## Supplemental Material

# FoxO6 regulates memory consolidation and synaptic function

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## Supplemental Figure Legends

**Supplemental Figure 1.** Generation of *FoxO6* mutant mice.

*(A)* Construct design: the first coding exon of *FoxO6* was substituted with a Neomycin cassette in a BAC construct using standard recombineering techniques in EL250 bacteria. The *FoxO6* targeting vector contained the Ampicillin (Amp) marker for positive selection in EL250 bacteria, a Neomycin (Neo) marker for positive section in ES cells, and the diphtheria toxin A (DTA) marker for negative selection in ES cells. The genomic position of the probe downstream of the first *FoxO6* coding exon used for Southern blotting is illustrated.

*(B)* Southern blot of genomic DNA digested with *Bam*H I and tested with a probe downstream of the first *FoxO6* exon to confirm accurate homologous recombination in F1 offspring. Endogenous *FoxO6* allele migrates at 4.9 kb, and the targeted allele migrates at 6.1 kb. A *FoxO6* wild-type (+/+), heterozygous (+/-) and homozygous (-/-) littermate are shown.

*(C)* Southern blot of genomic DNA digested with *Pac* I and *Bgl* II and tested with a probe upstream of the first *FoxO6* exon to confirm accurate homologous recombination in F1 offspring. Endogenous *FoxO6* allele migrates at 6.4 kb, and the targeted allele migrates at 8.7 kb.

*(D)* Southern blot of genomic DNA digested with *Bam*H I and tested with a probe towards Neomycin to confirm accurate homologous recombination in F1 offspring. The targeted *FoxO6* allele migrates at 6.1 kb.

*(E)* Full-length *FoxO6* mRNA is not expressed in the hippocampus of adult *FoxO6* mutant mice using RT-PCR. Two male *FoxO6* wild-type and mutant siblings at 4-5-months-old were tested. The *FoxO6* RT-PCR product migrates at 274 bp, and the *Hprt* RT-PCR product migrates at 109 bp.

*(F)* Protein levels of other FoxO isoforms (FoxO1, FoxO3, and FoxO4) are not obviously increased in the cortex or hippocampus of *FoxO6* mutant mice. Anterior brain from newborn mice (2-days-old), and cortex and hippocampus from 8-week-old FoxO6 mutant (-/-) and wild-type (+/+) siblings were tested by Western blotting with antibodies to full-length FoxO1, an N-terminal fragment of FoxO3, full-length FoxO4, full-length FoxO6, and GAPDH.

**Supplemental Figure 2.** The growth rate, brain weight and gross hippocampal anatomy of *FoxO6* mutant mice is normal.

*(A)* The bodyweight of *FoxO6* mutant mice is normal from birth to 8-weeks-old. Male *FoxO6* mutant and wild-type siblings were weighed at birth, and then on a weekly basis

from 3 weeks of age. Mean ± SEM. *n* = 5 mice (1-day-old) per genotype, and 20 mice (3-8-week-old) per genotype.

*(B)* The brain weight of *FoxO6* mutant mice is normal at birth and 8-weeks-old. Male *FoxO6* mutant and wild-type sibling brain weights were expressed as a percentage of the bodyweight. Mean ± SEM. *n* = 5 mice (1-day-old) per genotype, and *n* = 8 mice (8-week-old) per genotype.

*(C)* The gross morphology of the *FoxO6* mutant hippocampus appears normal. Cresyl violet staining on coronal brain sections from female 3-4-month-old *FoxO6* null and wild-type siblings. Scale bar represents 500 μm.

**Supplemental Figure 3.** *FoxO6* mutant male mice display normal thigmotaxis, motor coordination and neuromuscular capacity.

*(A) FoxO6* mutant mice display normal thigmotaxis levels compared to wild-type siblings in an open field assay. Results are shown for the time duration the mice remained in the border area of the open field arena (the border area was 50% of the total area of the open field arena). Mean ± SEM. *n* = 19 mice (6-7-month-old males) per genotype.

*(B) FoxO6* mutant mice display normal thigmotaxis levels compared to wild-type siblings in an open field assay. Results are shown for the time duration the mice remained in the middle area of the open field arena (the middle area was 50% of the total area of the open field arena). Mean ± SEM. *n* = 19 mice (6-7-month-old males) per genotype. *P* = not significant for *FoxO6* mutant vs wild-type, unpaired Student's *t*-test.

*(C) FoxO6* mutant mice display normal levels of rearing compared to wild-type siblings in an open field assay. Results are shown for the number of rearing events per mouse

in the 10 min exploration period. Mean ± SEM. *n* = 19 mice (6-7-month-old males) per genotype.

*(D) FoxO6* mutant mice run significantly less than wild-type siblings in an open field arena. Results are shown for the total distance traveled. Mean ± SEM. *n* = 19 mice (6-7-month-old males) per genotype. **P* < 0.05 for *FoxO6* mutant vs wild-type, unpaired Student's *t*-test.

*(E)* Grooming activity is increased for the *FoxO6* mutant mice compared to wild-type siblings in the open field arena. Results are shown for the number of grooming bouts per mouse in the 10 min exploration period. Mean ± SEM. *n* = 19 mice (6-7-month-old males) per genotype. **P* < 0.05 for *FoxO6* mutant vs wild-type, unpaired Student's *t*-test.

*(F)* The performance of *FoxO6* mutant mice on a one trial accelerating RotaRod assay is normal. Results are shown for the time the mice could maintain their balance on the accelerating spindle. Mean ± SEM. *n* = 19-21 mice (6-7-month-old males) per genotype.

*(G)* The inter-limb coordination is normal for *FoxO6* mutant mice using the CatWalk system. Results are shown for the regularity of the paw usage as mice ran from one end of the CatWalk apparatus to the other end. Mean ± SEM. *n* = 12-17 mice (3-4-month-old males) per genotype.

*(H)* A hanging wire test suggests that neuromuscular capacity is not defective for the *FoxO6* mutant mice. Results are shown for the time the mice could hang upside-down from a wire screen. Mean ± SEM. *n* = 19-21 mice (6-7-month-old males) per genotype.

*(I)* The grip strength performance of the *FoxO6* mutant mice is normal. Results are shown for the amount of weight the mice could grip with both of their front paws before

releasing the steel grid connected to a kilogram dynamometer (Grip strength meter). Mean ± SEM. *n* = 16-19 mice (6.5-9-month-old males) per genotype.

**Supplemental Figure 4.** The nociception, hearing, vision and novel object exploration of the *FoxO6* mutant mice are normal.

*(A)* The response to noxious stimuli is normal for the *FoxO6* mutant mice using a hot plate assay. Results are shown for time taken to display the first hind-paw licking or jumping. Mean ± SEM. *n* = 16-19 mice (6.5-9-month-old males) per genotype.

*(B)* The hearing of the *FoxO6* mutant mice is normal using a startle response assay. Results are shown for the peak mouse startle response, assessed with a piezoelectric accelerometer, following the presentation of startle stimuli at 5 different intensities in a pseudorandom sequence. Mean ± SEM. *n* = 18-20 mice (8-9-month-old males) per genotype.

*(C)* The vision of the *FoxO6* mutant mice is not impaired, assessed using a visible platform in a swimming pool. Results are shown for the escape latency to a visible platform. The mice were tested for 4 trials performed in one day. Mean ± SEM. *n* = 18-20 mice (7-8-month-old males) per genotype.

*(D)* The novel object exploration time is normal for *FoxO6* mutant mice. On the learning day (0 hr; Fig. 2E,F), the mice were allowed 10 min to explore two identical but novel objects. Results are shown for total time the mice explored the two objects. Mean ± SEM. *n* = 9-10 mice (2-3-month-old males) per genotype.

**Supplemental Figure 5.** The spatial learning and memory of *FoxO6* mutant mice is not defective in the Morris water maze.

*(A)* Scheme to test spatial learning and memory using the Morris water maze test.

*(B)* The swim velocity of *FoxO6* mutant mice is normal. The swim velocity is shown for trial blocks 1-8. Mean ± SEM. *n* = 18-20 mice (6-7-month-old males) per genotype.

*(C)* The escape latency of *FoxO6* mutant mice is significantly faster during the first 8 trial blocks in the spatial learning phase of the Morris water maze. The hidden platform was in quadrant 2. Mean ± SEM. *n* = 18-20 mice (6-7-month-old males) per genotype. **P* < 0.05 *FoxO6* mutant vs wild-type during trial blocks 1-8, two-way repeated measures ANOVA with genotype and trial as factors, $F_{1,36} = 4.32$.

*(D)* The thigmotaxis of the *FoxO6* mutant mice in the Morris water maze pool is decreased between trial blocks 5-8. The percentage of time spent in the border area of the water maze is shown for trial blocks 1-8. Mean ± SEM. *n* = 18-20 mice (6-7-month-old males) per genotype. **P* < 0.05 *FoxO6* mutant vs wild-type during trial blocks 1-8, two-way repeated measures ANOVA with genotype and trial as factors, $F_{1,36} = 4.63$.

*(E)* Spatial memory at day 5 is not impaired in *FoxO6* mutant mice. Mean ± SEM. *n* = 18-20 mice (6-7 month-old males) per genotype. Both *FoxO6* mutant and wild-type siblings spent significantly more time in the target quadrant (quadrant 2). ****P* < 0.0001, one-way repeated measures ANOVA with quadrant as the factor; wild-type, $F_{3,68} = 13.12$; *FoxO6* mutant, $F_{3,76} = 14.95$. In contrast, there was no significant difference between *FoxO6* mutant and wild-type siblings by two-way repeated measures ANOVA using genotype and quadrant as factors.

*(F)* Long term spatial memory is not impaired in *FoxO6* mutant mice. The platform was removed at 1, 4, 9 and 23 days after the end of reversed platform training (as described in Supplemental Fig. 5A), and the time spent in the target quadrant (quadrant 4) is shown. Mean ± SEM. *n* = 18-20 mice (6-7-month-old males) per genotype.

**Supplemental Figure 6.** Expression of FoxO6ΔCt in the hippocampus of adult mice.
*(A)* Validation of a dominant negative C-terminal deletion of FoxO6 (FoxO6ΔCt-GFP) in vitro. Western blotting was performed with an antibody to full-length FoxO6 using U2OS cell lysates transfected with constructs expressing GFP, wild-type FoxO6-GFP (FoxO6 WT-GFP), or FoxO6ΔCt-GFP.
*(B)* FoxO6 with a deletion of the C-terminal region acts as a dominant negative towards FoxO3 and FoxO4. Luciferase assays in U2OS cells using constructs expressing wild-type FoxO1-His-myc (FoxO1 WT), FoxO3-His-myc (FoxO3 WT), FoxO4-His-myc (FoxO4 WT) alone or with FoxO6ΔCt-M2 (FoxO6ΔCt), and a luciferase reporter driven by three consensus FoxO6 binding sites (p3xFoxO6). Results are normalized to renilla, and EV (empty vector, pcDNA Neo) construct alone. Mean ± SD from 2 independent experiments performed in triplicate.
*(C)* Microinjection of HSV vectors expressing FoxO6ΔCt-GFP or GFP into the CA1 region of the adult hippocampus does not result in expression in the amygdala (basolateral amygdala, BLA). Counterstained with the nuclear marker DAPI. Scale bar represents 1 mm.
*(D)* Disrupting FoxO6 function locally and acutely in the CA1 region of the dorsal hippocampus does not impair contextual fear memory by affecting shock reactivity.

