Supplemental Data

FoxO3 regulates neural stem cell homeostasis

Valérie M. Renault, Victoria A. Rafalski, Alex Morgan, Dervis A. M. Salih, Jamie O. Brett, Ashley E. Webb, Saul A. Villeda, Pramod U. Thekkat, Camille Guillerey, Nicholas C. Denko, Theo D. Palmer, Atul J. Butte, Anne Brunet*

***: to whom correspondence should be addressed.**

anne.brunet@stanford.edu

Supplemental Experimental Procedures

Animals

FoxO3$^{-/-}$ and *FoxO3*$^{lox/lox}$ mice in the FVB/N background were generously provided by Dr. Ron DePinho (Dana Farber Cancer, Boston). *Nestin-Cre* mice in the C57/Bl6 background were purchased from Jackson Laboratories. All care and procedures were in accordance with the Guide for the Care and Use of Laboratory Animals.

Antibodies

Antibodies to β-actin, GAPDH, and karyopherin β were obtained from Novus Biological, Abcam, and Santa Cruz Biotechnology respectively. Antibodies to GFAP were obtained from Dako, antibodies to CNPase and Sox2 (Y-17) were purchased from Santa Cruz, and antibodies to NeuN were purchased from Millipore. The antibody to myc (9E10) was purchased from Calbiochem and the antibody to BrdU was from AbD serotec. Antibodies to Nestin and cleaved caspase 3 were obtained from BD Pharmingen and Cell Signaling Technology respectively. Antibodies to phospho-T32 and to FoxO3 ('NFL' and 'Ct') were described previously (Brunet et al., 1999; Greer et al., 2007). 'NFL' is directed to full length human FoxO3. 'Ct' is directed to amino-acids 497 to 601 of mouse FoxO3.

Immunohistochemistry on mouse brain sections

Mice were anesthetized and perfused transcardially with PBS containing 5 U/ml of Heparin, then by 4% paraformaldehyde (PFA). Brains were fixed in 4% PFA for 4 hours at 4ºC, then in 30% Sucrose/4% PFA overnight at 4ºC, and embedded in Tissue-Tek (Sakura) at -80ºC. Coronal sections (40 µm) were obtained using a microtome (MICROM). The antigens were retrieved by a 30-min incubation in Sodium Citrate 10 mM pH 6.0/Triton 0.05% at 80ºC. Sections were incubated with primary antibodies (FoxO3 'Ct', 1:500; NeuN, 1:600; Sox2, 1:200; BrdU, 1:500) overnight at 4ºC, with secondary antibodies (biotinylated donkey anti-rabbit, 1:400; Texas Red donkey anti-mouse, anti-goat, or anti-rat, 1:400, Jackson ImmunoResearch) overnight at 4ºC, and then with Fluorescein-DFAT streptavidin (1:500, Jackson ImmunoResearch) overnight at 4ºC. Fluorescent images were taken with a Leica confocal with LCS Leica Confocal Software (Cell Sciences Imaging Facility, Stanford University).

Western blotting

NSC protein extracts were obtained by lysing NSC in lysis buffer (Tris HCl pH 8.0 (50 mM), NaCl (100 mM), EGTA (2 mM), NaF (10 mM), β-glycerophosphate (40 mM), Triton-X100 (0.4%), aprotinin (10 µg/ml), phenylmethylsulfonyl fluoride (PMSF, 1 mM)). Tissue protein extracts were obtained by lysing tissues in RIPA buffer (Tris-HCl pH 8.0 (50 mM), NaCl (150 mM), EDTA (1 mM), NP-40 (1%), Na-deoxycholate (0.25%), PMSF (10 mM), Aprotinin (10 µg/ml), NaF (10 mM), β-glycerophosphate (40 mM)). Thirty µg of proteins was resolved on SDS-PAGE

(10%) and transferred onto nitrocellulose membranes. The membranes were incubated with primary antibodies and the primary antibody was visualized using HRP-conjugated anti-mouse or anti-rabbit secondary antibodies and enhanced chemiluminescence (ECL, Amersham).

Luciferase assays

NSC at early passages were seeded at 10^5 cells/ml in poly-D-lysine-coated 24-well plates. The next day, NSC were transfected using Lipofectamine (Invitrogen) with 200 ng of a luciferase reporter construct driven by three tandem repeats of the FoxO binding element contained in the FasL promoter (FHRE pGL3) (Brunet et al., 1999), 200 ng of a *Renilla* luciferase reporter construct (pRL0), and for the positive controls, 200 ng of FoxO3 constructs in pECE plasmids (Brunet et al., 1999). Forty-eight hours after transfection, cells were lysed and luciferase and renilla luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

BrdU and cleaved caspase 3 immunocytochemistry

Freshly dissociated NSC were plated onto acid-treated glass coverslips (Bellco) coated with poly-D-lysine (Sigma, 50 µg/ml) at a density of 5 x 10^4 cells/ml in 24-well dishes. Twenty-four hours later, BrdU (10 µM) was added for 1 hr and the cells were fixed in 4% PFA and permeabilized in 0.4%Triton for 30 min. Coverslips were incubated with 2N HCl for 30 min, and washed extensively with PBS. Coverslips were incubated with primary antibodies (BrdU, 1:500) and with

secondary antibodies (Alexa 488 goat anti-rat, 1:400, Molecular Probes). For quantification, at least 1,000 cells per coverslip on three different coverslips from two independent experiments were counted in a blinded manner.

For cleaved caspase 3 staining, twenty-four hours after plating, cells were fixed in 4% PFA and permeabilized with 0.1% Triton X-100 for 30 min. Coverslips were incubated with antibodies to cleaved caspase 3 (1:400) and with the Alexa 555 goat anti-rabbit antibodies (1:400, Molecular Probes). Cleaved caspase 3-positive nuclei were quantified in a blinded manner from a total of more than 1,000 nuclei on three coverslips.

References for GSEA analysis (Figure 7C)

(1): (Mense et al., 2006);

(2): (Harris, 2002);

(3): (Kim et al., 2003);

(4): (Leonard et al., 2003);

(5): (Semenza, 2001);

(6): http://www.broad.mit.edu/gsea/msigdb/cards/V$HIF1_Q3.html;

(7): (Lu et al., 2004); (8): (Carter et al., 2005);

(9): http://www.broad.mit.edu/gsea/msigdb/cards/V$FOXO4_01.html;

(10): (Chang et al., 2004);

(11): (Kanehisa et al., 2008);

(12): http://wikipathways.org/index.php/Pathway:WP534.

Chromatin Immunoprecipitation

NSC from 3 month-old mice were plated at a density of 1 x 10^5 cells/ml. Twenty four hours later, dissociated NSC were switched to medium without EGF and bFGF for 4 hours and treated with 20 μM LY294002 (Calbiochem) for 1 hour to activate endogenous FoxO3 (Brunet et al., 1999). Cells were crosslinked with 1% PFA for 10 min. Crosslinking was stopped with 0.125 M glycine for 5 min. NSC were resuspended in swelling buffer (HEPES pH 7.8 (10 mM), $MgCl_2$ (1.5 mM), KCl (10 mM), NP-40 (0.1%), DTT (1 mM), PMSF (0.5 mM)), and incubated on ice for 15 min, and then dounced 25 times. Nuclei were pelleted and resuspended in RIPA buffer (10% NP-40, 10% sodium deoxycholate, 10% SDS in PBS, with protease inhibitor tablet [Roche]). Chromatin was sheared by sonication with a Vibra-Cell Sonicator VC130 (Sonics) six times for 30 s at 60% amplitude. Chromatin was cleared by centrifugation at 14,000 rpm for 15 min at 4°C. Immunoprecipitation was done as described (Mortazavi et al., 2006). Briefly, 5 μg of IgG antibody (Zymed) or 2.5 μg each of anti-FoxO3 'NFL' and H-144 (Santa Cruz Biotechnology) was coupled to sheep anti-rabbit IgG Dynabeads (Invitrogen). Chromatin from 5-8 x10^6 NSC was incubated with antibody-coupled beads. Beads were washed with solutions from Upstate Biotechnology (1 x low salt buffer, 2 x high salt buffer, 3 x LiCl Buffer, 2 x TE) and chromatin was eluted (NaHCO3 (0.1M), SDS (1%)) at 65°C for 1 hr. Crosslinks were reversed by an overnight incubation at 65°C. DNA was purified and concentrated with the PCR purification kit (Qiagen). For quantification of the ChIP products, real-time PCR

was performed using a BioRad CFX96 Real Time System and C1000 Thermal Cycler. The following primers were used with the following conditions: 0.5x SYBR Green (BioRad), 0.5 mM forward primer, 0.5 mM reverse primer, 2.5 μl DNA using the following program: 94°C for 3 minutes, then 40 cycles of 95°C for 20 seconds, 57°C for 30 seconds, 72°C for 30 seconds:

p27^{KIP1} (forward): 5' TTTTTACGCATCGCTGCTACT 3',

p27^{KIP1} (reverse): 5' GCTTAGCCGACCTCACTACG 3',

Ddit4 (forward): 5' CTTTCAGCAGCTGCCAAGGTC 3',

Ddit4 (reverse): 5' CAGAAGCTAGGGGTACCTTTCTC 3',

negative control (forward) 5' GGGGGATAATGATTGCAAAA 3'

negative control (reverse): 5' GCGTGGACAGAGATGTAGGC 3'.

