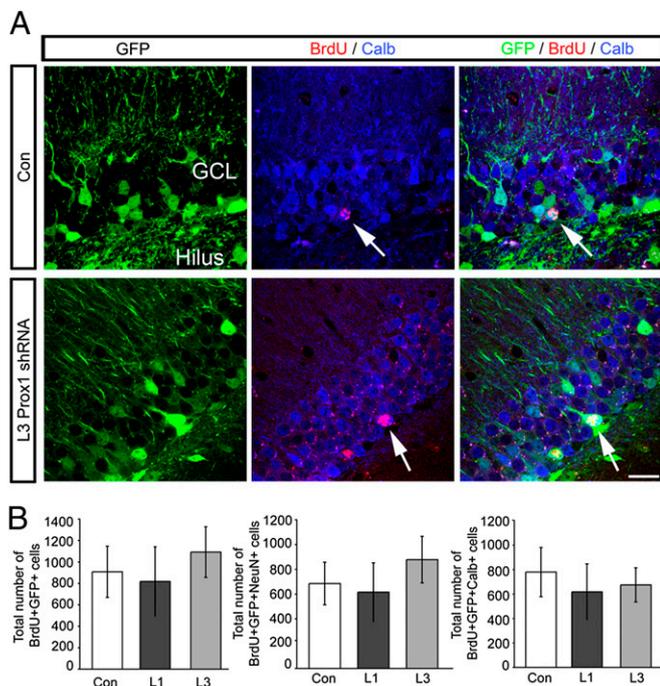


Fig. 6. Prox1 knockdown does not affect survival of mature granule cells. (A) Representative images of BrdU (red)-labeled, calbindin-positive (blue, arrows) cells in areas expressing control lentivirus (green, *Upper*) and after Prox1 knockdown (green, *Lower*). (B) The numbers of cells labeled with BrdU 16 wk before lentivirus injection were not different between control and Prox1 knockdown conditions. In analogy, the number of BrdU/NeuN colabeled cells was not different between groups. ML, molecular layer; GCL, granule cell layer. (Scale bar: 50 μm .)

munoprecipitation of the crosslinked protein-DNA complexes, 5 μ g of rabbit anti- β -catenin antibody (C2206; Sigma) or 1 μ g of rabbit IgG antibody (Sigma) were used. For qPCR detection, Power SYBR Green PCR Master Mix (Applied Biosystems) was used. Primer sequences are provided in *SI Materials and Methods*. The amount of DNA pulled down from each immunoprecipitation was calculated using Ct values and expressed as fold enrichment compared with the DNA amount pulled down by the IgG immunoprecipitation.

For luciferase assays, we cloned the genomic regions of the *Prox1* enhancer containing a consensus TCF/LEF site into the reporter plasmid pGL4.25 (Promega) using PCR. Primer sequences are provided in *SI Materials and Methods*. Plasmids used for β -catenin expression, TOPFLASH, and FOPFLASH reporters have been described previously (7). NSCs were electroporated with reporter plasmids and β -catenin plus/minus SOX9 by a Nucleofector device (Amaxa) according to the manufacturer's recommendations. pLR-TK (Promega) was coelectroporated as an internal control. Forty-eight hours after electroporation, luciferase activity in the lysis supernatant was measured with a Dual-Luciferase Reporter Assay System (Promega) using a MicroLumat Plus (Berthold).

Animals and Stereotactic Injections. Animal experiments were approved by the veterinary office of the Canton of Zurich, Switzerland, or by the Bezirksregierung, Braunschweig, Germany. For all experiments in the adult brain, the animals used were 6- to 8-wk-old female C57BL/6 mice (Janvier) or SOX2GFP transgenic mice (24). Mice were stereotactically injected with 1.5 μ L of lentiviral suspension (or 1 μ L retrovirus for overexpression experiments) into the DG as described previously (38). According to the experimental paradigm for lentiviral analysis, either BrdU (100 mg/kg) or CldU (85.5 mg/kg; Sigma) was injected to the animals intraperitoneally at the times indicated. Perfusion and preparation of brain sections were described previously (40). Group sizes for all experimental groups were greater than three. For qPCR of *Prox1* mRNA isolated from cerebral subregions, brains from 8-wk-old C57BL/6 mice ($n = 2$) were microdissected and RNA was obtained, reverse-transcribed, and used for expression analysis. For experiments in the embryonic and postnatal day 0 forebrain, we used the previously described mouse lines *Emx1^{IRESCre}*, β -catenin^{exo3} (28, 29).

Immunostaining, Western Blotting, in Situ Hybridization, and qPCR. Tissue and cells were prepared for immunostaining as described earlier (37). Briefly, mice were perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Brains were removed and postfixed overnight. Brains were then transferred into 30% sucrose overnight and cut on a dry ice-cooled microtome into 40- μ m-thick coronal sections. Cells were fixed for 15 min in 4% PFA followed by extensive washes in phosphate buffer. A list of antibodies is provided in *SI Materials and Methods*. For in vivo analyses, areas transduced by lentiviruses were identified by GFP coverage by using ImageJ. Virus-infected cells (50–100 cells per animal; $n \geq 3$) in the granule cell layer of one-in-12 series of sections were analyzed by using high-resolution confocal microscopy in sequential scanning mode (SP2; 20 \times objective, 0.7 N.A.; Leica). The presented total number of cells per DG was adjusted accordingly. Imaris version 6.1.x (Bitplane) was used to quantify colocalization of neurogenesis markers and thymidine analogues in lentivirus-infected areas. Radioactive and nonradioactive in situ hybridizations were performed as described before (37) or according to standard protocols (41). qPCR was performed using standard procedures. Further details are provided in *SI Materials and Methods*.

Statistical Analysis. All numerical analyses were performed using Excel (Microsoft) with appropriate add-ons or SPSS Statistics (IBM). For all comparisons, two-sample *t* tests or ANOVA if appropriate were used. Differences were considered significant at $P < 0.05$.

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