Supplementary information for

In vivo clonal analysis reveals self-renewing and multipotent adult neural stem cell characteristics

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Supplementary information includes

6 Supplementary figures and figure legends (corresponding to each of the first six main figures)

2 Supplementary movies

1 Supplementary table

Extended Experimental Procedures

References

Supplementary Figure Legends

Figure S1. A genetic sparse marking strategy for in vivo analysis of individual nestin$^+$ radial glia-like neural precursors in the adult mouse dentate gyrus, Related to Figure 1

(A) A schematic diagram illustrating the genetic animal model used for analysis of individual nestin-expressing precursors in the adult mouse hippocampus.

(B) A summary table of criteria used to define different cell types in this study. GCL: granule cell layer; ML: molecular layer; SGZ: subgranular zone.

(C) Computational simulation of the nearest distance between two cells in the 3D dentate gyrus. Shown are reverse cumulative distributions as a function of increasing distance from one precursor to the nearest precursor (top), or to the nearest astroglia (A; middle); or from one astroglia to the nearest astroglia (bottom). The numbers of precursors correspond to the expected number of precursors induced at 2 dpi in MADM (2), Z/EG at 0.5X tamoxifen (5), Z/EG and PTEN cKO at 1X tamoxifen (8) animals. The numbers of astroglia correspond to the expected number of astroglia induced at 2 dpi in MADM (2), 0.5X Z/EG (8), 1X Z/EG (14) and PTEN cKO (25) animals. The plots were used as standard curves to determine the P_c of each clone based on the clone composition.

(D) Summary of the number of given clones at different time points after induction with the 1X dose of tamoxifen. Each circle represents data from one dentate gyrus and each line represents the mean value.

Figure S2. Different modes of self-renewal by individual RGLs in the adult dentate gyrus, Related to Figure 2

(A) Sample confocal images of a GFP$^+$ cell clone consisting of a GFAP$^+$Sox2$^+$ RGL (arrow), and two GFAP$^-$Sox2$^+$ non-radial precursors (arrowheads). Scale bars: 10 μm.

(B-E) Lack of oligodendrocyte lineage differentiation from labeled RGLs in the adult dentate gyrus.

Shown are sample confocal images of GFP^+ cells displaying characteristics of an RGL (B), IPCs (C) and transition astroglia (D), all of which did not express NG2. On the other hand, numerous $NG2^+$ cells were present in the surroundings. Scale bars: 10 μm. Also shown is a summary diagram (E).

Figure S3. Lineage-tree analysis of RGLs that exhibited long-term self-renewal, Related to Figure 3

Shown are lineage-trees for clones shown in Figure 3H (A) and Figure 3I (B). R: RGL; A: astroglia; N: neuronal lineage, including IPC and neuron. For the purpose of simplicity, not all permutations of the lineage-trees are listed.

Figure S4. Summary of clone properties at different time points after labeling, Related to Figure 4

(A) A summary table of the number of clones according to their clone composition and number of progeny at 1 mpi. Clones with the neuronal lineage contain more cells than the astroglial lineage due to the presence of IPCs. R: RGL; A: astroglia; N: neuronal lineage (including IPCs and neurons).

(B and C) Distribution of numbers of neurons (B) or astrocytes (C) per clone at 1 or 2 mpi.

(D and E) A plot of measured longest distance from a GFP^+ cell to the clone center at 12 mpi (D) and calculated P_c value for each cell cluster (E). Data at 1 mpi (from Figures 1F and 1G) are also shown for comparison.

Figure S5. MADM-based analysis of $nestin^+$ radial glia-like neural precursors in the adult dentate gyrus, Related to Figure 5

(A) MADM-based reporter for genetic lineage tracing in vivo (adapted from Zong et al. 2005).

(B) Comparison of the number of labeled precursors per entire dentate between the MADM and Z/EG reporters after different doses of tamoxifen. The same data as in Figures 1B and 5B are re-

plotted in the same graph for direct comparison.