Standard curves were calculated for each primer set using a serial dilution of the input, and were in turn used to determine fold enrichment relative to the IgG control ChIP.

FoxO3 antibody specificity

HEK293T cells were transfected with empty vector or with myc-tagged mouse FoxO family members (FoxO1, FoxO3, FoxO4 and FoxO6 in pcDNA4.1). Briefly, 2.5 μg of DNA was transfected in each well of a 12-well plate using the calcium phosphate method. Fourty eight hours after plating, cells were fixed in 4% PFA and permeabilized with 0.1% Triton X-100 for 30 min. Coverslips were incubated with antibodies to FoxO3 ('Ct'; 1:500) or to myc (1:500) for 2 hours and washed with PBS. Cells were then incubated for 1 hr with Texas Red goat anti-rabbit

antibodies or FITC goat anti-mouse antibodies (1:400, Molecular Probes). Coverslips were mounted in Vectashield containing DAPI. Pictures were taken randomly using a fluorescent microscope and AxioVision 4 software (Zeiss).

Multipotency assay

After 7 days in a neurosphere assay (low cell plating density, 1 to 4,000 cells/ml), secondary or tertiary neurospheres were collected and transferred onto acid-treated glass coverslips coated with poly-D-lysine (50 µg/ml) in 24-well plate. Neurosphere differentiation was induced by incubation in differentiation medium (NeuroBasal-A medium supplemented with penicillin-streptomycin-glutamine, B27 supplement (2%) and 1% Fetal Bovine Serum (FBS)) for 7 days. Differentiation medium was changed every other day for a week. To simultaneously determine the ability of neurospheres to differentiate into astrocytes, neurons and oligodendrocytes, unfixed differentiated neurospheres were incubated with the antibody to O4 (1:1 in 10% goat serum/1% BSA/0.1M L-lysine) for 2 hrs at room temperature before fixation using 4% PFA for 15 min. Neurospheres were the incubated with secondary antibodies (biotinylated donkey anti-mouse, 1:400, Jackson Immunologicals) for 1 hr at room temperature, followed by an incubation with the ABC system (Vector Laboratories) for 30 min at room temperature, and the reaction with the DAB solution (Sigma, 0.05%) containing 0.015% H_2O_2 for 10 min. Differentiated neurospheres were next stained with antibodies to Tuj1 (1:1,000) and GFAP (guinea pig, 1:1,000). Secondary antibodies (Alexa 488 goat anti-rabbit and goat anti-guinea pig,

Molecular Probes) were used at a dilution of 1:400. The coverslips were mounted on slides using Vectashield containing DAPI (Vector laboratories) and examined under epifluorescent illumination using a Zeiss microscope digital camera with AxioVision 4 software. For quantification, 20 to 50 whole neurospheres were counted in a blinded manner.

Reverse-transcription followed by quantitative PCR

The expression of mouse transcripts was determined by reverse transcription of total RNA followed by quantitative PCR analysis (RT-qPCR). After a DNAse treatment, 2 μg of total RNA was reverse transcribed (High Capacity Reverse Transcription kit, Applied Biosystems) according to the manufacturer's protocol. Real-time PCR was performed on a Bio-rad iCycler using iQ SYBR Green mix (BioRad) with the following primers:

Cyclin G2: 5' TCTTGGCCCTTATGAAGGTGA 3' (forward) and
5' CATTTACACTGACTGATGCGGAT 3' (reverse),

Ero1l: 5' TCAAACCCTGCCATTCTGATG 3' (forward) and
5' TCAGCTTGCTCACATTCTTCAA 3' (reverse),

Mbp: 5' GGGCATCCTTGACTCCATCG 3' (forward) and
5' GCTCTGCTTTAGCCAGGGT 3' (reverse),

Myelin (plp1): 5' TGAGCGCAACGGTAACAGG 3' (forward) and
5' GGGAGAACACCATACATTCTGG 3' (reverse),

Otx2: 5' TATCTAAAGCAACCGCCTTACG 3' (forward) and
5' AAGTCCATACCCGAAGTGGTC 3' (reverse),

p27^{KIP1}: 5’ CAGAGTTTGCCTGAGACCCAA 3’ (forward) and

5’ GCAGGAGAGCCAGGATGTCA 3’ (reverse),

Pdk1: 5” GGACTTCGGGTCAGTGAATGC 3’ (forward) and

5’ TCCTGAGAAGATTGTCGGGGA 3’ (reverse),

Selenbp1: 5’ ATGGCTACAAAATGCACAAAGTG 3’ (forward) and

5’ CCTGTGTTCCGGTAAATGCAG 3’ (reverse),

Slc2a3: 5’ ATGGGGACAACGAAGGTGAC 3’ (forward) and

5’ GTCTCAGGTGCATTGATGACTC 3’ (reverse),

Vegfa: 5’ GTACCCCGACGAGATAGAGT 3’ (forward) and

5’ ATGATCTGCATGGTGATGTTG 3’ (reverse),

Xlr3a: 5’ TTGATGCTGGTAGGGAGGACA 3’ (forward) and

5’ AGAACTTTGTTAGGTGGCTCTTC 3’ (reverse),

β-actin: 5’ TGTTACCAACTGGGACGACA 3’ (forward) and

5’ CTCTCAGCTGTGGTGGTGAA 3’ (reverse).

The experiments were conducted in triplicate and the results were expressed as $2^{-(\text{Gene of interest Ct-}\beta\text{-actin Ct})}$. Control PCR reactions were also performed on total RNA that had not been reverse-transcribed to test for the presence of genomic DNA in the RNA preparation.

Histopathology

Histopathology was performed on 3 month-old *FoxO3*$^{-/-}$ and *FoxO3*$^{+/+}$ mice using the Luxol Fast Blue staining method. Briefly, 30 µm coronal brain sections were mounted on slides and were dehydrated and immersed in Luxol Fast Blue

solution (Sigma) overnight at 60ºC. Slides were rinsed in 95% ethanol for 5 min, in distilled water for 5 min, and then in 0.05% lithium carbonate, in 70% ethanol, and in distilled water. Slides were dehydrated and mounted using Permount (Fisher Scientific). The corpus callosa of *FoxO3*$^{-/-}$ and *FoxO3*$^{+/+}$ brains were measured at Bregma -1.34 mm by light microscopy and normalized by the brain section area.

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assays were performed as described (Greer et al., 2007). Briefly, the forward and reverse oligonucleotides surrounding FoxO canonical binding sites in the promoters of specific genes were annealed and labeled with polynucleotide kinase in the presence of [γ-^{32}P] ATP for 30 min. The following oligonucleotides containing FoxO canonical binding sites (underlined) were used:

FoxO3_01 (*Otx2*): 5' CGGACAGTGTTA<u>AATAAACAAGGG</u>TCTCTTTAAAAT 3' (forward) and 5' ATTTTAAAGAGA<u>CCCTTGTTTATT</u>TAACACTGTCCG 3' (reverse),

FoxO4_01 (*Ddit4*): 5' GGATCAAGGAAA<u>GACTTGTTTATTAT</u>AGGGGCGCG 3' (forward) and 5' CGCGCCCCT<u>ATAATAAACAAGTC</u>TTTCCTTGATCC 3' (reverse),

FoxO3_01 (*Ndrg1*): 5' GTAGCCTGGCAAGGTTTGTTTATGTCCGTGCGTGCGT AGGGC 3' (forward) and 5' GCCCTACGCACGCACGGACATAAACAAACCTTG CCAGGCTAC 3' (reverse)

The double-stranded probes were purified from a 15% native gel. 10,000 cpm of labeled probes were incubated for 20 min at room temperature with 100–500 ng of recombinant GST-FoxO3 protein and 6 μg of salmon sperm DNA in electrophoretic mobility shift assay binding buffer (Tris-HCl, pH 7.5 (50 mM), KCl (250 mM), dithiothreitol (5 mM), $MgCl_2$ (25 mM), glycerol (50%), and Nonidet P-40 (0.25%)). The reactions were then resolved by electrophoresis on 4% native acrylamide gels. Gels were dried and autoradiographed.