Results are expressed as a motion index in response to one foot-shock during the learning phase. Mean ± SEM. *n* = 7 mice (2-3-month-old) per viral construct.

*(E)* Disrupting FoxO6 function locally and acutely in the CA1 region of the dorsal hippocampus does not impair the expression of a stronger contextual fear memory by affecting shock reactivity. Results are expressed as a motion index in response to each of three foot-shocks during the learning phase. Mean ± SEM. *n* = 7 mice (2-3-month-old) per viral construct.

**Supplemental Figure 7.** *Grp* and *Crym* are differentially expressed in the hippocampus between *FoxO6* mutant and wild-type siblings, and genes regulated by FoxO6 in the hippocampus play a role in synaptogenesis before learning and synaptic function after learning.

*(A) Grp* and *Crym* mRNA levels are decreased in the hippocampus of *FoxO6* mutant mice following object learning. Hippocampal mRNA was purified from *FoxO6* mutant and wild-type siblings after the novel object learning task (Fig. 5A) and RT-qPCR was performed to quantify *Grp* and *Crym* mRNA levels to confirm the microarray results. Normalized against *Gapdh*, *Rps28* and *Rps29* mRNA levels. Mean ± SEM of samples assayed in triplicate. *n* = 6 mice (8-9-week-old males) per genotype. **P* < 0.05, ***P* < 0.01 for *FoxO6* mutant vs wild-type, unpaired Student's *t*-test.

*(B) Grp* mRNA levels are decreased in the hippocampus of basal *FoxO6* mutant mice. Hippocampal mRNA was purified from *FoxO6* mutant and wild-type siblings before the novel object learning task (Fig. 5A) and RT-qPCR was performed to quantify *Grp* mRNA levels to confirm the microarray results. The *Grp* mRNA levels were normalized against

*Gapdh*, *Rps28* and *Rps29* mRNA levels. Mean ± SEM of samples assayed in triplicate. *n* = 8 mice (8-9-week-old males) per genotype. **P* < 0.05 for *FoxO6* mutant vs wild-type, unpaired Student's *t*-test.

*(C) Crym* mRNA levels are decreased in the hippocampus of neonatal *FoxO6* mutant mice. The *Crym* mRNA levels were normalized against *Gapdh* mRNA levels. Mean ± SEM of samples assayed in triplicate. *n* = 6 mice (3 males and 3 females at 1-day-old) per genotype. **P* < 0.05 for *FoxO6* mutant vs wild-type, unpaired Student's *t*-test.

*(D)* Genes regulated by FoxO6 before novel object learning are enriched for genes involved in synapse formation. Enrichment of genes differentially expressed in the *FoxO6* mutant mice under basal (white bars) or learning conditions (black bars) belonging to selected Gene Set Enrichment Analysis (GSEA) gene categories. GSEA category information is presented in Supplemental Table 1. Dashed lines: *P* = 0.05 and *P* = 0.01, Familywise-error rate.

*(E)* Genes differentially expressed in the *FoxO6* mutant mice under basal (white bars) or learning (black bars) were analyzed by PANTHER (*P* < 0.05 for *FoxO6* mutant vs wild-type, one-way ANOVA). PANTHER category information is presented in Supplemental Table 1. Dashed lines: *P* = 0.05, *P* = 0.01 and *P* = 0.001, Binomial statistic.

*(F)* Genes regulated by FoxO6 are enriched for genes involved in glutamate and insulin/IGF/PI3K signaling. Genes differentially expressed in the *FoxO6* mutant mice under basal (white bars) or learning (black bars) were analyzed by PANTHER (*P* < 0.05 for *FoxO6* mutant vs wild-type, one-way ANOVA). PANTHER category information is presented in Supplemental Table 1. Dashed lines: *P* = 0.05, *P* = 0.01 and *P* = 0.001, Binomial statistic.

**Supplemental Figure 8.** FoxO6-regulated genes are enriched for genes involved in pathways of glutamate signaling and Alzheimer's disease.
*(A)* Gene set enrichment analysis plot for FoxO6-regulated genes and genes involved in glutamate signaling (obtained from the HEFalMp database).
*(B)* Gene set enrichment analysis plot for FoxO6-regulated genes and genes involved in Alzheimer's disease (obtained from the HEFalMp database). NES, normalized enrichment score; FWER *P* value, familywise-error rate; FDR q value, false discovery rate.

**Supplemental Figure 9.** FoxO6-regulated genes are enriched for genes involved in the pathway of p53 signaling. Gene set enrichment analysis plot for FoxO6-regulated genes and genes involved in p53 signaling (obtained from the HEFalMp database). NES, normalized enrichment score; FWER *P* value, familywise-error rate; FDR q value, false discovery rate.

**Supplemental Figure 10.** FoxO6-regulated genes were visualized within the glutamate signaling pathway. Pathway diagram adapted from Ingenuity. Green: genes down-regulated in *FoxO6* mutant mice. Red: genes up-regulated in *FoxO6* mutant mice.

**Supplemental Figure 11.** FoxO6-regulated genes were visualized within the Alzheimer's disease pathway. Pathway diagram adapted from Ingenuity. Green: genes down-regulated in *FoxO6* mutant mice. Red: genes up-regulated in *FoxO6* mutant mice.

**Supplemental Figure 12.** FoxO6-regulated genes were visualized within the p53 signaling pathway. Pathway diagram adapted from Ingenuity. Green: genes down-regulated in *FoxO6* mutant mice. Red: genes up-regulated in *FoxO6* mutant mice.

**Supplemental Figure 13.** The dendritic spine width of the *FoxO6* mutant hippocampus is normal, and the length of dendrites and axons is normal in *FoxO6* mutant hippocampal neurons cultured in vitro.

*(A)* Spine width is not altered in the *FoxO6* mutant hippocampal neurons cultured from e18 embryos. Quantification of dendritic spine width in neurons prepared as in Fig. 7A. Mean ± SEM. *n* = 7-8 mice per genotype, 8-10 neurons per mouse and 50 spines per neuron resulting in 2950-3200 spines per genotype.

*(B)* Spine width is normal in adult *FoxO6* mutant hippocampal CA1 pyramidal neurons in vivo compared to wild-type siblings. Quantification of dendritic spine width in neurons visualized as in Fig. 7C. Mean ± SEM. *n* = 4 mice (4-4.5-months-old) per genotype, 8-10 neurons per mouse and 50 spines per neuron resulting in 1600-1650 spines per genotype.

*(C)* Hippocampal neurons were cultured from embryonic day 18 *FoxO6* mutant mice and wild-type siblings, and were transfected with a GFP expression construct and a plasmid expressing the anti-apoptotic gene *Bcl-xl* at DIV1. The hippocampal neurons were subjected to immunocytochemistry at DIV5 with an antibody against GFP. Representative images are shown. Arrows, arrowheads and asterisks indicate dendrities, axons, and the cell body, respectively. Scale bar represents 50 μm.

*(D)* Morphometric analysis of the total length of dendrites (left) or axons (right) per neuron as in prepared in (C). Mean ± SEM. *n* = 3 mice per genotype, 27-63 neurons per mouse.

## Supplemental Table Legend

**Supplemental Table 1** Differential gene expression of the hippocampus of *FoxO6* mutant mice before and after novel object learning using whole genome microarrays. (1) Differential gene expression of the hippocampus of *FoxO6* mutant mice under basal conditions. (2) Differential gene expression of the hippocampus of *FoxO6* mutant mice after object learning. (3) Genes induced or repressed differentially in the hippocampus of wild-type siblings relative to *FoxO6* mutant mice after object learning. (4) Gene set enrichment analysis reveals that molecular signatures for synaptogenesis and synapse organization are regulated by FoxO6 under basal conditions. (5) GO analysis reveals that molecular signatures for synaptic function are regulated by FoxO6 after object learning. (6) PANTHER analysis reveals that molecular signatures for synaptic function, the glutamate and PI3K signaling pathways are regulated by FoxO6 after object learning. (7) Co-occurrence of the FoxO consensus matrix with MEF2, EGR and/or STAT consensus matrices in the promoters of genes down-regulated in the *FoxO6* null hippocampus following novel object learning. (8) DAVID analysis reveals that molecular signatures for the synapse compartment and cell-cell signaling are regulated by genes differentially expressed after object learning in *FoxO6* mutant mice and that contain a FoxO binding site.

## Supplemental Materials and Methods

### Generation of *FoxO6* null mice

BAC constructs (RP24-238C2 and RP24282C13) containing genomic mouse *FoxO6* sequence from the C57BL/6J background were obtained from BACPAC Resources (Children's Hospital and Research Center, Oakland, CA). The following primer sequences (generated by Operon Biotechnologies), contained approximately 50 bp of homology to the genomic sequence of *FoxO6* immediately upstream and downstream of the first exon, and were used to amplify the lox-Neomycin-lox (lox-Neo-lox) cassette from the PL452 vector (Pentao Liu, Wellcome Trust Sanger Institute, UK) by high-fidelity PCR (Pfx):

Forward: 5'

CTTTGCCTCCCTCCGTGCTCGCCAGTTTGTCCAGCCTCCTGCCTCCGCTATAACTT

CGTATAATGTATG 3'

Reverse: 5'

TGCCCCACGCCCTGGGCGCGCACTCCATGACCCCTAGGGGTGTTCCAGAGATAA

CTTCGTATAGCATAC 3'

The lox-Neo-lox cassette flanked by the short *FoxO6* homology arms was used to substitute the first coding exon of *FoxO6* in the BAC constructs in recombination permissive EL250 bacteria. The pBKS-DTA vector contained the diphtheria toxin A (DTA) negative selection marker for use in subsequent ES cell culture. The following primer sequences were used to sub-clone around 300 bp of *FoxO6* genomic sequences located 3-5 kb upstream and downstream of the first *FoxO6* coding exon into the pBKS-

DTA vector with the endonucleases *Eco*R I, *Hind* III and *Kpn* I using high fidelity PCR (Pfx):

*FoxO6* upstream homology arm *Eco*R I:

5’ ACATGAATTCCAACCTGGAGGCCTACTCTGGACAC 3’

*FoxO6* upstream homology arm *Hind* III:

5’ CTTCACTAAGCTTGTTGGCTGCTGTCACCTGCACCCAC 3’

*FoxO6* downstream homology arm *Hind* III:

5’ AGGATTGAAGCTTGCAGAGAGGAGGAACTGTGCCTGCTG 3’

*FoxO6* downstream homology arm *Kpn* I:

5’ TTCGAGGTACCCTGAGCCCAGGCTTGATACTCTCTGTC 3’

The pBKS-DTA vector containing around 300 bp of upstream and downstream *FoxO6* genomic sequence sub-cloned with the *Eco*R I, *Hind* III and *Kpn* I enodnucleases was linearized with *Hind* III. The linear pBKS-DTA vector containing 300 bp flanking sequences of upstream and downstream genomic *FoxO6* sequence was used to transfer the lox-Neo-lox cassette flanked by 3-5 kb genomic *FoxO6* homology arms in the BAC construct to the pBKS-DTA vector in recombination permissive EL250 bacteria. The pBKS-DTA vector containing the lox-Neo-lox cassette flanked by approximately 3-5 kb of genomic sequence from the *FoxO6* locus was linearized with *Pme* I digestion to produce the *FoxO6* targeting vector (Supplemental Fig. 1A). The linear targeting vector was purified using a Zymo DNA clean and concentrator protocol (Zymo Research, Orange, CA). At all stages of generation the *FoxO6* targeting vector was validated by sequencing and endonuclease restriction digestion.