(C) A plot of measured longest distance of a labeled cell to the clone center at 2 mpi for Z/EG and MADM reporters.

(D) Individual confocal images of the same clone as shown in Figure 5E to better illustrate the co-localization of GFP, RFP and GFAP. Scale bars: 10 μm.

Figure S6. Clonal analysis of PTEN function in regulating RGLs in the adult dentate gyrus, Related to Figure S6

(A) The animal model used for analysis of *Pten* deletion in individual nestin-expressing precursors within the adult mouse hippocampus. Shown are schematic diagram (top) and percentages of subtypes of labeled precursors after induction with 1X dose of tamoxifen in adult control and PTEN cKO mice at 2 dpi (bottom).

(B) Confirmation of *Pten* deletion in GFP^+ RGLs (left at 2 dpi) and neurons (right) in adult PTEN cKO mice. Shown are sample confocal images of immunostaining for GFP, PTEN and Prox1. Scale bars: 10 μm.

(C- E) Quantitative analysis of clonality at 1 mpi after induction with the 1X dose of tamoxifen in PTEN cKO mice. Shown in (C) is the distribution of measured distance from a GFP^+ precursor to its nearest GFP^+ cells in the dentate gyrus at 2 dpi (histograph) and data from computational simulation of distances (8 precursors and 25 astroglia; red line). Shown in (D) is a plot of measured longest distance of a GFP^+ cell to the clone center. Shown in (E) is calculated P_c value for each cell cluster.

(F) Sample confocal images of a transition astroglia exhibiting increased processes and a loss of nestin expression. Scale bars: 10 μm.

(G) A summary of the frequency of different clone types without an RGL in control (n = 7) and PTEN cKO mice (n = 6) at 1 mpi. Note that *Pten* deletion diminishes the RGL pool in the adult brain

and increases astroglial differentiation. TAs: transition astroglia; A: astroglial; N: neuronal lineage. Values represent mean ± SEM (**: $P < 0.01$; *: $P < 0.05$; student's t-test).

Supplementary Movie Legends

Movie S1. 3D reconstruction of dentate gyrus showing the distribution of labeled cells at 2 days after induction with 1X dose of tamoxifen.

The dots indicate the position of different cell types that were labeled at 2 dpi. Red: RGL; Green: IPC; Blue: mature astrocyte. The 3D reconstruction was also used for computational simulation in Figure S1C.

Movie S2. A clone indicating RGL self-renewal and multi-lineage differentiation at 1 month after induction.

The same clone as in Figure 3B is shown. The clone consists of a single RGL with a long radial process, a bushy astroglia positioned in the granule cell layer and a cluster of neuronal progeny. The neuronal cluster contains 18 cells with many possessing tangential processes.

Supplementary Table

Table S1. Detailed information on antibodies, including species, dilution, source and catalog numbers.

Primary Antibody	Species	Dilution	Source	Catalog #
Doublecortin (DCX)	Goat	1:500	Santa Cruz	SC-8066
GFAP	Mouse	1:2000	Millipore (clone GA5)	MAB360
GFAP	Rabbit	1:2000	DAKO	Z0334
GFP	Chicken	1:1000	Aves	GFP-1020
GFP	Goat	1:1000	Rockland	600-101-215
GFP	Rabbit	1:1000	Invitrogen	A11122
Ki67	Rabbit	1:1000	Novocastra	NCL-Ki-67p
Mcm2	Mouse	1:500	BD (BM28)	610701
Myc (for RFP)	Goat	1:200	Novus	NB600-335
Nestin	Chicken	1:500	Aves	NES
Nestin	Mouse	1:500	Chemicon	MAB353
NG2	Rabbit	1:500	Dr. Stallcup	N/A
Prox1	Rabbit	1:1000	Abcam	Ab11941
PTEN	Rabbit	1:200	Cell Signaling	9559
S100β	Mouse	1:1000	Sigma	S2532
Sox2	Goat	1:400	Santa Cruz (Y-17)	SC-17320
Tbr2/Eomes	Rabbit	1:1000	Abcam	Ab23345