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Supplemental Table Legend

Table S1: List of genes down-regulated and up-regulated in *FoxO3*$^{+/+}$ and *FoxO3*$^{-/-}$ NSC

Subset of genes that were differentially expressed between *FoxO3*$^{+/+}$ and *FoxO3*$^{-/-}$ NSC with a q-value (false discovery rate) less than 10% as reported by SAM and ranked by fold change in expression. A q-value of 0.000 indicates that this q-value is $<10^{-6}$. The correspondence with a selection of molecular signatures (MSigDB), or with a signature derived from genes that contain the FoxO motif RWAAACAANNN (V$FOXO4-01), or with a signature derived from genes that contain the HIF1 motif GNNKACGTGCGGNN (V$HIF1_Q3) from GSEA, is indicated by the presence of a 1. NA: not attributed.

Supplemental Figure Legends

Figure S1. Specificity of the antibodies to FoxO3

(A) Specificity of the 'Ct' antibody to FoxO3 by immunocytochemistry. HEK293T cells expressing myc-tagged forms of mouse FoxO1, FoxO3, FoxO4, and FoxO6 were immunostained with antibodies to FoxO3 'Ct' and to the myc epitope. Cell nuclei were stained with DAPI. **(B-C)** Specificity of the 'NFL' and 'Ct' antibodies to FoxO3 by Western blotting. **(B)** Western blotting of protein extracts of 293T cells expressing myc-tagged forms of mouse FoxO1, FoxO3, FoxO4, and FoxO6 using antibodies to FoxO3 ('NFL' or 'Ct') and to the myc epitope. Empty arrowhead: Overexpressed myc-FoxO3. Filled arrowhead: endogenous FoxO3. **(C)** Western blots of protein lysates from secondary neurospheres in self-renewal

conditions from 3 month-old *FoxO3*$^{+/+}$ and *FoxO3*$^{-/-}$ littermates probed with antibodies to FoxO3 ('Ct') and to β-actin as a loading control. Molecular weights are indicated in kDa.

Figure S2. FoxO3 is expressed in NSC niches *in vivo*

(A) FoxO3 expression in NSC niches *in vivo*. Immunohistochemistry of FoxO3 in the DG and the SVZ of adult *FoxO3*$^{+/+}$ and *FoxO3*$^{-/-}$ brains, showing the specificity of the FoxO3 antibody ('Ct'). Note that there is remaining staining in *FoxO3*$^{-/-}$ brain sections that is likely background staining. Scale bar: 50 µm. (B) FoxO3 expression in adult brain regions. Western blots of lysates from the SVZ, DG, and cortex isolated from adult wild-type brain probed with antibodies to FoxO3 ('NFL'), to phospho-Threonine 32 (FoxO3 T32-P: filled arrowhead), and to GAPDH as a loading control. The T32-P antibodies recognize a band of lower molecular weight in these brain tissues, which likely corresponds to another FoxO family member (empty arrowhead), as this phosphorylation site and the surrounding amino-acids are conserved in other FoxO family members and as this band is still seen in *FoxO3*$^{-/-}$ extracts (data not shown). Note that in protein extracts from dissected brain regions, it is not possible to distinguish NSC from progenitors and differentiating progeny. (C) FoxO3 is expressed in BrdU-positive label-retaining NSC *in vivo*. Immunohistochemistry with antibodies to FoxO3 ('Ct'), to NeuN, and to BrdU in the DG and SVZ of 3 month-old mice one month after 7 days of daily BrdU injections. BrdU-positive/NeuN-negative/FoxO3-positive nuclei in the SGZ and in the SVZ are shown by white arrowheads. Scale

bar: 100 µm.

Figure S3. FoxO3 is expressed in NSC *in vitro*

(A) FoxO3 is expressed in NSC in culture. Immunocytochemistry on NSC isolated from 3 month-old *FoxO3*$^{+/+}$ and *FoxO3*$^{-/-}$ littermate neonate mice with antibodies to FoxO3 ('NFL') and to Nestin (NSC/progenitor marker). NSC were either grown as neurospheres (whole NS) (top panels) or freshly dissociated NSC (Diss. NSC) (bottom panels). Note that there is remaining staining in *FoxO3*$^{-/-}$ NSC that is likely background staining. Similar results were obtained with adult NSC. Scale bars: 200 µm. (B) Differentiation marker expression during NSC differentiation. Western blots of protein lysates of dissociated wild-type NSC in self-renewal conditions (day 0) or in differentiation conditions for increasing lengths of time (day 1-6). Western blots were probed with antibodies to Tuj1 (neurons), GFAP (astrocytes), CNPase (oligodendrocytes), and β-actin as a loading control, to verify that NSC differentiated under these conditions.

Figure S4. Quantification of label-retaining NSC in *FoxO3*$^{+/+}$ and *FoxO3*$^{-/-}$ brains *in vivo*

(A) Label-retaining NSC in the SVZ of *FoxO3*$^{+/+}$ and *FoxO3*$^{-/-}$ mice. Coronal sections of adult *FoxO3*$^{+/+}$ and *FoxO3*$^{-/-}$ mouse brains showing BrdU-positive nuclei in the SVZ one month after 7 days of daily BrdU injections. Label-retaining NSC are indicated by a filled arrowhead. St: striatum, Sp: septum, CC: corpus callosum. Scale bar: 200 µm. (B) Label-retaining cells are NeuN-negative in the

SGZ and the SVZ. Immunohistochemistry of BrdU and NeuN (neuronal marker) in the SVZ and the DG one month after 7 days of daily BrdU injection. BrdU-positive nuclei were negatively labeled for NeuN in the SGZ (arrowhead) and in the SVZ, while BrdU-positive nuclei were positively labeled for NeuN in the granular cell layer (GCL, arrow) and in the olfactory bulb (OB). Scale bar: 100 μm. (C) Volume of the granule cell layer in 6 month-old adult *FoxO3*$^{+/+}$ and *FoxO3*$^{-/-}$ littermates 4 weeks after 7 daily BrdU injections. Quantification of the granular cell layer (GCL) area of the DG in 10-12 coronal sections using Metamorph. Values represent mean ± SD of 5 animals for *FoxO3*$^{+/+}$ and 4 animals for *FoxO3*$^{-/-}$ mice. Mann-Whitney test, p=0.11. (D) Volume of the granule cell layer in 5 month-old adult *FoxO3*$^{+/+}$ and *FoxO3*$^{-/-}$ littermates one day after 7 daily BrdU injections. Quantification of the granular cell layer (GCL) area of the DG in 10-12 coronal sections using Metamorph. Values represent mean ± SD of 3 animals for *FoxO3*$^{+/+}$ and 3 animals for *FoxO3*$^{-/-}$ mice. Mann-Whitney test, **: $p<0.01$.