The *FoxO6* targeting vector was electroporated into R1 ES cells (129X1/SvJ x 129S1 hybrid cells) by the Stanford Transgenic Research Center (Dept. of Pathology, Stanford University, CA). ES cells from two independent positive colonies were expanded and microinjected into C57BL/6 blastocysts, which were implanted into pseudopregnant C57BL/6 females. Seven males were born with >80% chimerism from two independent ES clones, of which three chimeric males provided germ-line transmission representing two independently targeted ES cell clones. The F1 offspring were tested by Southern blotting and PCR and then interbred to produce *FoxO6* null mice and wild-type siblings.

**Southern blotting**

Three independent Southern probes were used to verify accurate homologous recombination (Supplemental Fig. 1). The downstream *FoxO6* probe was designed from the genomic DNA region immediately downstream of the homologous recombination site using the following primers in a PCR:

Forward *FoxO6* downstream primer: 5' AGTGAGTTCCATATTCTGGGGAGTC 3'

Reverse FoxO6 downstream primer: 5' CGCTACTCTCCCAACCGCTGTTCAG 3'

The upstream *FoxO6* probe was designed from the genomic DNA region immediately upstream of the homologous recombination site using the following primers in a PCR:

Forward FoxO6 upstream primer: 5' GACAGAGCTGTGAATAGATTGG 3'

Reverse FoxO6 upstream primer: 5' GCTTCTCTGAGTCCTGATGTGTG 3'

The neomycin probe was developed by excising a 571 bp fragment from a PCR product generated using the following primers, and then using the *Nco* I endonuclease.

Forward Neo primer: 5’ GAAGCTGGGCTGGGCGAGGTGTGTG 3’

Reverse Neo primer: 5’ GAATGAATACAGCTGCTGCCAGCATG 3’

The downstream and upstream *FoxO6* probes were cloned into the pCR2.1 vector (Invitrogen) and excised by *Eco*R I endonuclease digestion. Tail genomic DNA was digested with *Bam*H I for the downstream probe, *Pac* I and *Bgl* II for the upstream probe and *Bam*H I for the neomycin probe. Blots were hybridized with random-primed, $^{32}$P labeled probes (Rediprime II random prime labeling system, and Rapid-hyb buffer, Amersham Biosciences, UK).

**Genotyping**

For genotyping *FoxO6* null and wild-type sibling mice, three primers were used in the same PCR reaction:

Forward *FoxO6* genotyping primer 1: 5’ CCAGTTTGTCCAGCCTCCT 3’

Reverse *FoxO6* genotyping primer 1: 5’ CAGAGGCCAGGTACACGAG 3’

Reverse *FoxO6* genotyping primer 2: 5’ CTAAAGCGCATGCTCCAGAC 3’

The PCR reaction mixture contained 1x *Taq* reaction buffer, 0.6 mM dNTPs, 1.0 μM of each primer, 10% DMSO and 2.5 units *Taq* DNA polymerase enzyme in a 25 μl total reaction volume with an annealing temperature of 58ºC and 32 cycles of amplification [94°C for 30 sec, annealing at 58°C for 60 sec and extension at 72°C for 90 sec]. The *FoxO6* wild-type allele produced a band of 257 bp, and the *FoxO6* mutant allele produced a band of 174 bp.

**Antibodies**

Antibodies to full-length FoxO1 and FoxO4 were generated by injection of GST fusion proteins into rabbits, and the antibodies were purified by affinity (Quality Controlled Biochemicals). The antibodies to full-length human FoxO3 ('NFL') are described previously (Greer et al. 2007; Renault et al. 2009).

**Basic Behavioral Assays**

Male *FoxO6* mutant mice were studied alongside wild-type littermate controls in six cohorts. The mice were handled daily for at least 7 days in the procedure room prior to behavioral testing. The open field test was performed for 10 min in a 70 x 70 x 70 cm black plastic box with a white PVC vinyl material on the base in a dimly lit room. A video tracking system (Videotrack Automated Behavioral Analysis System, Viewpoint Life, Science Inc., France), was used to record and analyze the data (mouse location and running path with time). The wire hang task is the ability to hang upside down from a wire screen, and measures neuromuscular function and grip strength (Sango et al. 1996). A modified wire cover to a rat cage was used. The wire bars were around 2 mm in diameter and spaced 1 cm apart. A rectangular area of the screen (12 x 18 cm) was taped off with duct tape to confine the mice to the wire screen. The mice were placed on the screen, and the screen was gently waved in the air three times to force the mice to grip the wires. The screen was then immediately turned upside down, 60 cm above a large rodent housing cage. The latency to fall into the cage was recorded. Mice that fell in under 10 sec were given a second trial. Mice that did not fall during the 60 sec trial period were given a maximum score of 60 sec. The accelerating RotaRod assesses the

motor coordination of mice. The mice were acclimatized to the RotaRod (Economex RotaRod, Columbus Instruments, Columbus, Ohio) using a pre-trial period. The pre-trial consisted of gently placing the mice onto the stationary spindle (4 cm diameter) for 60 sec. If a mouse fell within 60 sec it was given a second trial. The mice were then placed in the RotaRod collection chambers, and the spindle acceleration was set to 3 rpm using the computer software. The mice were placed on the spindle for a period of 90 sec. After the 90 sec had elapsed, the mice were placed in the RotaRod collection chambers for 90 sec rest. Each mouse was given 3 trials at 3 rpm spindle rotation for 90 sec with an inter-trial interval (ITI) of 90 sec. If a mouse could not maintain its balance for at least one trial at 3 rpm for 90 sec, it was not included in the study. After the pre-trial the mice were rested for at least 30 min in the home cage. The accelerating RotaRod trial consisted of rotating the spindle at 3 rpm and placing the mice onto the spindle. The spindle was then steadily accelerated to 40 rpm over a 330 sec period. The time the mice could maintain their balance on the accelerating spindle before falling 40 cm into the collection chambers was recorded. Mice that did not fall in 330 sec were given the maximum score of 330 sec.

**Hot plate assay**

Mice were handled for 2 min each and habituated to the testing environment 24 hours before testing. On the testing day, the hot plate apparatus (Model 39, ITC Life Science Inc., Woodland Hills, CA) was set to a temperature of 55 ± 0.2°C. The mice were placed on the surface of the hot plate and covered by a glass transparent cylinder (12 cm diameter, 25 cm high). Each mouse was tested one time only. A cut-off time of 30 sec

was assigned but not employed since the mice never reached that value. A remote foot-switch pad was used to control the start/stop/reset function. The latency time was recorded when the first hind-paw licking or jumping occurred.

**Startle response assay**

Mice were handled for 3 days and habituated to the testing environment for 15 min before testing. On the testing day, each mouse was habituated to the testing environment for 5 min before the startle response was tested. Startle reflexes were measured as Heldt et al. (2004), with the following modifications using a startle response system (Med Associates Inc., St. Albans, VT). The system consisted of an animal holder of steel grid construction (ENV-264C, Med Associates Inc., St. Albans, VT), mounted on a platform located in a well-ventilated, sound-proof chamber. The startle stimulus was presented through a high-frequency speaker located 15 cm above the startle chamber. The mice were placed in the holder and after 5 min were given 2 startle stimuli at each of 5 different startle stimulus intensities (90, 100, 110, 120 and 130 dB) with an inter-stimulus interval of (ISI) of 30 sec. The startle stimuli were presented in a pseudorandom sequence with each stimulus intensity occurring only once during the 5 trial block. A total of 2 trial blocks were presented. Mouse movements were detected by a piezoelectric accelerometer mounted under the platform and were digitized and stored by a computer interface. Movements were sampled every millisecond (ms) and startle amplitude was defined as the peak accelerometer voltage that occurred during the first 100 ms after the onset of the startle stimulus.

**Catwalk**

Mice were handled for 2 min each and habituated to the testing environment 24 hours before testing. The Catwalk apparatus (Noldus) provides an objective and quantitative assessment of gait. Mice are gently placed at one end of the walkway and allowed to traverse to the other end where the home cage is placed. Data is acquired using a video camera positioned underneath the glass floor to capture the illuminated areas, and the data is sent to a computer running the CatWalk software. Three runs were collected for each mouse, and the equipment was cleaned between mice. A wide range of time-related parameters, such as the swing and stance duration and swing speed were calculated.

**Grip strength**

Mice were handled for 2 min each and habituated to the testing environment 24 hours before testing. A grip strength meter (Grip strength system, SDI) was used to measure the grip strength of the forepaws of mice. The procedure was carried out by gently lowering the mouse over the base-plate by the tail so that its front paws could reach to grasp the steel grid. Then the mouse was then gently pulled backward by the tail until it released the steel grid. The grid was connected to a kilogram dynamometer. The grip strength of the front paws was measured when the mouse released the grid grasped by both front paws. The maximal grip strength of three successful trials was recorded. Between trials, the animals were held in their cages for a 30 sec inter-trial interval (ITI).

**Evaluation of mouse vision**

To evaluate the vision of the mice, a water maze consisting of a circular black tank (180 cm diameter, 60 cm deep) filled with water at 22 ± 1°C containing tempera paint (Elmer's Products Inc., Ohio). The platform (18 cm diameter) was submerged 1-2 cm under the water surface. The platform was indicated using a table-tennis ball attached to a stick rising above the water line to make the platform visible from all areas within the pool. Mice were habituated by handling each mouse for 120 sec daily for 3 days before the training. Mice were allowed to acclimatize to the procedure room for at least 1 hour during each day of handling and training. Four trials were conducted with the visible platform during one day, using 2 blocks of trials with 2 consecutive trials per block with an inter-trial interval (ITI) of 50-120 min within a block of trials, and 50-90 min between the 2 blocks. For each trial, the mouse was placed into the water with its head facing the wall of the pool. For each individual set of trials, a start location from 5 different positions spaced evenly around the pool was chosen, and the release location of the mouse was changed for each individual set of trials. On each trial the mouse was given 60 sec to find the platform. A video tracking system (Ethovision v3.1; Noldus Information Technology, The Netherlands) was used to record and analyze the data (latency to find platform, mouse location, swim path and swim velocity). The experimenter was blind to the genotypes of the mice.