EXPERIMENTAL PROCEDURES

Animals and Tamoxifen Administration

Nestin-CreER^{T2} mice (Balordi and Fishell, 2007) were backcrossed to the C57BL/6 background (Charles River) for at least six generations. The following genetically modified mice were purchased from Jackson Labs: Z/EG$^{f/+}$ (stock #3920) (Novak et al., 2000), RG$^{f/f}$ (stock #6080) and GR$^{f/+}$ (stock #6075) (Zong et al., 2005), and *Pten*$^{f/+}$ (stock 4597) (Groszer et al., 2001). Nestin-CreER$^{T2+/}$;Z/EG$^{f/+}$ animals were generated by crossing nestin-CreER^{T2} and Z/EG$^{f/+}$ mice or nestin-CreER^{T2};Z/EG with wild-type C57BL/6 mice. *Pten*$^{f/+}$ mice were backcrossed to the C57BL/6 background over six generations and bred into nestin-CreER^{T2};Z/EG mice to generate nestin-CreER$^{T2+/-}$;*Pten*$^{f/f}$;Z/EG$^{f/+}$ mice. Nestin-CreER^{T2};MADM mice (nestin-CreER$^{T2+/-}$;RG$^{f/+}$;GR$^{f/+}$) were generated by crossing nestin-CreER$^{T2+/-}$;RG$^{f/f}$ mice to GR$^{f/+}$ mice. Since adult neurogenesis is particularly sensitive to the genetic background (Kempermann et al., 2006; Kempermann et al., 1997), all mice used in the study were back-crossed to the C57BL/6 background to ensure the reproducibility of clonal induction with specific doses of tamoxifen. Animals were housed in a 14 hr light/10 hr dark cycle with free access to food. All animal procedures were in accordance with institutional guidelines.

Genetically modified mice were identified using primer sets from original publications (Balordi and Fishell, 2007; Groszer et al., 2001; Novak et al., 2000; Zong et al., 2005). Genomic DNA were isolated in 25 mM NaOH, 0.2 mM EDTA and processed for 35 PCR cycles. Z/EG mice were phenotyped using an X-gal reaction [50 mM $K_3Fe(CN)_6$, 50 mM $K_4Fe(CN)_6 3H_2O$, 1 M $MgCl_2$, 10 mg/ml X-Gal in PBS] for 4-16 hrs.

A stock of tamoxifen (62 mg/ml; Sigma; T5648) was prepared in a 5:1 ratio of corn oil (Sigma) to ethanol at 37^{o}C with occasional vortexing. A single tamoxifen or vehicle dose was i.p. injected into 8-10 week-old mice at various concentrations: 31 - 248 mg/kg for the Z/EG reporter and 186 - 373 mg/kg for the MADM reporter. Animals injected with tamoxifen at these doses showed no signs of distress.

Immunostaining and Confocal Imaging

Coronal brain sections (45 μm in thickness) were prepared through the entire dentate gyrus. Sections were maintained in the serial order using custom-built processing chambers and immunostaining was performed as previously described (Ge et al., 2006). Antibodies used in this study are listed in Table S1. For immunostaining of nestin and MCM2, an antigen retrieval procedure was performed after GFP staining using a microwave. Citrate buffer (1.8 mM citric acid, 8.2 mM tri-sodium citrate) was pre-heated for 5 min at the maximum power. Sections were then placed in the hot citrate buffer and incubated for another 7 min at the maximum power. Sections were then allowed to cool at room-temperature in the citrate buffer for 1 hr. For immunostaining of PTEN, sections were permeabilized overnight in 3% Tween-20 and 7.5% Glycine in phosphate buffer and then penetrated with incubation in 10%, 20%, 40%, 20%, 10% ethanol in phosphate buffer prior to primary antibody application (Tokuda et al., 2011). Antibodies were diluted in Blocking Buffer (3%-Tween-20, 7.5% glycine, 0.5% Igepal and 5% donkey serum). Buffer washes contained 3% Tween-20, 7.5% glycine and 0.5% Igepal. Sections were dehydrated again after secondary antibody application by incubation in 70%, 90%, 95%, 100% ethanol in phosphate buffer and then washed one final time in PBS.

