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Supplemental Information

Restoration of hippocampal neural precursor function by ablation of senescent cells in the aging stem cell niche

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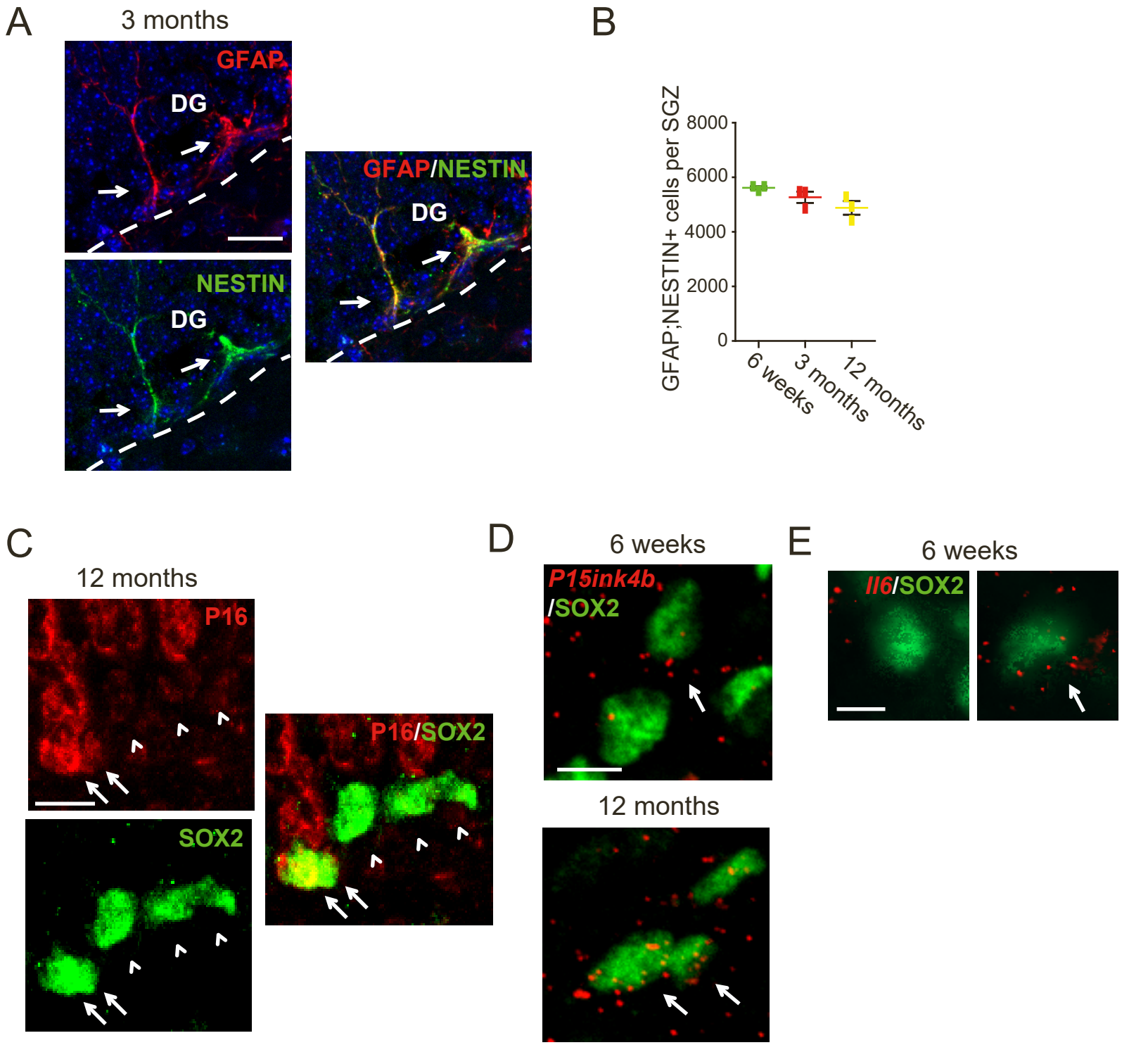
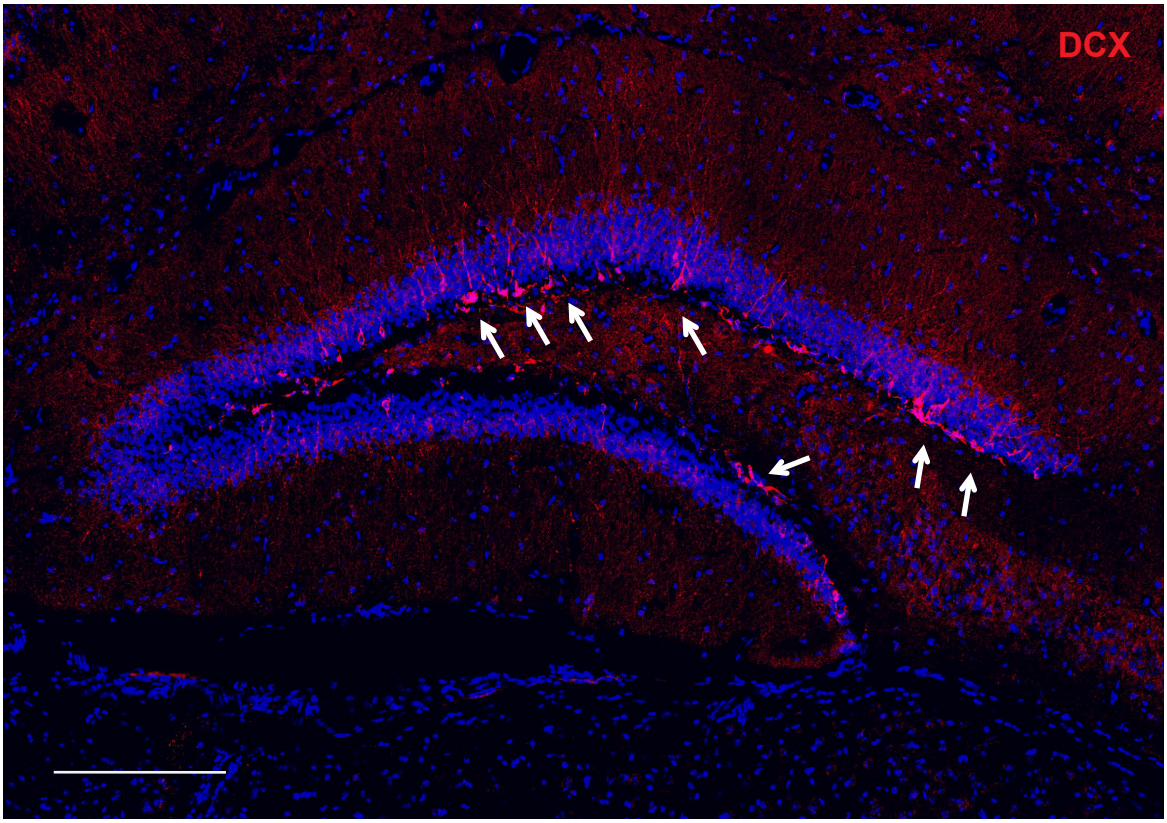