**Morris water maze**

The Morris water maze was performed according to Morris et al. (1982) and Zhang et al. (2008), with the modifications listed below. The water maze consisted of a circular

black tank described above as used for ‘Evaluation of mouse vision.’ The platform (18 cm diameter) was submerged 1-2 cm under water surface in the center of quadrant 2 after dividing the pool into 4 virtual quadrants. Distal cues (posters) were placed on the walls of the room to provide spatial references. Mice were habituated by handling each mouse for 120 sec daily for 3 days before the training. Mice were allowed to acclimatize to the procedure room for at least 1 hour during each day of handling and training. The training consisted of 4 days of training with 2 blocks of trials per day with 2 consecutive trials per block (total of 4 trials per day) with an inter-trial interval (ITI) of 50-120 min within a block of trials, and 50-90 min between the 2 blocks with the hidden platform in quadrant 2. For each trial, the mouse was placed into the water with its head facing the wall of the pool. For each individual set of trials, a start location from 5 different positions spaced evenly around the pool was chosen. On each trial the mouse was given 60 sec to find the platform and the mouse was allowed to stay on the platform for 15 sec. If a mouse did not find the platform, it was placed on the platform by the experimenter for 15 sec. A video tracking system (Ethovision v3.1; Noldus Information Technology, The Netherlands) was used to record and analyze the data (latency to find platform, mouse location, swim path and swim velocity). After 4 days of training, the platform position was ‘reversed’ to the quadrant opposite the original location (quadrant 4), and mice were trained for a further 5 days. For memory probe trials, the platform was removed from the maze, and the animals were allowed 60 sec to search the pool. The first probe trial was conducted the morning after 4 days of hidden platform training, the second trial was conducted the morning after the 5 days of reversed platform training, the third, fourth and fifth probe trials were conducted 4, 9 and 23 days after the

end of reversed platform training respectively, to assess the decay of the reference memory. The time spent (%) in each quadrant of the maze was recorded. The experimenter was blind to the genotypes of the mice.

**Luciferase assays**

U20S cells were plated at a density of 8.0 x $10^4$ cells $ml^{-1}$ in 24-well plates. The next day, each well of cells was transfected using Polyethyleneimine (PEI; Polysciences), with 400 ng of a luciferase reporter construct driven by three tandem repeats of the FoxO6 consensus binding motif (p3xFoxO6) and 100 ng of a renilla luciferase reporter construct (pRL0). For the constructs to be tested 50 ng of each was transfected: wild-type FoxO1-His-myc (FoxO1 WT), FoxO3-His-myc (FoxO3 WT) or FoxO4-His-myc (FoxO4 WT) in the pcDNA Neo vector. To test for dominant negative activity of FoxO6ΔCt-M2, 50 ng of FoxO1 WT, FoxO3 WT or FoxO4 WT were spiked with 150 ng of the FoxO6ΔCt-M2 construct. For the transfection of each well of cells the total amount of DNA was made up to 200 ng with empty pcDNA Neo vector. Fifty two hours after transfection, the cells were lysed, and the luciferase and renilla luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

## Supplemental References


Greer EL, Oskoui PR, Banko MR, Maniar JM, Gygi MP, Gygi SP, Brunet A. 2007. The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor. *J Biol Chem* **282**: 30107-30119.

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Morris RG, Garrud P, Rawlins JN, O'Keefe J. 1982. Place navigation impaired in rats with hippocampal lesions. *Nature* **297**: 681-683.

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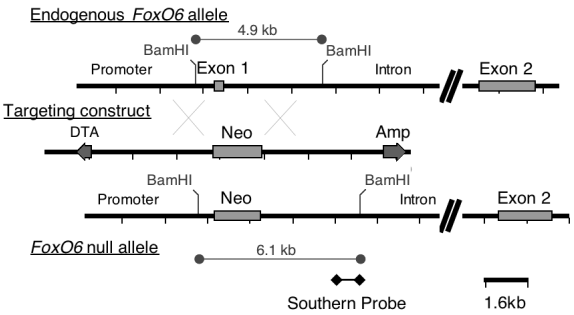
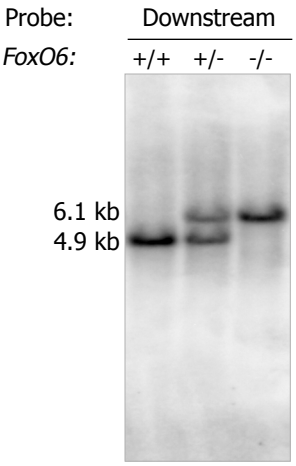
Sango K, McDonald MP, Crawley JN, Mack ML, Tifft CJ, Skop E, Starr CM, Hoffmann A, Sandhoff K, Suzuki K et al. 1996. Mice lacking both subunits of lysosomal beta-hexosaminidase display gangliosidosis and mucopolysaccharidosis. *Nat Genet* **14**: 348-352.

Zhang CL, Zou Y, He W, Gage FH, Evans RM. 2008. A role for adult TLX-positive neural stem cells in learning and behaviour. *Nature* **451**: 1004-1007.

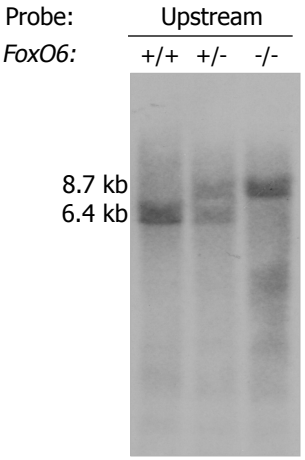
A


Endogenous FoxO6 allele
4.9 kb
BamHI
BamHI
Promoter
Exon 1
Intron
Exon 2
Targeting construct
DTA
Neo
Amp
BamHI
BamHI
Promoter
Neo
Intron
Exon 2
FoxO6 null allele
6.1 kb
Southern Probe
1.6kb

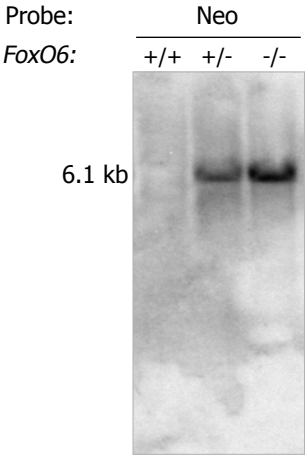
B


Probe:
Downstream
FoxO6:
+/+ +/- -/-
6.1 kb
4.9 kb

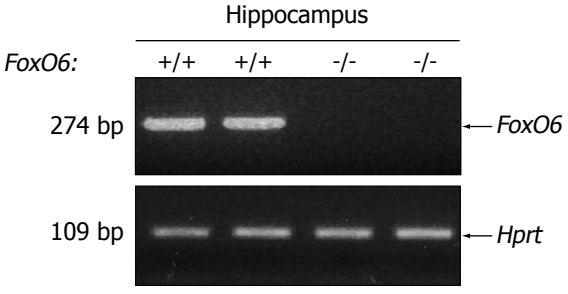
C


Probe:
Upstream
FoxO6:
+/+ +/- -/-
8.7 kb
6.4 kb

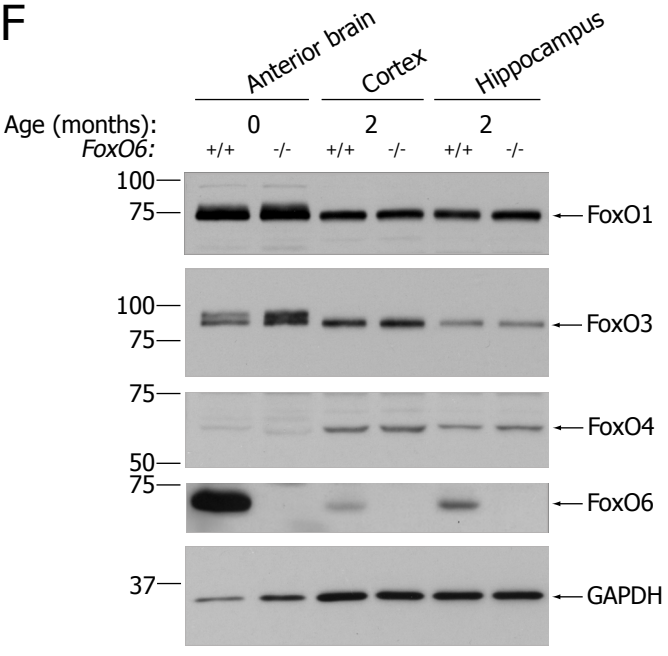
D


Probe:
Neo
FoxO6:
+/+ +/- -/-
6.1 kb

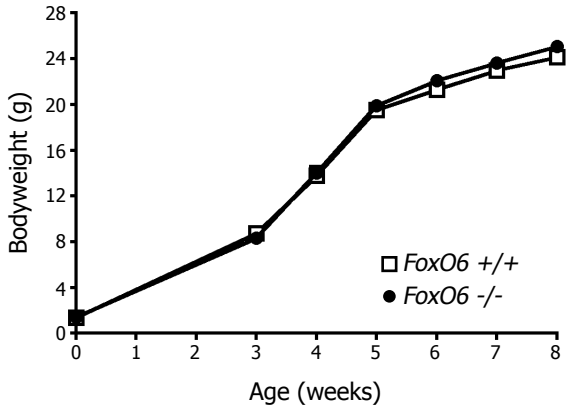
E


Hippocampus
FoxO6:
+/+ +/+ -/- -/-
274 bp
FoxO6
109 bp
Hprt

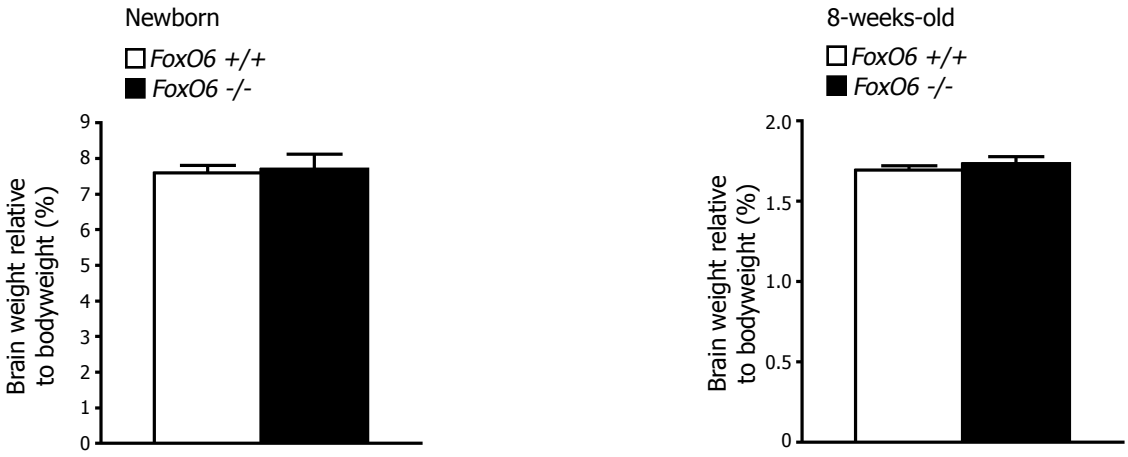
F


Anterior brain
Cortex
Hippocampus
Age (months):
0
2
2
FoxO6:
+/+ -/- +/+ -/- +/+ -/-
100
75
FoxO1
100
75
FoxO3
75
FoxO4
50
75
FoxO6
37
GAPDH