Figure S5. Quantification of the neurosphere-forming ability of *FoxO3*$^{+/+}$ and *FoxO3*$^{-/-}$ NSC

(A-B) *FoxO3*$^{-/-}$ NSC from middle-aged adult mice, but not from neonate mice, display a defect in primary neurosphere formation. NSC freshly isolated from *FoxO3*$^{+/+}$ or *FoxO3*$^{-/-}$ littermates at two different ages were seeded in triplicate at a density of 1,000 to 32,000 cells/ml and the number of neurospheres formed after one week was counted. (A) 1 day-old. Values represent mean ± SD of 2

independent experiments conducted with two littermates for each genotype. Two-way ANOVA, p=0.9766 for the genotype variable; (B) 1 year-old. Values represent mean ± SD of 3 independent experiments conducted with 3 to 5 littermates for each genotype. Two-way ANOVA, p<0.0001 for the genotype variable, Bonferroni post-tests, **: p<0.01; ***: p<0.001. (C-E) Adult *FoxO3*$^{-/-}$ NSC display a defect in secondary neurosphere formation. Dissociated primary neurospheres formed after NSC isolation from *FoxO3*$^{+/+}$ or *FoxO3*$^{-/-}$ littermates at 3 different ages (1 day-old, C; 3 month-old, D; 1 year-old, E), were seeded at 1,000 to 4,000 cells/ml and the number of secondary neurospheres formed after one week was counted. Values represent mean ± SEM of 2 independent experiments with two littermates (C), 4 independent experiments with 5 littermates (D), and 4 independent experiments with 3-5 littermates (E) for each genotype. Two-way ANOVA, p=0.3587 (C), p=0.0028 (D), and p=0.0004 (E) for the genotype variable, Bonferroni post-tests, *: p<0.05. (F) Self-renewal is not affected at later passages in *FoxO3*$^{-/-}$ NSC versus *FoxO3*$^{+/+}$ NSC. NSC from 3 month-old *FoxO3*$^{+/+}$ or *FoxO3*$^{-/-}$ littermates were seeded at a density of 1,000 to 4,000 cells/ml, and the number of neurospheres formed after one week was counted. Values represent mean ± SEM of 2 independent experiments conducted with 5 littermates for each genotype. Two-way ANOVA, p=0.5501 for the genotype variable.

Figure S6. *FoxO3*$^{-/-}$ NSC from adult mice have defects in multipotency compared to *FoxO3*$^{+/+}$ NSC

(A) *FoxO3*$^{+/+}$ and *FoxO3*$^{-/-}$ NSC from 9 month-old mice were grown as secondary or tertiary neurospheres at low density and placed in differentiation medium for 7 days. Neurospheres were stained simultaneously with antibodies to Tuj1 (neurons), GFAP (astrocytes), and O4 (oligodendrocytes). A multipotent neurosphere (Tuj1-positive/GFAP-positive/O4-positive) is shown. (B) Neurospheres that contained all three types of progeny (Tuj1+/GFAP+/O4+), two types of progeny (Tuj1+/GFAP+ or O4+/GFAP+), or just one progeny (GFAP+) were counted in a blinded manner as a percentage of the total number of neurospheres. Values represent mean ± SEM of two independent experiments (secondary and tertiary neurospheres) on 2 littermates for each genotype. Two-way ANOVA, Bonferroni post-tests, ***: $p<0.001$. Note that in Figure 4, the O4-positive neurospheres that were counted correspond to the sum of the Tuj1-positive/GFAP-positive/O4-positive and the GFAP-positive/O4-positive neurospheres.

Figure S7. Validation of the differences in gene expression for selected genes differentially regulated between *FoxO3*$^{-/-}$ and *FoxO3*$^{+/+}$ NSC

RT-qPCR analysis of RNA from *FoxO3*$^{-/-}$ and *FoxO3*$^{+/+}$ secondary neurospheres from 3 month-old mice with primers to specific genes. The results are normalized by β-actin expression. Values represent mean ± SEM of two independent samples conducted in triplicate. These results were also observed in an independent experiment. Student's t-test, *: $p<0.05$.

Figure S8. FoxO3-regulated genes are enriched for genes expressed in cell quiescence, hypoxia, and aging

(A) Gene set enrichment analysis plot for FoxO3-regulated genes and genes expressed in quiescent cells (Chang et al., 2004). (B) List of genes enriched for high levels of expression in quiescent cells and in *FoxO3*$^{+/+}$ NSC compared to *FoxO3*$^{-/-}$ NSC. (C) Gene set enrichment analysis plot for FoxO3-regulated genes and genes expressed in hypoxic astrocytes and HeLa cells (Mense et al., 2006). (D) List of genes enriched for high levels of expression in hypoxic astrocytes and HeLa cells and in *FoxO3*$^{+/+}$ NSC compared to *FoxO3*$^{-/-}$ NSC. (E) Gene set enrichment analysis plot for FoxO3-regulated genes and genes expressed in human aging brains (Lu et al., 2004). (F) List of genes enriched for high levels of expression in human aging brains and in *FoxO3*$^{+/+}$ NSC compared to *FoxO3*$^{-/-}$ NSC.

Figure S9. The corpus callosum area is increased relative to the brain area in adult *FoxO3*$^{-/-}$ mice compared to *FoxO3*$^{+/+}$ littermates

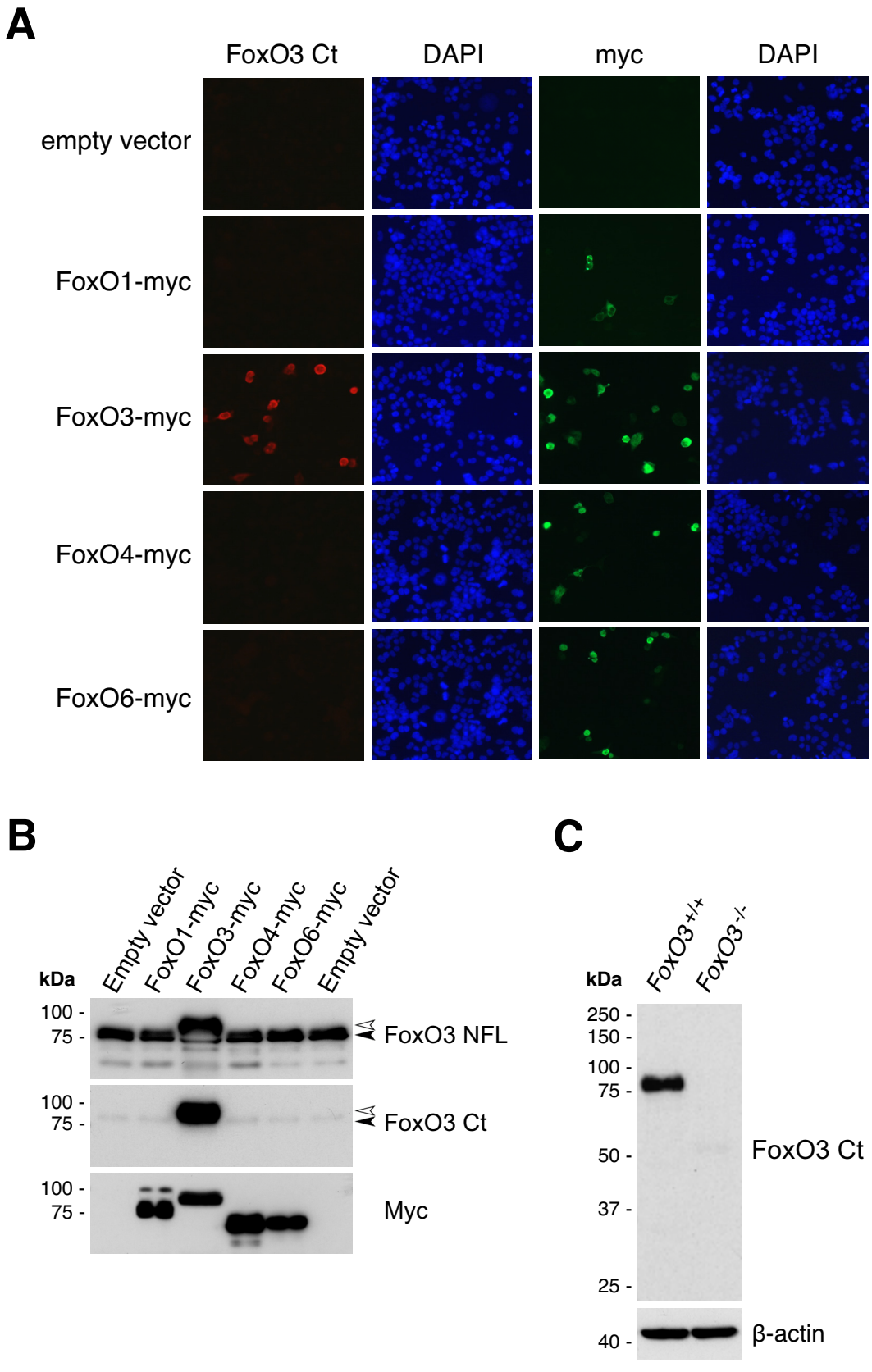
Histopathology of sections of 3 month-old *FoxO3*$^{-/-}$ and *FoxO3*$^{+/+}$ male brains using Luxol Fast Blue staining. Representative sections are shown. Co: cortex; DG: dentate gyrus; CC: corpus callosum; Th: thalamus; Hy: hypothalamus.