A
3 months
GFAP
DG
NESTIN
DG
GFAP/NESTIN
DG
B
GFAP;NESTIN+ cells per SGZ
8000
6000
4000
2000
0
6 weeks
3 months
12 months
C
12 months
P16
SOX2
P16/SOX2
D
6 weeks
P15ink4b
/SOX2
12 months
E
6 weeks
Il6/SOX2

Figure S1: ***Senescent NPCs accumulate in the SGZ with age, while neural stem cell numbers are unchanged.*** **Related to Figures 1 and 2.** Coronal sections through the hippocampal dentate gyrus (DG) of mice 6 weeks, 3 months, and 12 months of age were analyzed by immunostaining and FISH. (A) Image of the dentate gyrus from a mouse aged 3 months immunostained for GFAP (red) and NESTIN (green), counterstained for Hoechst 33258 (blue). Arrows denote double positive cells, and the dashed line indicates the boundary of the SGZ with the hilus. Scale bar = 20μm. (B) Quantification of sections as in (A) for the total number of GFAP;NESTIN double-positive cells in the dentate gyrus. n = 3 mice per timepoint. (C) Representative images of the SGZ from a mouse aged 12 months immunostained for p16 (red) and SOX2 (green). Arrows denote P16-positive, SOX2-positive cells, while arrowheads indicate SOX2-positive P16-negative cells. Scale bar = 10μm. (D) Representative images from the SGZ of 6 week and 12 month old mice analyzed by combined immunostaining for SOX2 (green) and FISH for *P15ink4b* (red). Arrows indicate cells positive for both SOX2 and *P15ink4b*. Scale bar = 10μm. (E) Images of the SGZ of a 6 week old mouse analyzed by combined immunostaining for SOX2 (green) and FISH for *Il6* (red) showing a SOX2-positive cell that is negative for *Il6* (left panel) and a SOX2-positive, *Il6*-positive cell (right panel, arrow). Scale bar = 5μm. In all cases, error bars indicate standard error of the mean.