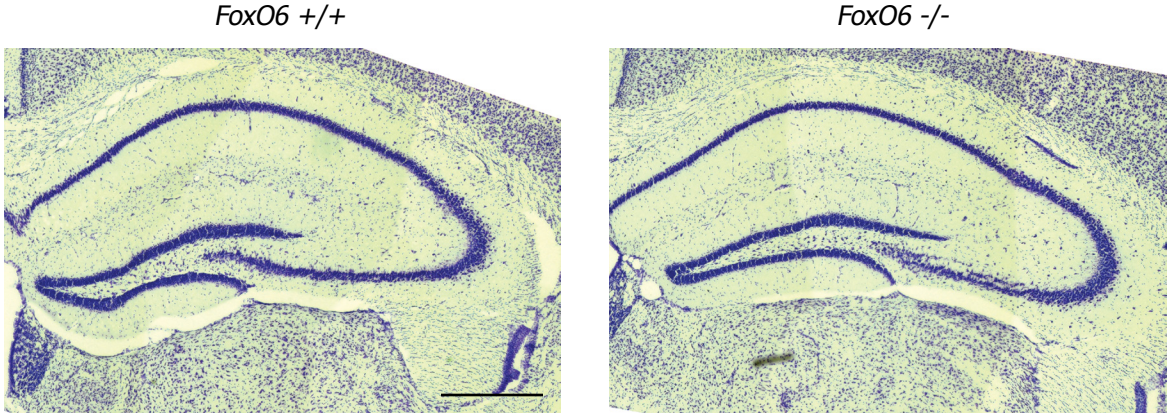
A

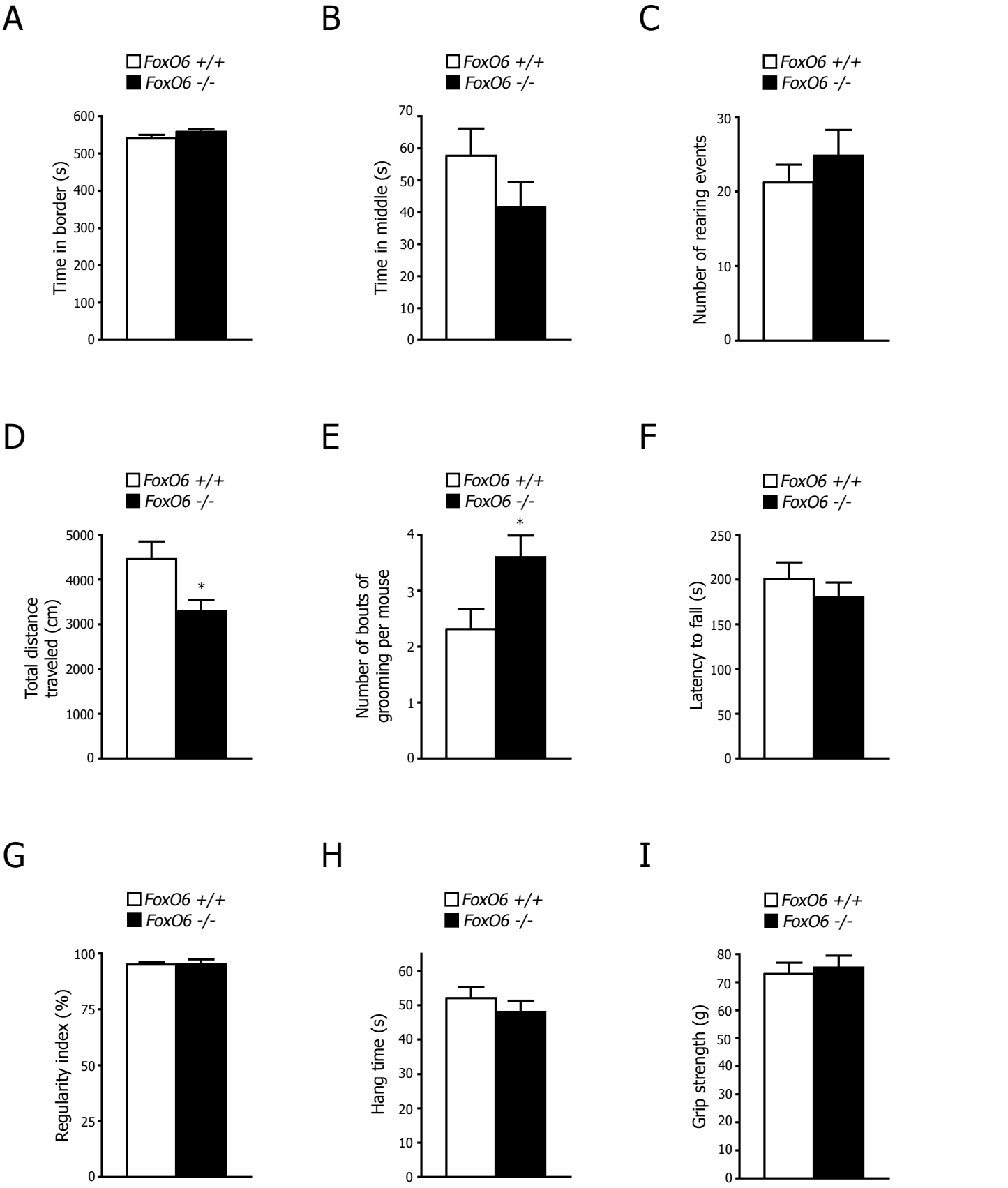

Bodyweight (g)
28
24
20
16
12
8
4
0
0 1 2 3 4 5 6 7 8
Age (weeks)
FoxO6 +/+
FoxO6 -/-

B

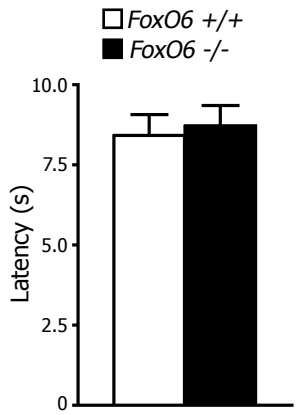

Newborn
FoxO6 +/+
FoxO6 -/-
Brain weight relative to bodyweight (%)
9
8
7
6
5
4
3
2
1
0
8-weeks-old
FoxO6 +/+
FoxO6 -/-
Brain weight relative to bodyweight (%)
2.0
1.5
1.0
0.5
0

C

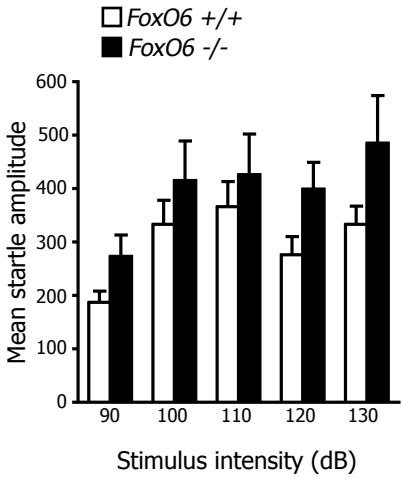

FoxO6 +/+
FoxO6 -/-


A
FoxO6 +/+
FoxO6 -/-
Time in border (s)
0
100
200
300
400
500
600
B
FoxO6 +/+
FoxO6 -/-
Time in middle (s)
0
10
20
30
40
50
60
70
C
FoxO6 +/+
FoxO6 -/-
Number of rearing events
0
10
20
30
D
FoxO6 +/+
FoxO6 -/-
Total distance traveled (cm)
0
1000
2000
3000
4000
5000
*
E
FoxO6 +/+
FoxO6 -/-
Number of bouts of grooming per mouse
0
1
2
3
4
*
F
FoxO6 +/+
FoxO6 -/-
Latency to fall (s)
0
50
100
150
200
250
G
FoxO6 +/+
FoxO6 -/-
Regularity index (%)
0
25
50
75
100
H
FoxO6 +/+
FoxO6 -/-
Hang time (s)
0
10
20
30
40
50
60
I
FoxO6 +/+
FoxO6 -/-
Grip strength (g)
0
10
20
30
40
50
60
70
80