Figure S10. FoxO3 binds to FoxO binding sites in the regulatory regions of target genes

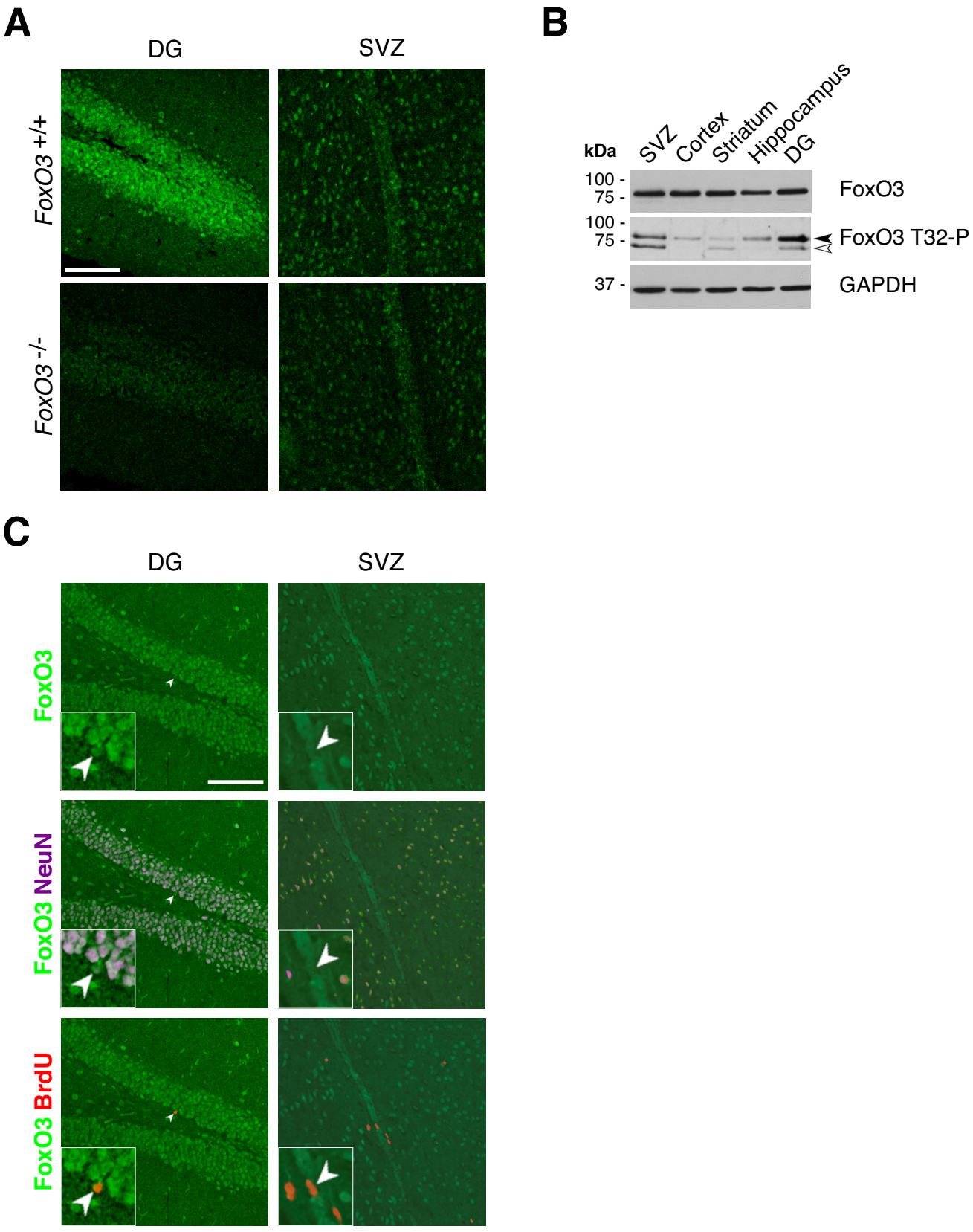
(A) Location of conserved FoxO binding sites in the regulatory regions of *Otx2, Ddit4,* and *Ndrg1* genes. Alignment of human, mouse, and rat sequences are shown. FoxO binding sites are bold and underlined. *: conserved nucleotides in the human, mouse, and rat sequences. (B) FoxO3 binds to FoxO binding sites in the regulatory regions of the genes encoding *Otx2, Ddit4,* and *Ndrg1.* Electrophoretic mobility shift assays (EMSA) using probes corresponding to FoxO binding sites in the regulatory regions of the *Otx2, Ddit4*, and *Ndrg1* genes and a purified form of human FoxO3 (GST-FoxO3). (C) Chromatin Immunoprecipitation (ChIP) of FoxO3 from adult NSC shows significant recruitment of FoxO3 at the promoters of $p27^{KIP1}$ and *Ddit4*. This enrichment of FoxO3 was not found at control regions that did not have FoxO binding sites and did not occur in $FoxO3^{-/-}$ neurospheres. The values presented correspond to the fold enrichment of ChIP with the FoxO3 antibodies over ChIP with control IgG in two independent experiments.

Author contributions

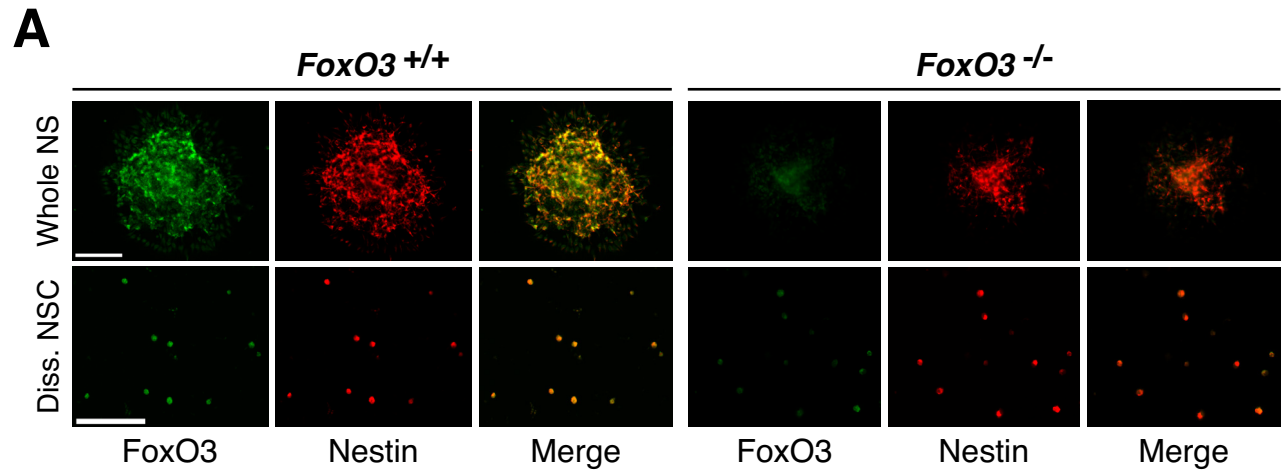
V.M.R. designed, performed and analyzed all experiments, except the ones indicated below, and supervised J.O.B and C.G. V.A.R. designed, performed and analyzed NSC differentiation markers by Western blot and self-renewal assays in low oxygen conditions. A.M. analyzed the microarray data. D.A.S. helped characterize *FoxO3*$^{lox/lox}$;*Nestin-Cre* mice, measure mouse brain weight, and stain corpus callosum. J.O.B. performed and analyzed RT-qPCR and EMSA experiments. A.E.W. designed, performed, and analyzed ChIP experiments. S.A.V. initiated a similar project in rat NSC and provided ideas. P.U.T. helped with Western blotting, immunocytochemistry, and the mouse colony. C.G. performed and analyzed luciferase experiments. N.C.D. helped with low oxygen experiments and provided ideas. T.D.P helped set up NSC cultures and provided ideas. A.J.B. supervised A.M., helped analyze the microarray data and provided ideas. A.B. helped design and analyze the experiments. V.M.R. and A.B. wrote the paper, with the help of V.A.R., J.O.B., A.E.W., and A.M..