A

Vehicle



ABT263

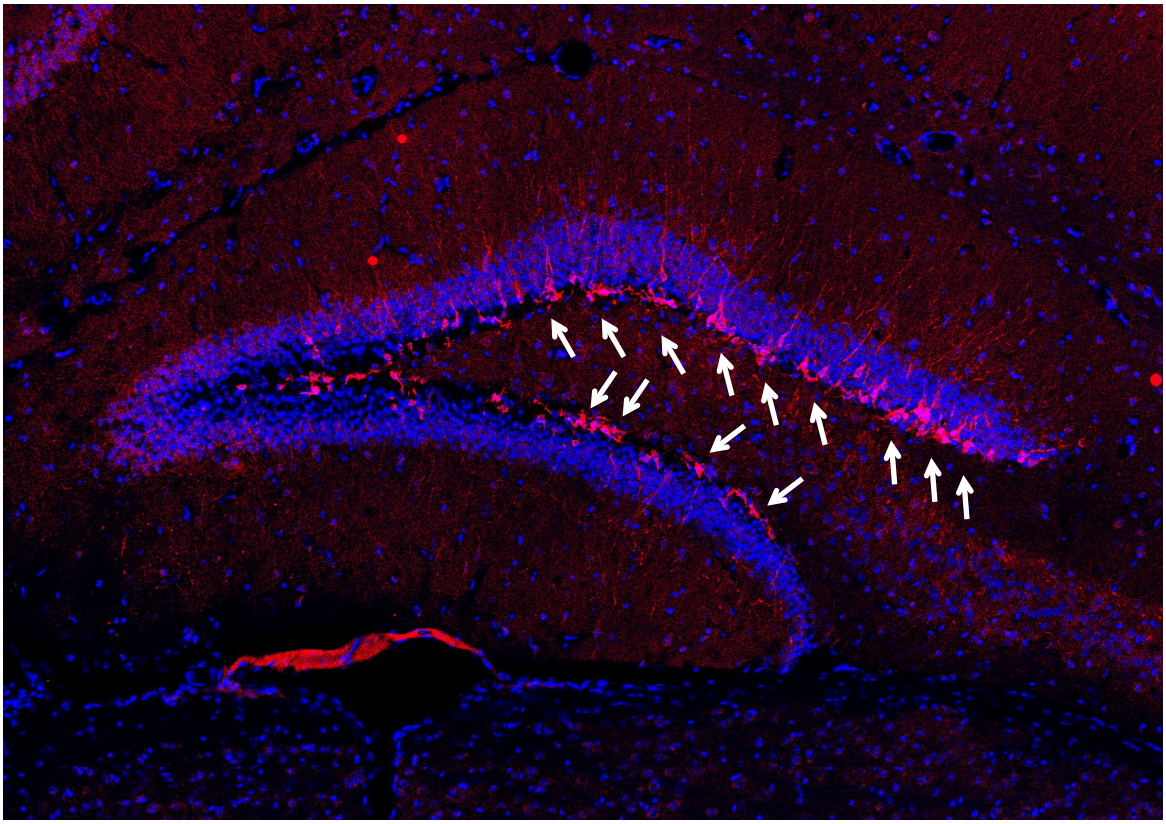


Figure S2: ***ABT-263 treatment enhances hippocampal neurogenesis in 3 month old mice.*** **Related to Figure 4.** Stitched images of coronal sections showing the dentate gyrus of 3 month old mice injected 5 days previously with either vehicle or ABT-263, immunostained for DOUBLECORTIN (DCX, red) and counterstained with Hoechst 33258 (blue). Arrows denote positive cells. Scale bar = 200 μm.