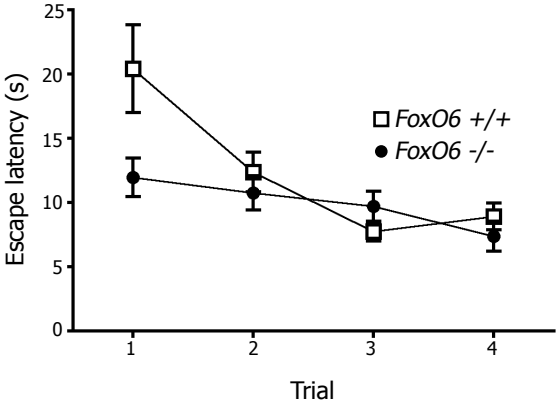
A


FoxO6 +/+
FoxO6 -/-
Latency (s)
10.0
7.5
5.0
2.5
0

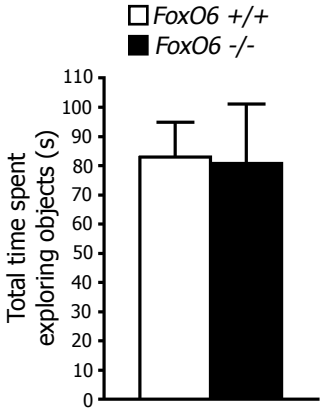
B

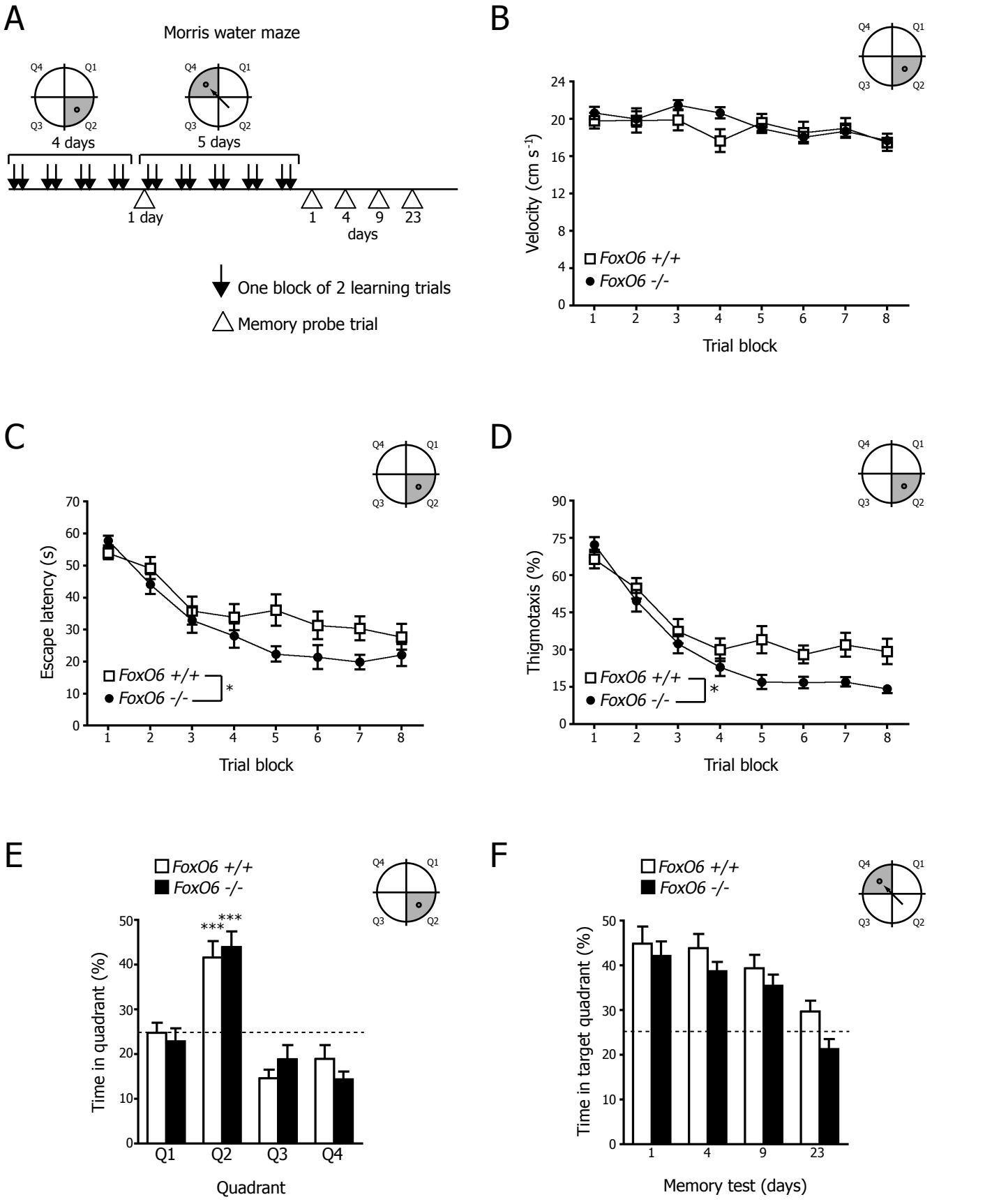

FoxO6 +/+
FoxO6 -/-
Mean startle amplitude
600
500
400
300
200
100
0
90
100
110
120
130
Stimulus intensity (dB)

C

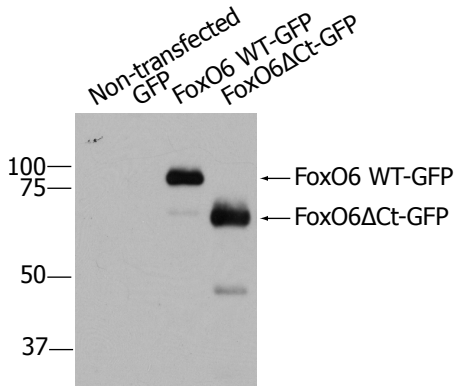

Escape latency (s)
25
20
15
10
5
0
FoxO6 +/+
FoxO6 -/-
1
2
3
4
Trial

D

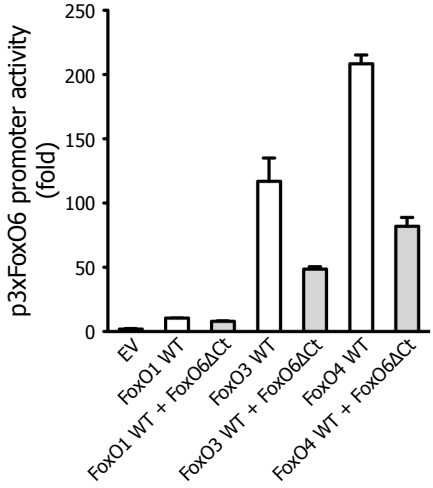

FoxO6 +/+
FoxO6 -/-
Total time spent exploring objects (s)
110
100
90
80
70
60
50
40
30
20
10
0


A
Morris water maze
Q4
Q1
Q3
Q2
4 days
5 days
1 day
1
4
9
23
days
One block of 2 learning trials
Memory probe trial
B
Velocity (cm s-1)
FoxO6 +/+
FoxO6 -/-
Trial block
C
Escape latency (s)
FoxO6 +/+
FoxO6 -/-
*
Trial block
D
Thigmotaxis (%)
FoxO6 +/+
FoxO6 -/-
*
Trial block
E
FoxO6 +/+
FoxO6 -/-
***
***
Time in quadrant (%)
Q1
Q2
Q3
Q4
Quadrant
F
FoxO6 +/+
FoxO6 -/-
Time in target quadrant (%)
1
4
9
23
Memory test (days)

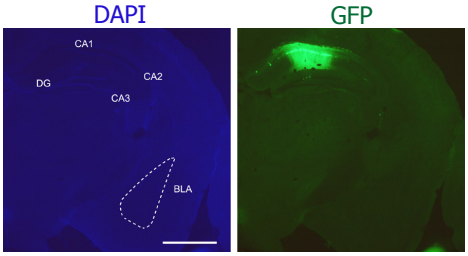
A


Non-transfected
GFP
FoxO6 WT-GFP
FoxO6ΔCt-GFP
100
75
50
37
FoxO6 WT-GFP
FoxO6ΔCt-GFP

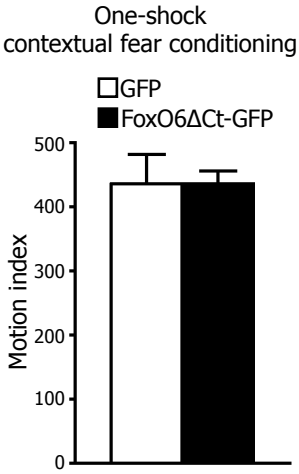
B


p3xFoxO6 promoter activity (fold)
250
200
150
100
50
0
EV
FoxO1 WT
FoxO1 WT + FoxO6ΔCt
FoxO3 WT
FoxO3 WT + FoxO6ΔCt
FoxO4 WT
FoxO4 WT + FoxO6ΔCt

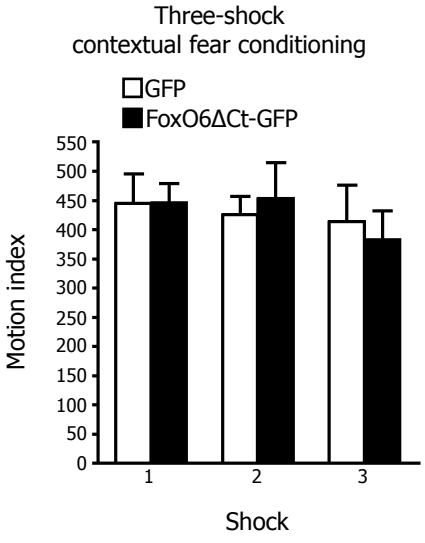
C

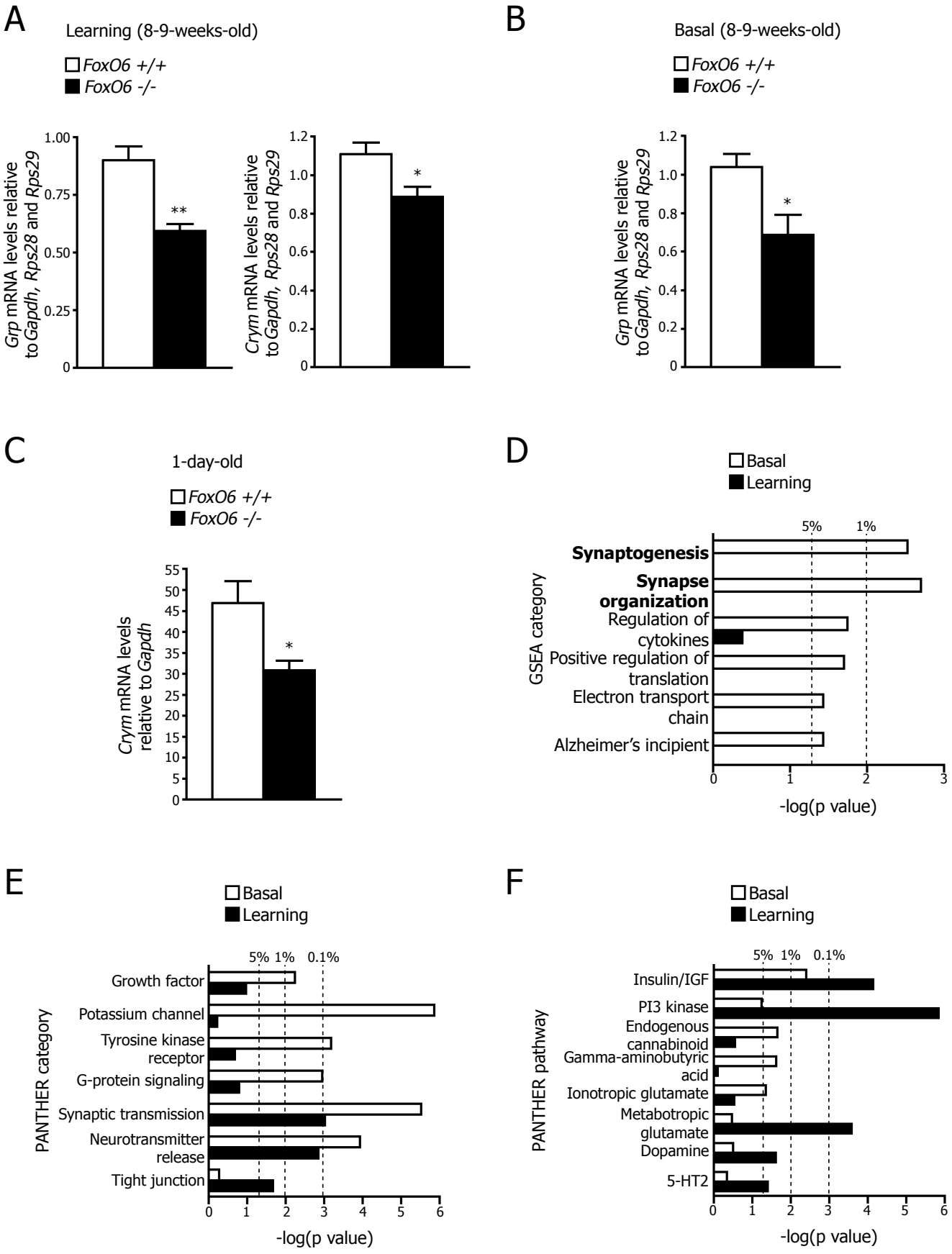

DAPI
GFP
CA1
CA2
CA3
DG
BLA

D


One-shock
contextual fear conditioning
GFP
FoxO6ΔCt-GFP
Motion index
500
400
300
200
100
0