GFP^+ cells were identified using an Axiovert 200M microscope (Zeiss) and acquired as a z-stack on Zeiss510 multi-photon or Zeiss710 single-photon confocal systems using multi-track configurations. Cell clones/clusters were acquired at 40X or 63X, while sections used to reconstruct the entire dentate gyrus were acquired at 10X magnification using the tiling function in the Zen 2008 software (Zeiss).

Image Processing, Reconstruction, Rendering and Movie Generation

Confocal images were viewed using LSM Image Browser 4.2.0 (Zeiss) to confirm cell identity according to immunohistological and morphological properties (Figure S1B). For 3D reconstruction,

optical stacks from the entire dentate gyrus were serially aligned using Reconstruct 1.1.0 (John C. Fiala, Human Brain Project, the National Institutes of Health). Briefly, 2D optical sections were translated and rotated in the X-Y plane to match the orientation of the subsequent serial section. Recorded keystrokes were propagated to preceding images within the z-stack. This process was repeated across stacked files to reconstruct clones or the entire dentate gyrus. Aligned images were exported at full resolution into Imaris 7.1.1 (Bitplane) for 3D visualization. Voxel sizes were adjusted according to acquired image dimensions specified in the LSM file.

Surface renderings were created on a series of aligned dentate sections containing DAPI nuclei staining and GFP immunostaining using the "magic wand" feature in Photoshop CS4 Extended (Adobe) to isolate the granule cell layer. These images were refined using an in-house MATLAB script (The MathWorks, Inc.) to convert images into logical values, number contiguous regions, extract dentate gyrus-containing regions, and fill gaps. For clone distance simulations and confidence, a parallel set of segmented images was created to include the ~130 μm above and below the dentate granule cell layer, corresponding to the hilus and molecular layer regions. Segmented images were scaled and viewed in 3D using Imaris. A "surface" was generated from segmented images and the original DAPI/GFP images were overlaid. Imaris spots were added corresponding to each labeled cell type (precursors, astrocytes) as mapped from the original data set to the surface. 3D movies were created using the "animation" features to generate a 360° perspective of the 3D image (Movie S1).

Clonal Analysis

Analyzed dentate volume included the molecular layer, stratum granulosum (granule cell layer), SGZ and hilus, but excluded the polymorphic layer (CA4) protruding into the posterior dentate gyrus. Serial sections were first screened for candidate clones, which were defined as possessing at least (1) an RGL, (2) neuronal cell(s) in close spatial proximity, or (3) astroglia in close spatial

proximity to other astroglia or neuronal cells. The definition of 'spatial proximity' was purely subjective to provide a starting point for the analysis and did not bias the final results.