Supplemental Experimental Procedures

Neuroanatomy and Immunostaining: Mice were sacrificed by an overdose of sodium pentobarbital, and then transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were post-fixed overnight in 4% paraformaldehyde, dehydrated in 30% sucrose-PBS, cryoprotected, and coronally sectioned at 20μm using a Leica CM 1850 cryostat (Leica Biosystems, Concord, ON, Canada). Immunostaining was performed as described previously (Cancino et al., 2013) and in detail in the Supplemental Methods. SA-*β*-Gal staining was performed using the Senescence β-Galactosidase Staining Kit (9860, Cell Signaling Technology, Danvers MA, USA) following the manufacturer's instructions. To combine with immunostaining, sections were first stained with SA-*β*-Gal, permeabilized, blocked and incubated with primary antibody as above. Secondary antibody labelling and Diaminobenzidine (DAB) staining were performed using the Rabbit specific HRP/DAB (ABC) Detection IHC Kit (Ab64261, Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. Sections were washed with TBS, permeabilized with 0.4% Triton X-100 in TBS, and blocked with 5% BSA, 0.4% Triton X-100 in TBS. Slides were then incubated overnight at 4 °C with primary antibody diluted in blocking solution. Slides were washed with TBS, incubated with secondary antibody in blocking solution for 1 hour at room temperature, and mounted with Permount solution (Thermo Fisher Scientific, Waltham, MA, USA). For immunostaining of cultured cells, cells were fixed with 4% PFA or 2% formaldehyde/0.2%glutaraldehyde, blocked, and permeabilized with 10% normal goat serum (NGS) and 0.3% Triton-X. Fixed cells were then incubated with primary antibodies at 4ºC overnight, with secondary antibodies at room temperature for 1 hour, counterstained with Hoechst 33258.

Antibodies: The following antibodies were used for the immunofluorescent and western blot experiments in this study: goat anti-SOX2 (Y-17, 1 : 500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-DOUBLECORTIN (C-18, 1 : 250, Santa Cruz Biotechnology), rat anti-BrdU (OBT0030, 1 : 200, ABD Serotec, Raliegh, NC, USA), AlexaFluor 555-conjugated mouse anti-NeuN (MAB377A5, 1 : 100, EMD Millipore), mouse anti-KI67 (556003, 1 : 200, BD Pharmingen, San Diego, CA, USA), mouse anti-P16 (F-12, 1 : 1000, Santa Cruz Biotechnology), mouse anti-*γ*H2AX (05–636, 1 : 250, EMD Millipore), rabbit anti-GFAP (Z0334, 1 : 1000, Dako, Santa Clara, CA, USA), mouse anti-GFAP (A21282, 1 : 1000, Life Technologies, Grand Island, NY, USA), mouse anti-ASCLI1/MASH1 (556604, 1 : 1000, BD Pharmingen), rabbit anti-LAMIN B1 (AB16048, 1 : 500, Abcam), rabbit anti-IBA1 (019-19741, 1 : 500, Wako), rabbit anti-DOUBLECORTIN (AB18723, 1 : 500, Abcam), mouse anti-NESTIN (ab6142, 1:100, Abcam), rabbit anti-S100 (Z0311, 1 : 1000, Wako), and rabbit anti-ERK1 (K-23, 1:10 000, Santa Cruz Biotechnology). Secondary antibodies were AlexaFluor 488/555/647/HRP-conjugated donkey anti-goat, anti-rabbit, anti-rat, and anti-mouse IgG and anti-chicken IgY (1 : 1000, Life Technologies).

BrdU/EdU Labeling: For proliferation studies, mice were injected i.p. with a single dose of 100mg/kg BrdU (Sigma-Aldrich) or EdU (Thermo Fisher Scientific),and sacrificed and perfused as above 24 hours

later. To detect BrdU, coronal sections were incubated in 2N HCl for 30 minutes at 37°C, rinsed with TBS, incubated with anti-BrdU antibody at 4°C overnight, and detected using an anti-rat secondary antibody. To detect EdU, the Click-iT Plus EdU Alexa Fluor 555 Imaging Kit (C10638, Thermo Fisher Scientific) was used according to manufacturer's instructions. To label adult-born neurons, mice were injected i.p. with 100mg/kg BrdU every 2 hours for a total of 8 hours (5 injections) and sacrificed 30 days later. The day following BrdU immunostaining, sections were incubated with AlexaFluor 555 conjugated anti-NeuN antibody for 1 hour at room temperature. In both cases, labelled cell numbers were determined as described below. For olfactory neurogenesis, olfactory bulbs were sectioned at 18µm and 10 sections, sampled every 10th section, were immunostained for BrdU and NeuN, as described above.