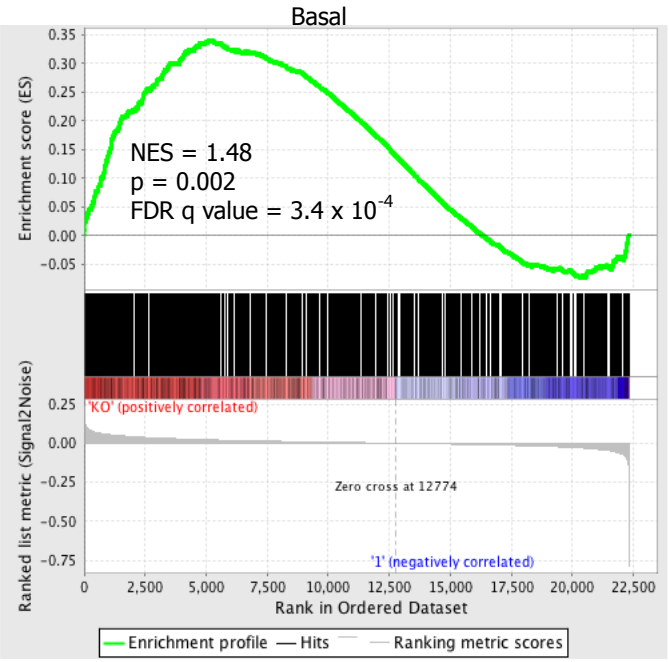
E

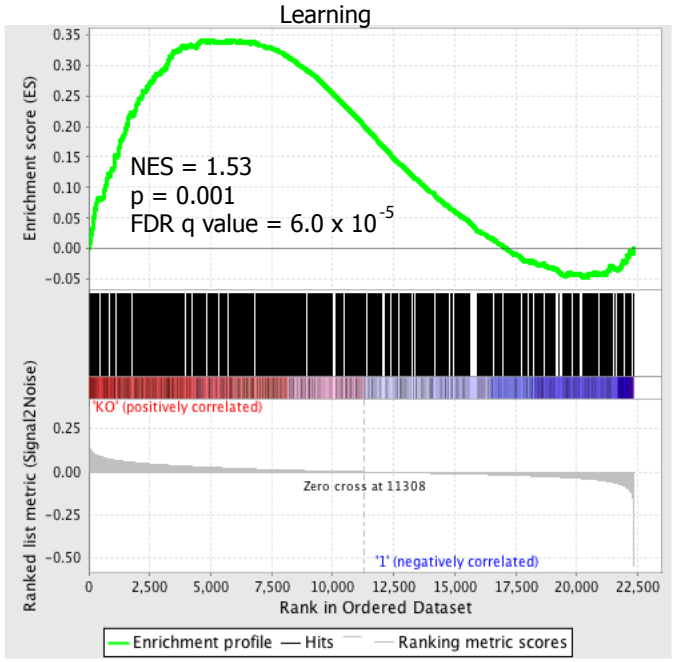

Three-shock
contextual fear conditioning
GFP
FoxO6ΔCt-GFP
Motion index
550
500
450
400
350
300
250
200
150
100
50
0
1
2
3
Shock


A
Learning (8-9-weeks-old)
FoxO6 +/+
FoxO6 -/-
Grp mRNA levels relative to Gapdh, Rps28 and Rps29
1.00
0.75
0.50
0.25
0
**
Crym mRNA levels relative to Gapdh, Rps28 and Rps29
1.2
1.0
0.8
0.6
0.4
0.2
0
*
B
Basal (8-9-weeks-old)
FoxO6 +/+
FoxO6 -/-
Grp mRNA levels relative to Gapdh, Rps28 and Rps29
1.2
1.0
0.8
0.6
0.4
0.2
0
*
C
1-day-old
FoxO6 +/+
FoxO6 -/-
Crym mRNA levels relative to Gapdh
55
50
45
40
35
30
25
20
15
10
5
0
*
D
Basal
Learning
5%
1%
GSEA category
Synaptogenesis
Synapse organization
Regulation of cytokines
Positive regulation of translation
Electron transport chain
Alzheimer's incipient
0
1
2
3
-log(p value)
E
Basal
Learning
5%
1%
0.1%
PANTHER category
Growth factor
Potassium channel
Tyrosine kinase receptor
G-protein signaling
Synaptic transmission
Neurotransmitter release
Tight junction
0
1
2
3
4
5
6
-log(p value)
F
Basal
Learning
5%
1%
0.1%
PANTHER pathway
Insulin/IGF
PI3 kinase
Endogenous cannabinoid
Gamma-aminobutyric acid
Ionotropic glutamate
Metabotropic glutamate
Dopamine
5-HT2
0
1
2
3
4
5
6
-log(p value)