Distance measurements were performed in Imaris. Spots were placed in the location of GFP^+ cells and their coordinates were exported to an Excel 2007 (Microsoft) file. An in-house MATLAB script was developed to determine the distances among every spot and exported the data as a matrix. For analysis at 2 dpi, the distance between each GFP^+ precursor to the nearest GFP^+ cell was recorded. For analysis of the cluster domain at 1, 2 and 12 mpi, the maximum distance from a GFP^+ progeny to the clone center was recorded, independent of the cell number within the cluster. In clusters lacking an RGL, the largest distance was measured from the center astroglia or neuronal lineage (in that order), depending on the cluster composition. For each candidate clone at 1, 2 or 12 mpi, a clone center was first defined by the location of the RGL. In candidate clones with more than one RGL or no RGLs, the clone center was defined by the location of the centermost RGL, astroglia, or neuronal cell - in that order. A ring with a radius of 150 μm from the clone center was used to determine the clone composition at 1 and 2 mpi. The radius was initially determined based upon the distribution of GFP^+ precursor-precursor and precursor-astroglia distances measured using the Z/EG reporter at 2 dpi (Figure 1E) and based on analysis of the MADM reporter at 2 mpi (Figure S5C), in both cases when clones can be unambiguously identified. Remaining neuronal progeny not assigned to any clone were then deemed clones themselves and the analyses were repeated. At 2, 4 and 7 dpi, clones had to contain at least an RGL or IPC(s) located in the SGZ. An individual astrocyte without other progeny (astrocyte, IPC or RGL) was not included in the analysis. Under control conditions, RGLs were largely single cells, while IPCs occurred as singlet or doublets at 2 dpi or clusters by 7 dpi (Figure 1). Clones were categorized according to the presence or absence of an RGL and the type of progeny (Figures 3 and 4). Quiescent RGL clones contained only a single RGL. Unipotential self-renewing clones contained at least one RGL and either the neuronal (IPCs, neuroblasts, neurons) or astroglial (transition astroglia, astrocytes) lineages. Multipotential and self-renewing clones contained at least one RGL

and cells of both neuronal and astroglial lineages. Symmetric self-renewing clones contained more than one RGL and no other cells. Clones lacking an RGL were defined as ‘non-self-renewing’ and classified according to composition permutations. The oligodendrocyte lineage was not observed in this analysis and was therefore omitted from classification (Figures S2B to S2E).

The criteria for clone assignment were validated with several independent assays. First, the numbers of candidate clones do not significantly change over time (Figure S1D). Second, the results from Z/EG reporter with 8 clones on average per dentate gyrus are very similar to those of MADM reporter with 2 clones per dentate gyrus (Figure 5F). Third, the distance analysis of progeny within a candidate clone reveals a similar distribution among three different experimental groups (Z/EG at 1 and 2 mpi and MADM at 2 mpi; Figures 1F and S5C). Finally, to obtain a statistical assessment of the probability whether each cell cluster was a clone, we developed a computational model for simulation and quantitative assessment. Segmented reconstructed dentate granule cell layer with and without hilus and molecular layer regions were loaded as 3D matrices in MATLAB. The number of starting precursor cells and astroglia was based upon data from 2 dpi under each experimental condition (reporter, tamoxifen concentration and genetic deletion). Each precursor cell was randomly placed in the model dentate granule cell layer lacking hilus and molecular layer regions. If applicable, each astroglia was randomly placed in the model dentate granule cell layer including hilus and molecular layer regions. Distance between each precursor cell and the nearest precursor cell (or astroglia, if applicable) was calculated and scaled according to voxel dimensions of the model dentate. This process of random cell placement and distance measurement was repeated for 5000 iterations to generate a distribution of distance measurements (Figures 1E and S6C, red lines) and reverse cumulative distribution plots (Figure S1C). Based upon its radius, each putative clone was assigned a probability as a clone (P_c) equal to 1 minus the probability that two cells could have been induced at 2 dpi within the clone's radius of each other (x 100%). This probability was taken directly from matching clone radius size with frequency on the appropriate clone distance simulated cumulative distribution plot (Figure S1C). Clones with RGLs only,

neuronal lineage only, or RGLs plus the neuronal lineage utilized precursor-only simulated distributions; clones with astroglia only utilized astroglia-only simulated distributions. Clones with RGLs and astroglia, or neuronal lineage and astroglia, utilized a simulated distribution with both precursors and astroglia. Multipotential clones used a simulated distribution with both precursors and astroglia, and probability as a clone (P_c) was calculated to both the neuronal and astroglial progeny with the lesser value taken as the clone probability.

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