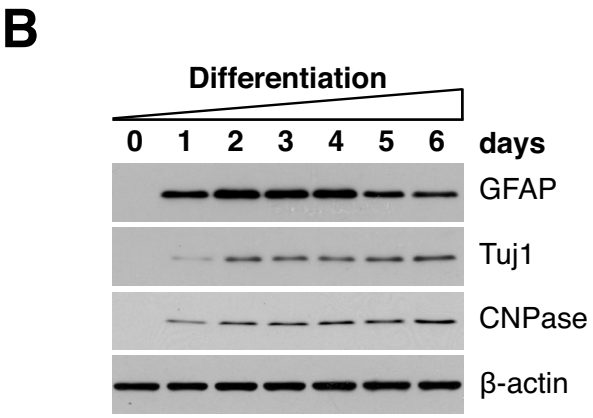


Supplemental Figure 1 - Renault *et al*.

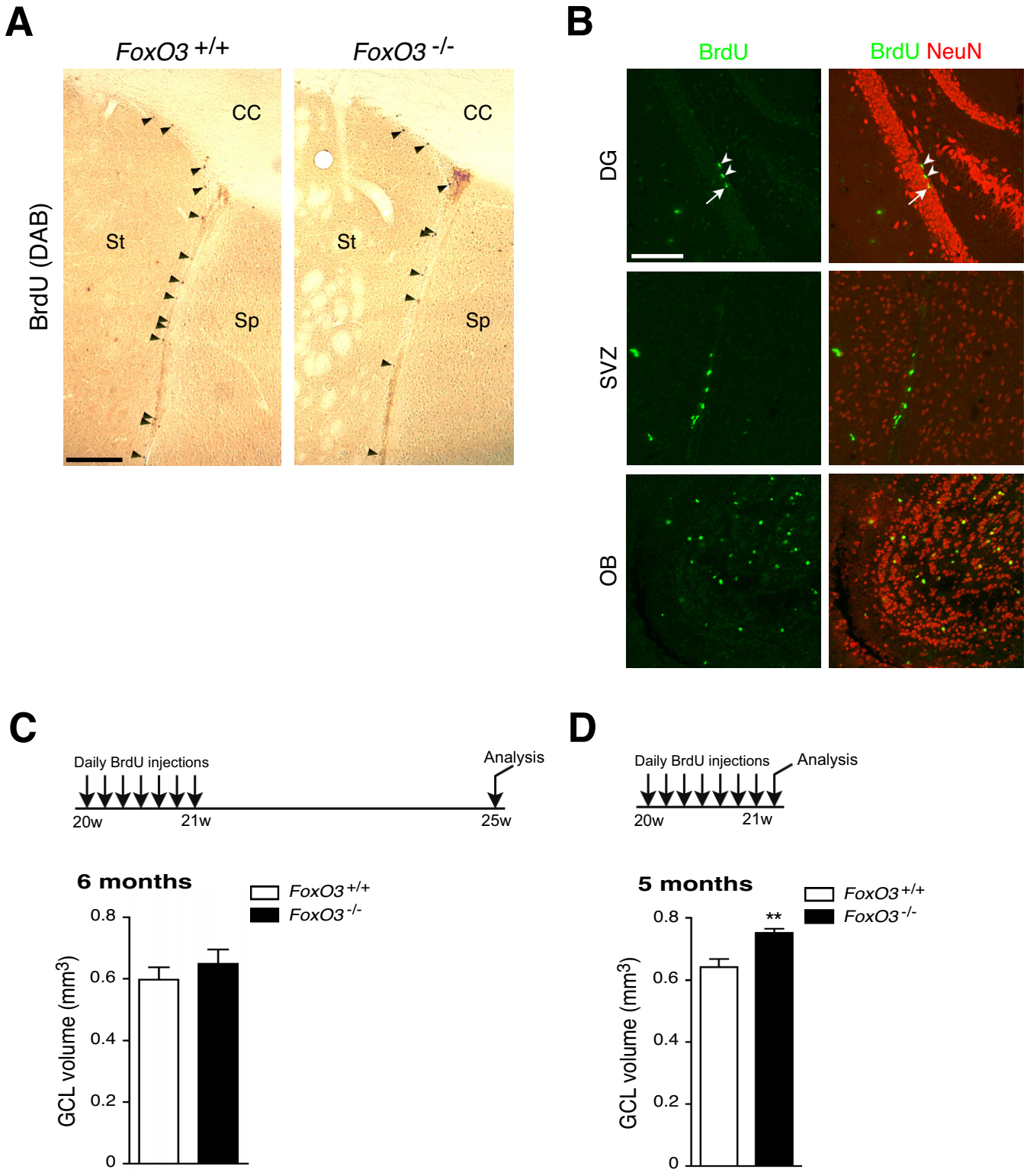


Supplemental Figure 2 - Renault *et al*.