Single molecule Fluorescence In Situ Hybridization (FISH): Single molecule FISH experiments were performed using the RNAScope® Multiplex Fluorescent Assay kit (Bio-techne, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, 20µm brain sections were dehydrated with ethanol, pretreated with protease, and probe hybridization and signal amplification performed. Sections were then washed with TBS and immunohistochemistry was performed as above. Positive signals were identified as punctate dots present in the nucleus and/or cytoplasm. Z stacks were obtained through the entire thickness of the section, and in some cases the number of RNA granules per cell was quantified. The following probes were used: *Cdkn2b* (458341, Bio-techne), and *Il6* (315891, Bio-techne).

Neurosphere Assay: For adult cultures, the V-SVZ of the lateral ventricles was dissected out as previously described (Fujitani et al., 2010). Cell density and viability were determined using trypan blue exclusion and a hemocytometer. Freshly isolated cells were then plated at a density of 10 cells/µl in six-well (2 ml per well) ultra-low attachment plates (Corning, Corning, NY, USA) in serum-free medium containing 20ng/ml EGF (Sigma-Aldrich), 10ng/ml FGF2 (Sigma-Aldrich), and 2µg/ml heparin (Sigma-Aldrich). Primary neurospheres were mechanically dissociated, passed through a 40-µm cell strainer, plated at a density of 2 cells/µl. To induce senescence, secondary neurospheres were grown for 2 days, treated with 25nM Camptothecin (Sigma-Aldrich) for 72 hours, washed, and then replated in fresh medium for an additional 2 days. DMSO was used as a vehicle control for all drug treatment experiments.

Quantitative PCR: RNA was extracted from tissue or cultured cells using the E.Z.N.A. Total RNA Kit I and was treated with DNase I. cDNA was synthesized from 200ng total RNA, and quantitative PCR was performed. *Gapdh* mRNA was used as an internal control for all reactions, and all reactions were run in triplicate. Quantitative PCRs were run on C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA), and analyzed using Bio-Rad CFX Manager Software (Bio-Rad). The following primers were used to for QPCR analysis: *mRFP* (Nam et al., 2007; Forward:CCAAGCTGAAGGTGACCAAG, Reverse: TCAAGTAGTCGGGGATGTCG), *Adam10* (Murthy et al., 2012; Forward: GATGCCAACCAGCCAGAGGG, Reverse: CAGATGCTGGGCAAAGGGCT), *Adam17* (Murthy et al.,

2012; Forward: CGGAGGAAGCAGGCTCTG, Reverse: GTTTCTAAGTGTGTCGCAGACTG), *Gapdh* (Forward: GGGTGTGAACCACGAGAAATA, Reverse: CTGTGGTCATGAGCCCTTC), and *Mmp2* (Forward: TAACCTGGATCCCGTCGT, Reverse: TTCAGGTAATAAGCACCCTTGAA). *Il6* transcript was detected using PrimePCR SYBR Green Assay primers (qMmuCED0045760) obtained from Bio-Rad.

Western Blot Analysis: Western blots were performed as described previously (Fatt et al., 2014). Briefly, secondary neurospheres were treated with either DMSO or camptothecin (as described above) and were collected via centrifugation. Samples were lysed in Radioimmunoprecipitation assay buffer (RIPA) supplemented with Aprotinin, Leupeptin, PMSF, Sodium Fluoride, and Sodium Orthovanadate. For each sample, 20mg of protein were loaded and run on a 15% SDS-PAGE gel. Following PAGE, the samples were then transferred to a 0.2-mm nitrocellulose membrane, blocked for 30 min with 5% BSA, 0.1%Tween-20 in TBS, and incubated overnight at 4 ˚C with primary antibody in blocking buffer. After several washes with 0.1%Tween-20 in TBS, membranes were incubated with secondary antibody at room temperature for 1 h. After several more washes, immunolabelling was detected via chemiluminescence.