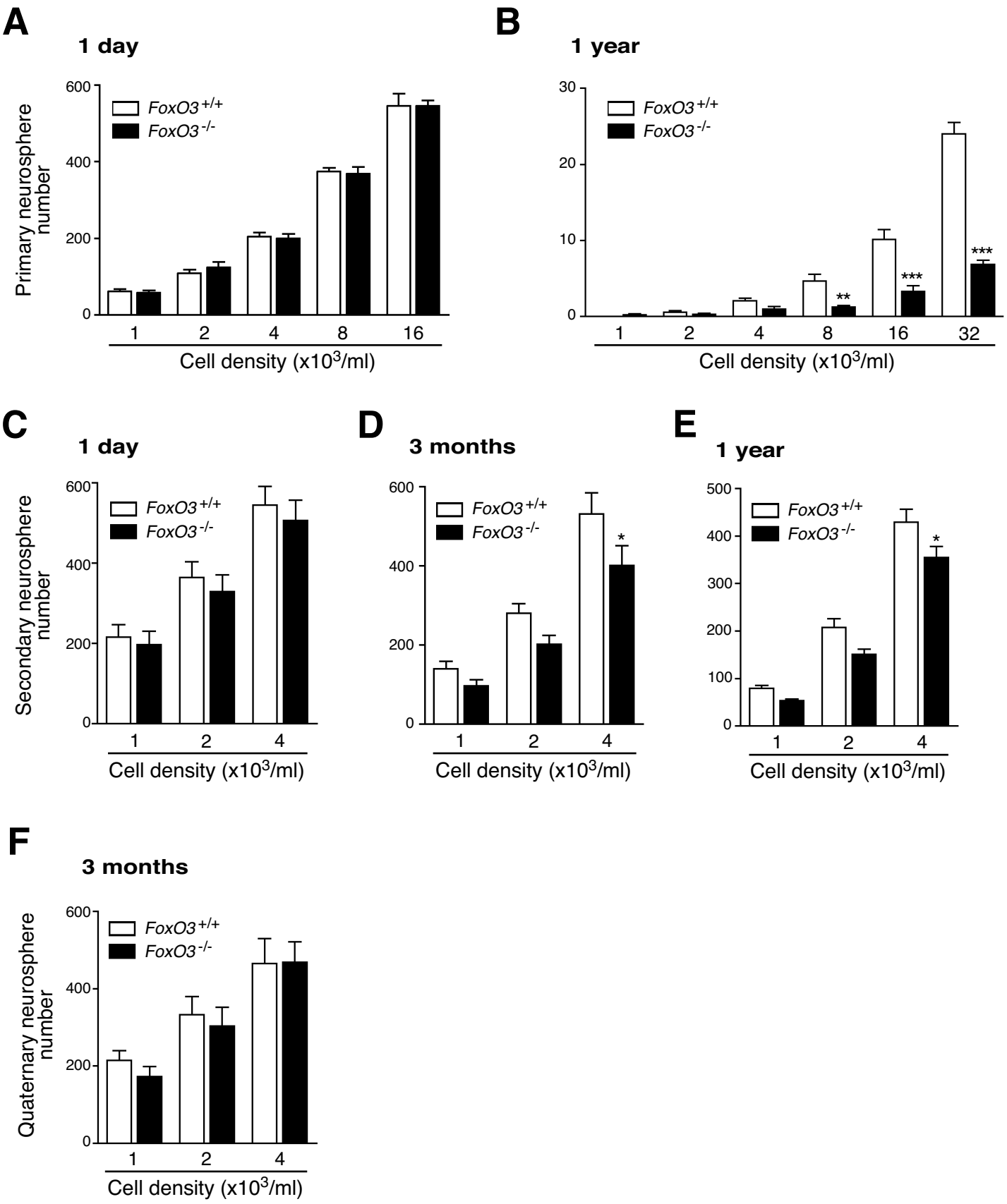




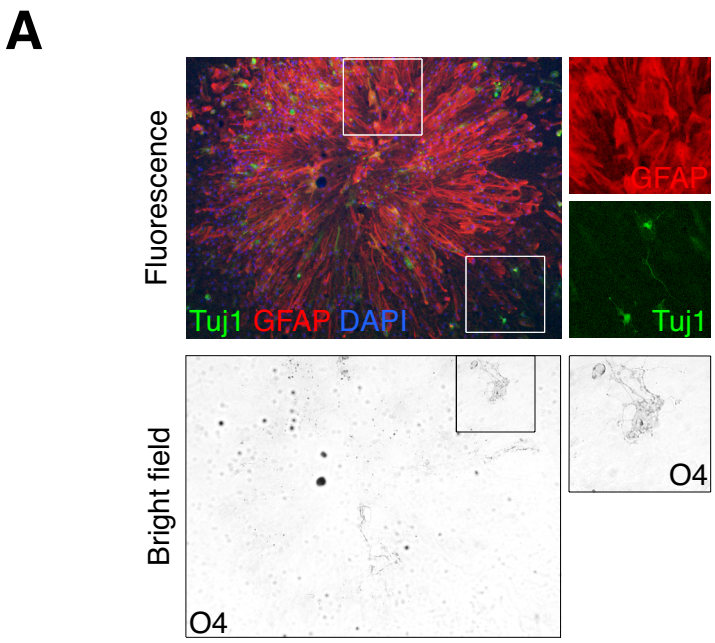
Supplemental Figure 3 - Renault *et al*.

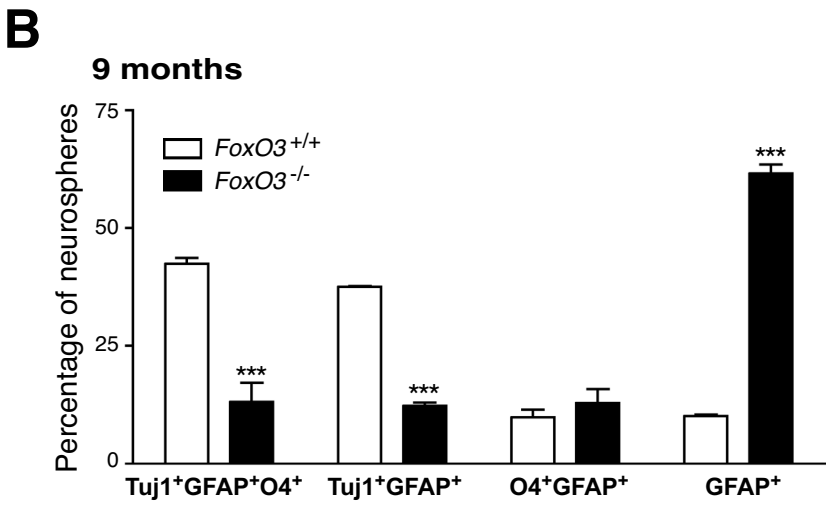


Supplemental Figure 4 - Renault *et al.*

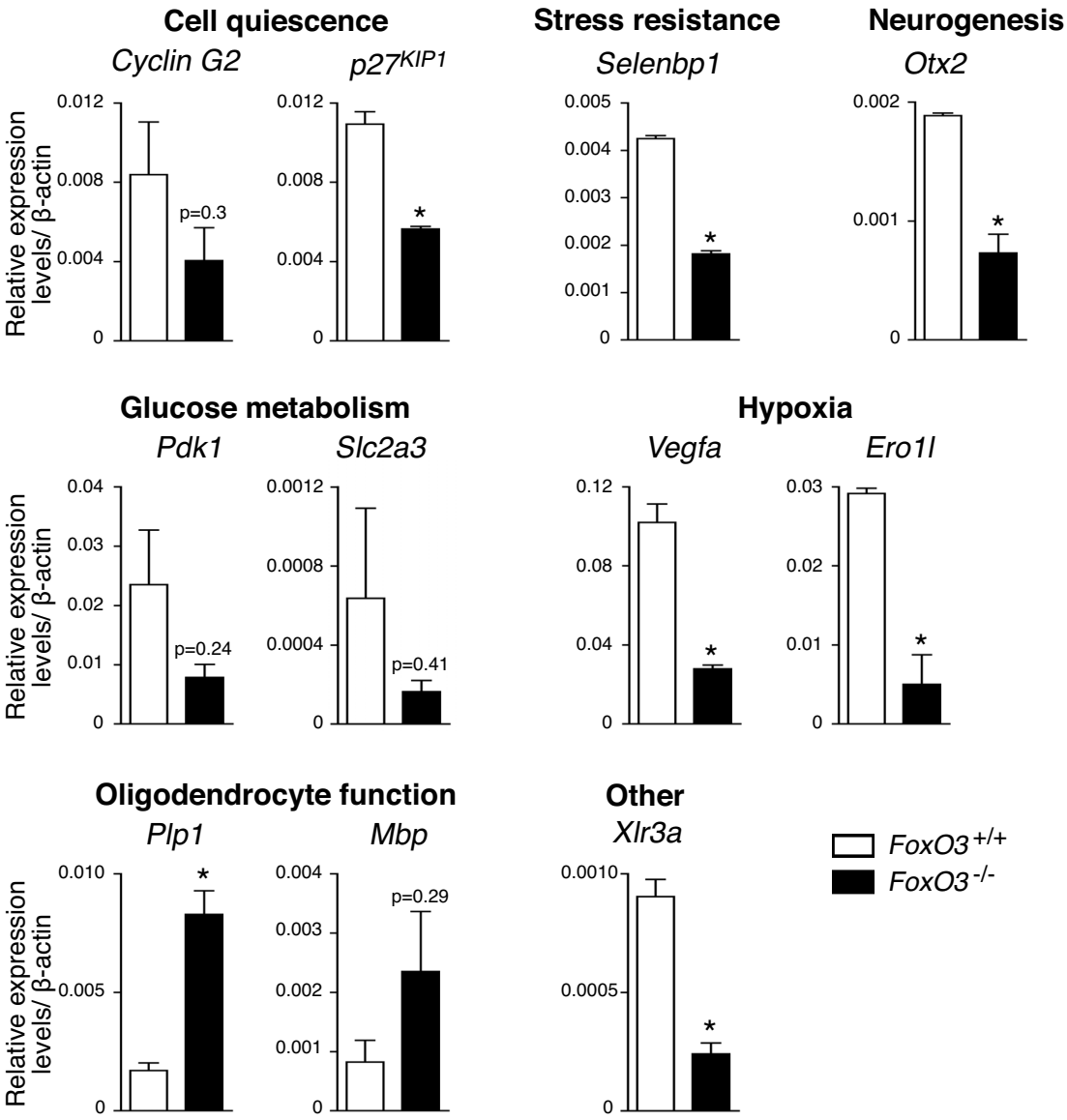


Supplemental Figure 5 - Renault *et al*.



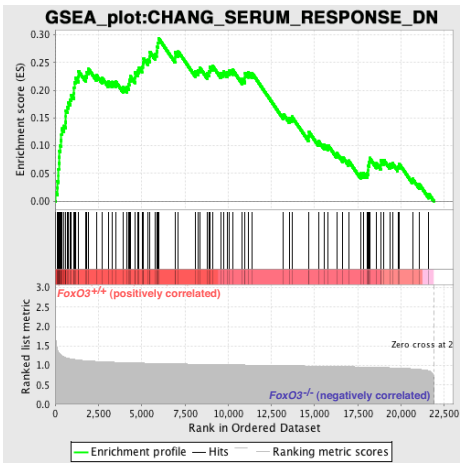


Supplemental Figure 6 - Renault *et al*.



Supplemental Figure 7 - Renault *et al.*

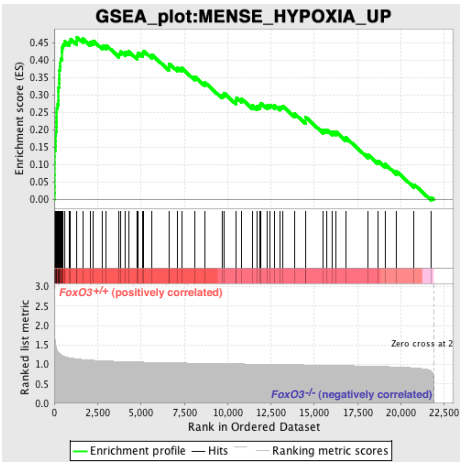
A



B

Gene Title	Symbol
selenium binding protein 1	Selenbp1
Max interacting protein 1	Mxi1
cyclin G2	Ccng2
gelsolin	Gsn
myelin basic protein	Mbp
apolipoprotein D	Apod

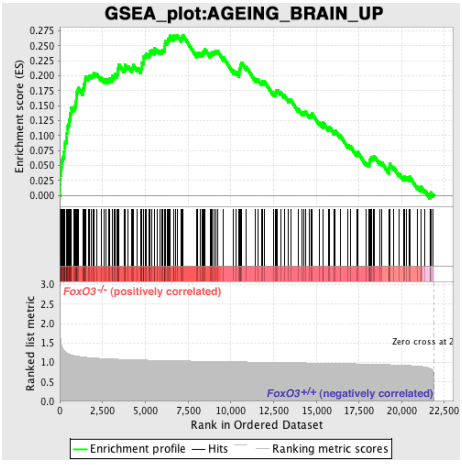
C



D

Gene Title	Symbol
solute carrier family 2 (facilitated glucose transporter), member 3	Slc2a3
N-myc downstream regulated gene 1	Ndrg1
ERO1-like (S. cerevisiae)	Ero1l
enolase 2, gamma neuronal	Eno2
protein phosphatase 1, regulatory (inhibitor) subunit 3C	Ppp1r3c
pyruvate dehydrogenase kinase, isoenzyme 1	Pdk1
ankyrin repeat domain 37	Ankrd37
BCL2/adenovirus E1B interacting protein 3	Bnip3
procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha II polypeptide	P4ha2
glucan (1,4-alpha-), branching enzyme 1	Gbe1
adrenomedullin	Adm
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	Pfkfb3
Max interacting protein 1	Mxi1
thymic stromal lymphopoietin	Tslp
procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha 1 polypeptide	P4ha1
insulin induced gene 2	Insig2

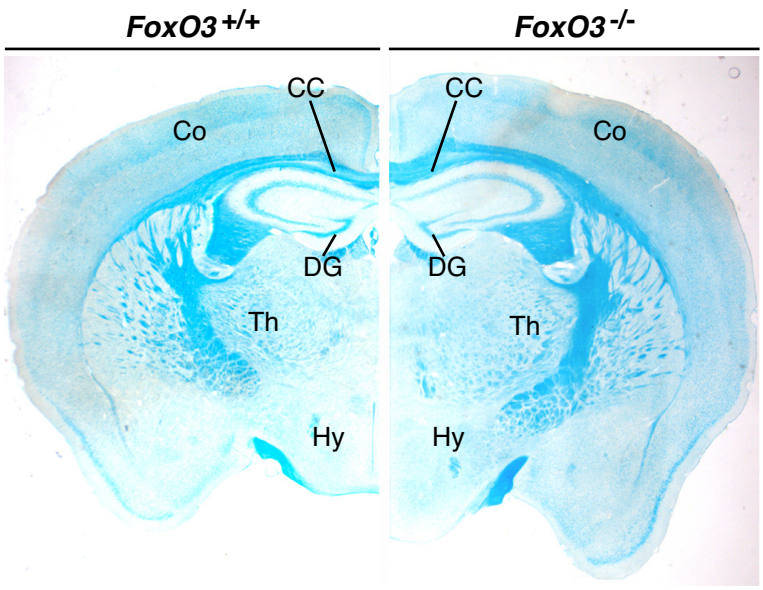
E



F

Gene Title	Symbol
DNA-damage-inducible transcript 4	Ddit4
N-myc downstream regulated gene 1	Ndrg1
PDZ and LIM domain 3	Pdlim3
caveolin, caveolae protein 1	Cav1
WD repeat domain, phosphoinositide interacting 1	Wipi1
regulator of G-protein signaling 20	Rgs20
spondin 1, (f-spondin) extracellular matrix protein	Spon1
caveolin 2	Cav2
angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	Agt
apolipoprotein D	Apod
proteolipid protein (myelin) 1	Plp1
cytochrome P450, family 1, subfamily b, polypeptide 1	Cyp1b1

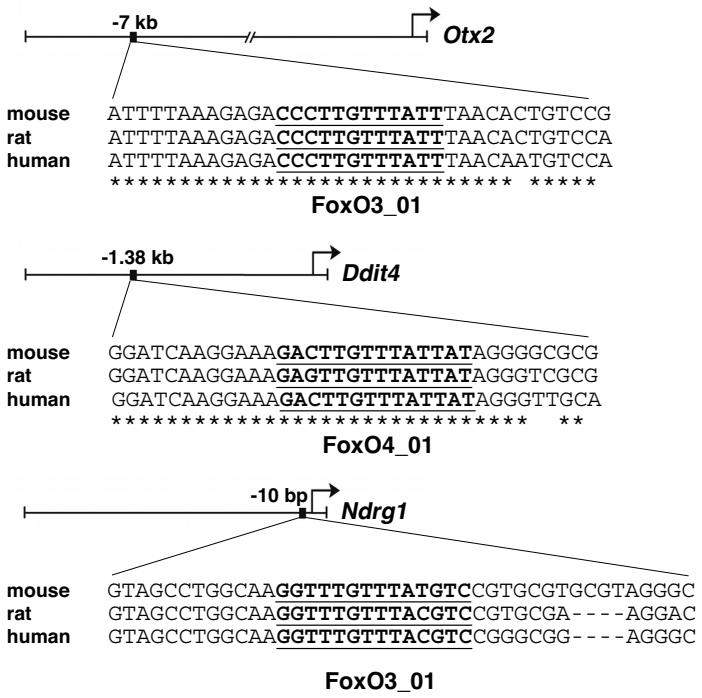
Supplemental Figure 8 - Renault *et al*.



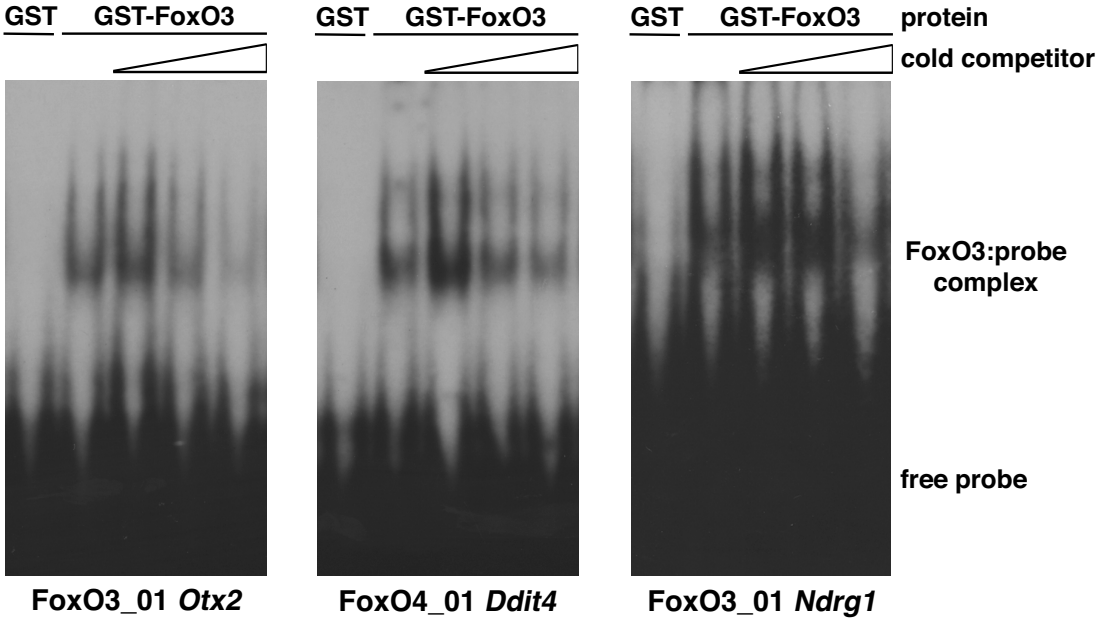
Ratio CC/brain area: 0.0112 0.0162

Supplemental Figure 9 - Renault *et al*.

A



B



C

	Fold enrichement (compared to IgG)			
	Experiment #1		Experiment #2	
Target promoter	*FoxO3*$^{+/+}$	*FoxO3*$^{-/-}$	*FoxO3*$^{+/+}$	*FoxO3*$^{-/-}$
(-)	2.17	2.75	0.79	1.70
p27^{KIP1}	30.67	2.00	5.01	1.09
Ddit4	16.30	2.90	19.11	1.43

Supplemental Figure 10 - Renault *et al*.