Mini-Pump Implantation and ICV Injections: Mice were pre-treated with atropine sulfate (0.1mg/kg, ip), anesthetized (chloral hydrate, 400mg/kg, ip) and placed in a stereotaxic frame. Osmotic Minipumps (Model 1007D and Brain Infusion Kit 000851, Alzet Osmotic Pumps, Cupertino CA, USA) were implanted at the following coordinates – AP -0.5, ML -1.1, DV -2.0. The minipump was filled with the GCV (2.5mg/mL) or PBS and subcutaneously inserted for 7 days. All mice were treated with analgesia (ketoprofen, 5mg/kg, sc) following surgery. For ICV injections, mice were anesthetized and placed in a stereotaxic frame. Intracerebroventricular (ICV, coordinates: AP -0.5, ML -1.1, DV -2.5) microinjections of the ABT-263 or vehicle were performed (1ul/side, 0.12ul/minute) via glass micropipettes connected via polyethelene tubing to a microsyringe (Hamilton, Reno, NV). Micropipettes remained in place for 5 minutes after microinjection to ensure drug diffusion.

Quantification: Positive cell numbers in stained sections were quantified either by direct counts on the microscope or by counting cells in images captured using either Axiovision or Zen software (Zeiss, Oberkochen, Germany) on a Zeiss Axioplan 2 microscope with a Hamamatsu (Bridgewater, NJ, USA) Orca-R2 CCD camera, or with Volocity software (Perkin-Elmer, Waltham, MA, USA) on an Olympus (Center Valley, PA, USA) IX81F-3 confocal microscope with a Hamamatsu EM-CCD camera. Similar results were obtained with both approaches. To obtain total relative numbers of stained cells within the dentate gyrus, the entire hippocampus was sectioned, and all positive cells were counted in 10 sections, sampled every tenth section, after staining. The number of positive cells was then multiplied by 10 (to compensate for sampling frequency) to obtain the total relative number of positive cells in the dentate gyrus. To obtain the proportion of double-labeled cells, all single- and double-labeled cells were counted

in 10 sections spanning the entirety of the hippocampus. The SGZ was defined as the two cell layers beneath the granular cell layer of the hippocampus. Analyses of the lateral ventricle were performed in a similar manner to those described above for the hippocampus. For analysis of FISH, Z-stack images were taken from random regions of the SGZ and the number of punctate dots per cell was quantified from at least 200 Sox2-positive cells per animal. For *P15ink4a* mRNA, cells with at least 6 punctate dots were counted as positive while for *Il6* mRNA, cells with at least 2 dots were counted as positive.

Water Maze Training and Test Probes: Mice were trained in the hidden platform version of the water maze. A circular pool (120cm diameter, 50cm height) was filled with water (28^{o}C) to a depth of 40cm. Water was made opaque by the addition of nontoxic paint. A circular escape platform (10cm diameter) was submerged approximately 0.5cm below the surface of the water in the centre of one of the pool quadrants (N, S, E, W). The pool was surrounded by a curtain painted with 5 large, distinct geometric shapes located 1–1.5m from the pool wall. Mice received 3 consecutive block-training trials per day for 6 consecutive days. Each trial began by placing the mouse into the pool, facing the wall, from one of four possible start positions. The order of the release points varied pseudorandomly across days. The trial ended when the mouse reached the hidden escape platform or after 60 seconds had elapsed. If the mouse failed to locate the hidden platform, the experimenter's hand was placed over the platform (to serve as a visual cue) and the mouse was given an additional 15 seconds to find the platform. If the mouse failed to do so, it was gently guided to the platform. The mouse stayed on the platform for 15 seconds after which it was placed on a heated blanket for an additional 15 seconds (total inter-trial interval of approximately 30 seconds). Memory was tested using a probe test 24 hours following the last training session. During the probe test, the escape platform was removed from the water and the mouse was allowed to swim freely for 60 seconds. The mouse's behavior in the pool was recorded by an overhead video camera and tracked using automated software (Watermaze 3.0, Actimetrics). During training, we analyzed escape latency, distance travelled, and swim speed. In the probe test, we quantified spatial memory by measuring amount of time mice spent searching in the target zone (20cm radius, centered on location of platform during training, corresponding to 11% of pool surface) versus average time spent in three other equivalent zones in other areas of pool (Moser et al, 1998).

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, David R. Kaplan (dkaplan@sickkids.ca).

Supplemental References

Cancino, G.I., Yiu, A.P., Fatt, M.P., Dugani, C.B., Flores, E.R., Frankland, P.W., Josselyn, S.A., Miller, F.D., Kaplan, D.R., 2013. p63 regulates adult neural precursor and newly born neuron survival to control hippocampal-dependent behavior. J. Neurosci. 33, 12569–12585.