A

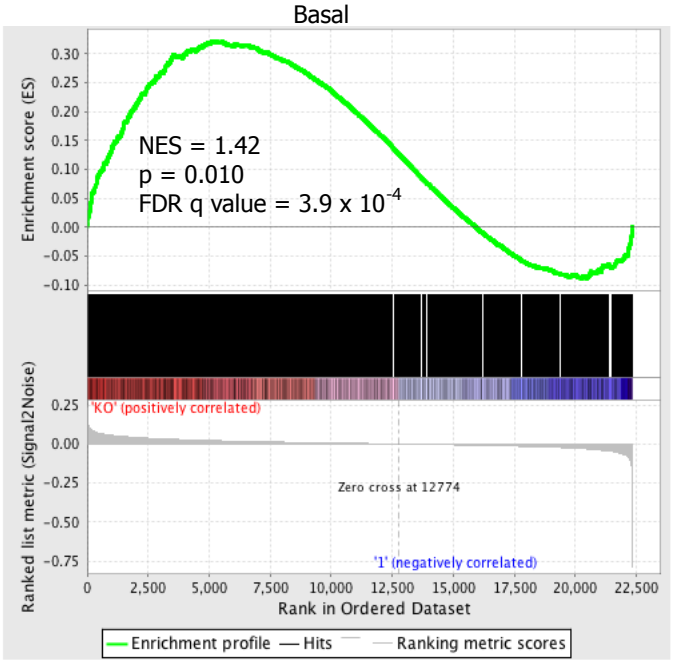
Glutamate pathway

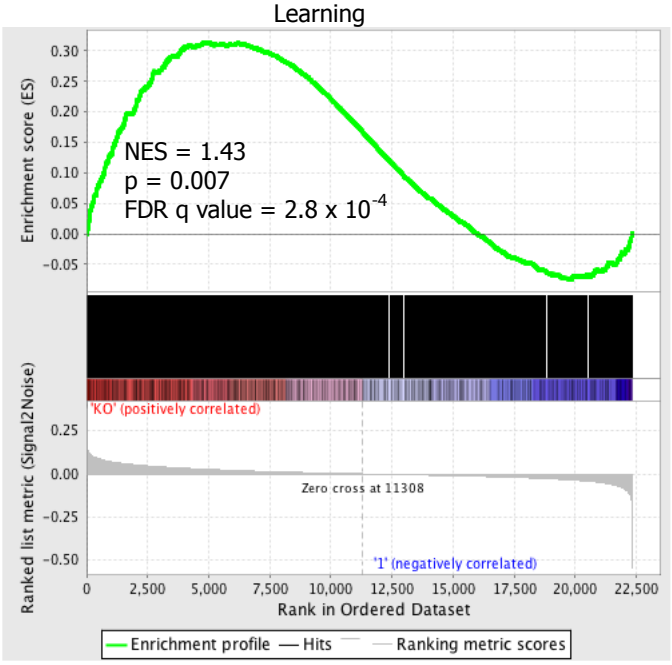



B

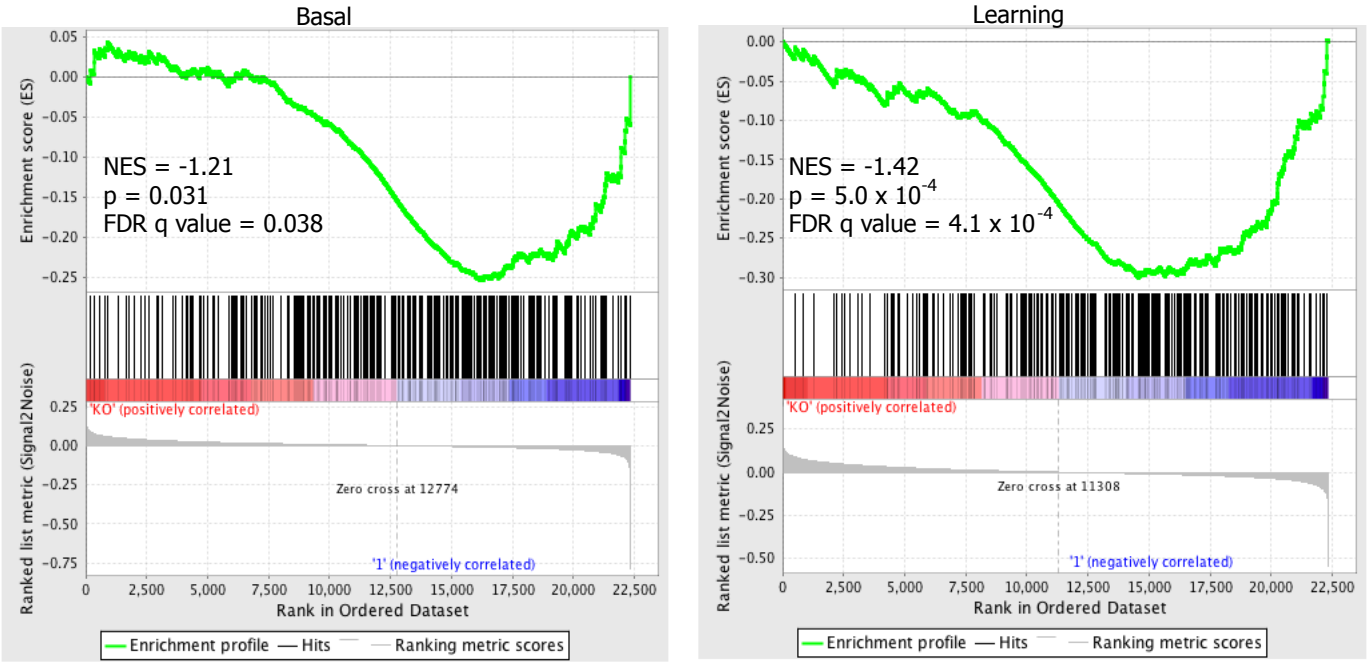
Alzheimer's disease



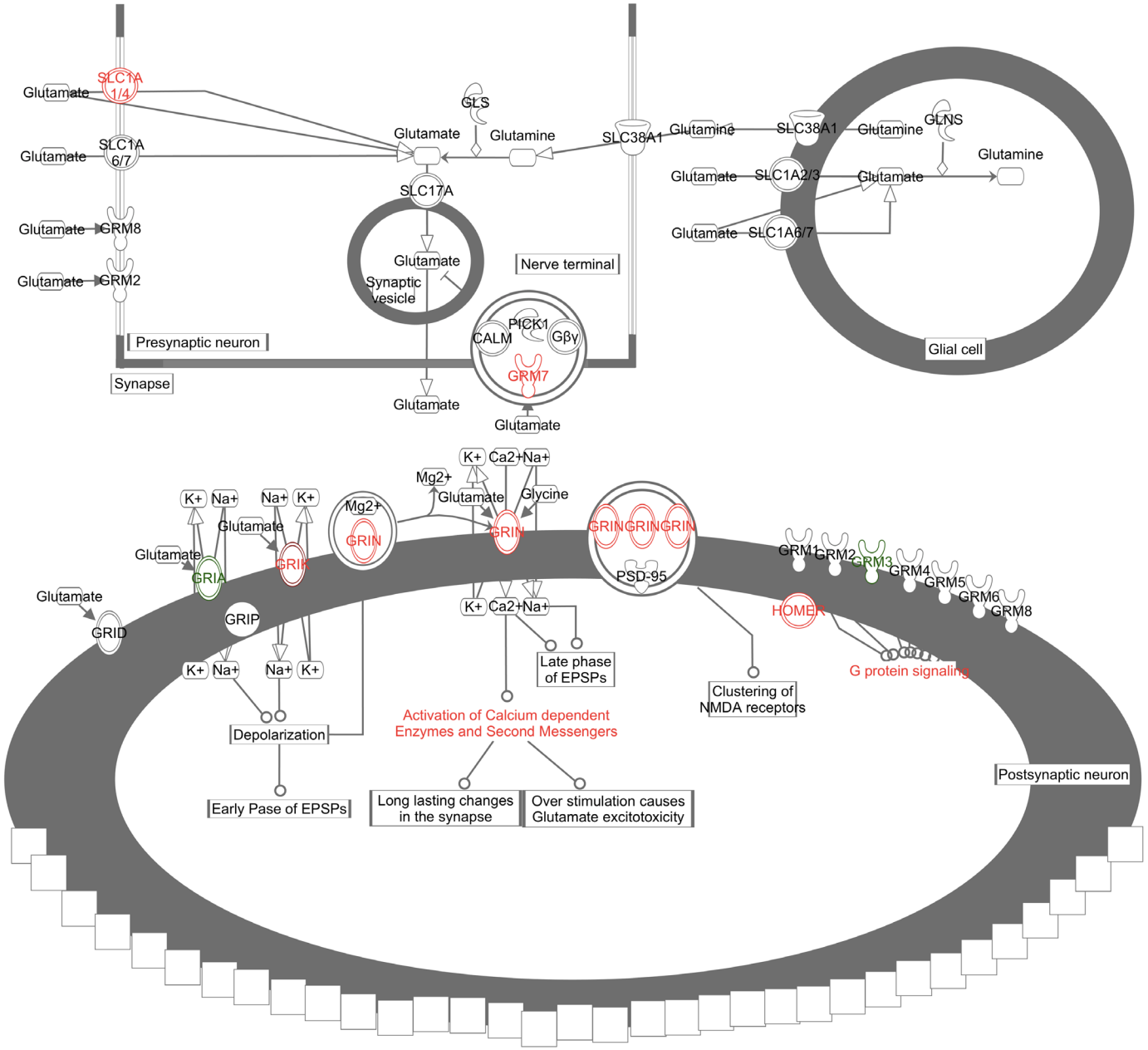



Salih Supplemental Figure 9

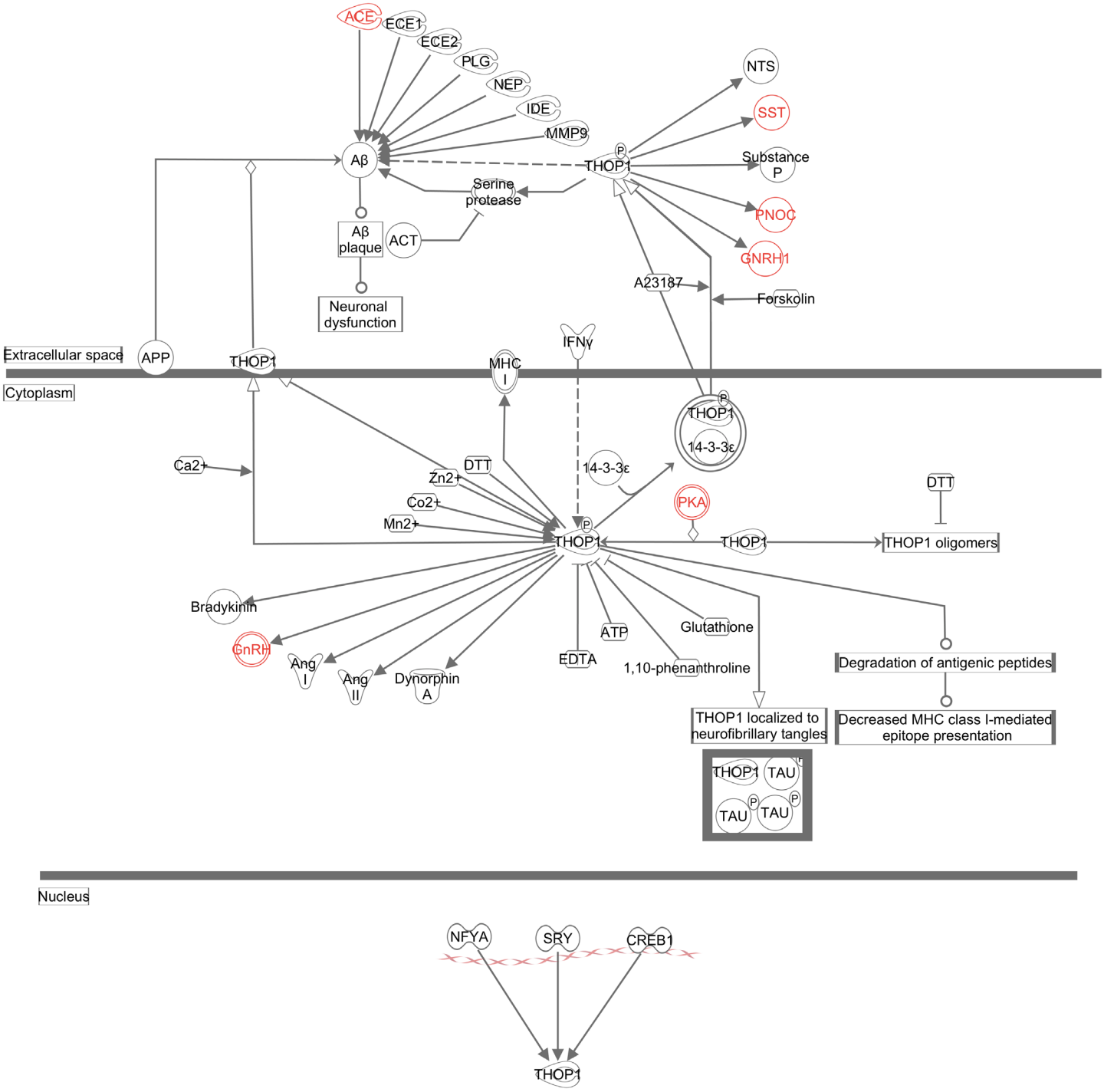
p53 pathway



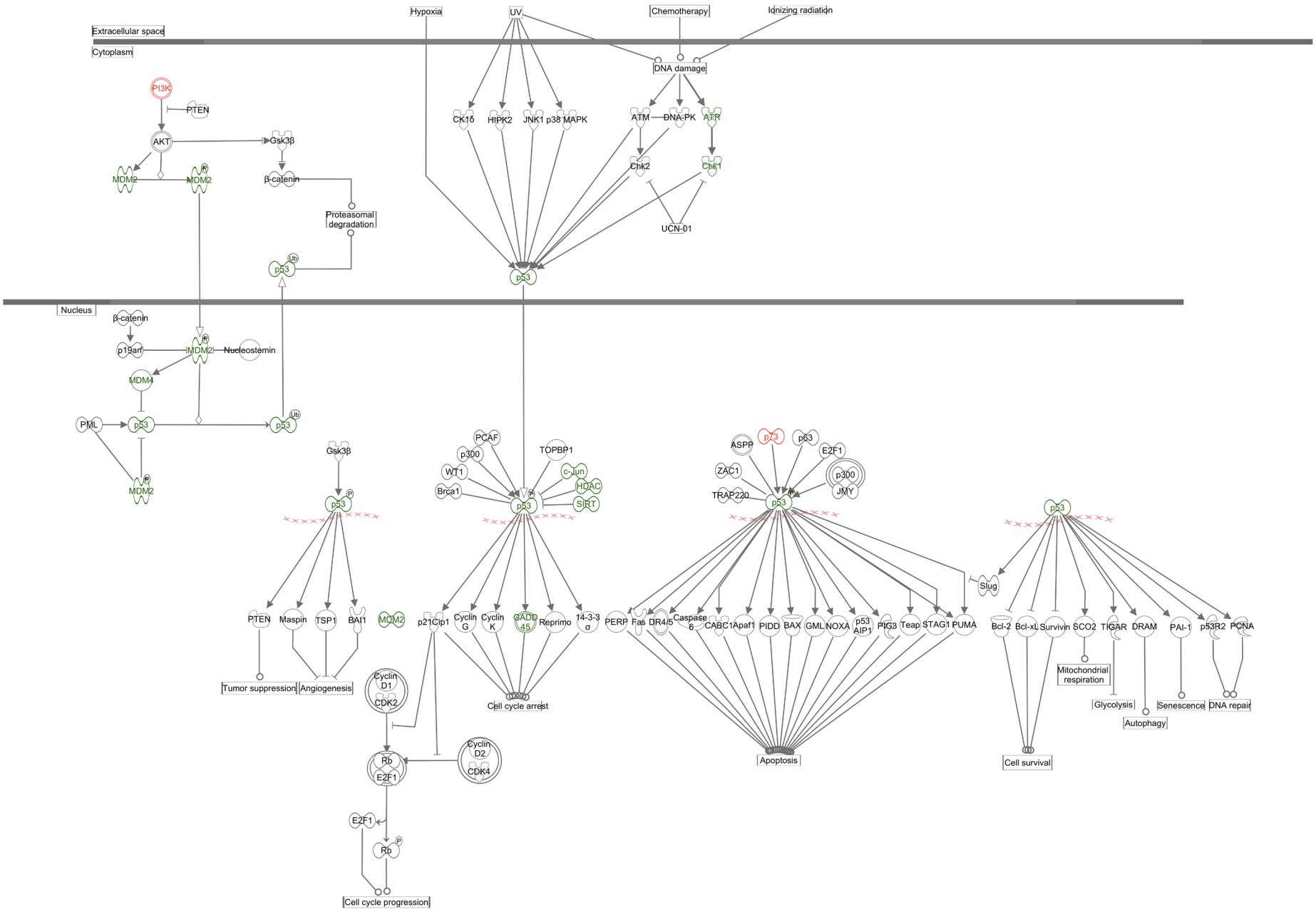
Salih Supplemental Figure 10



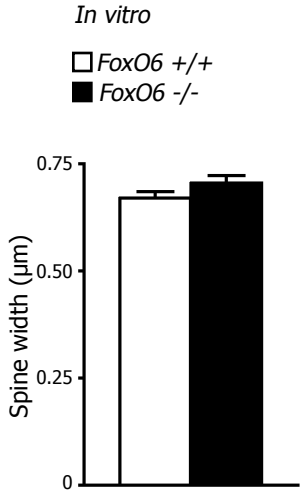
# Salih Supplemental Figure 11



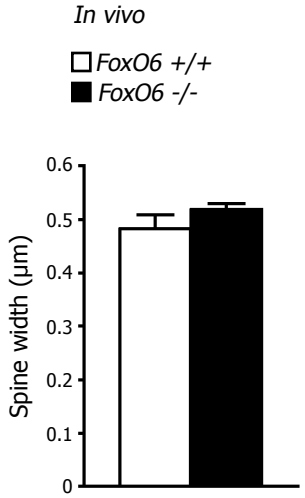
# Salih Supplemental Figure 12



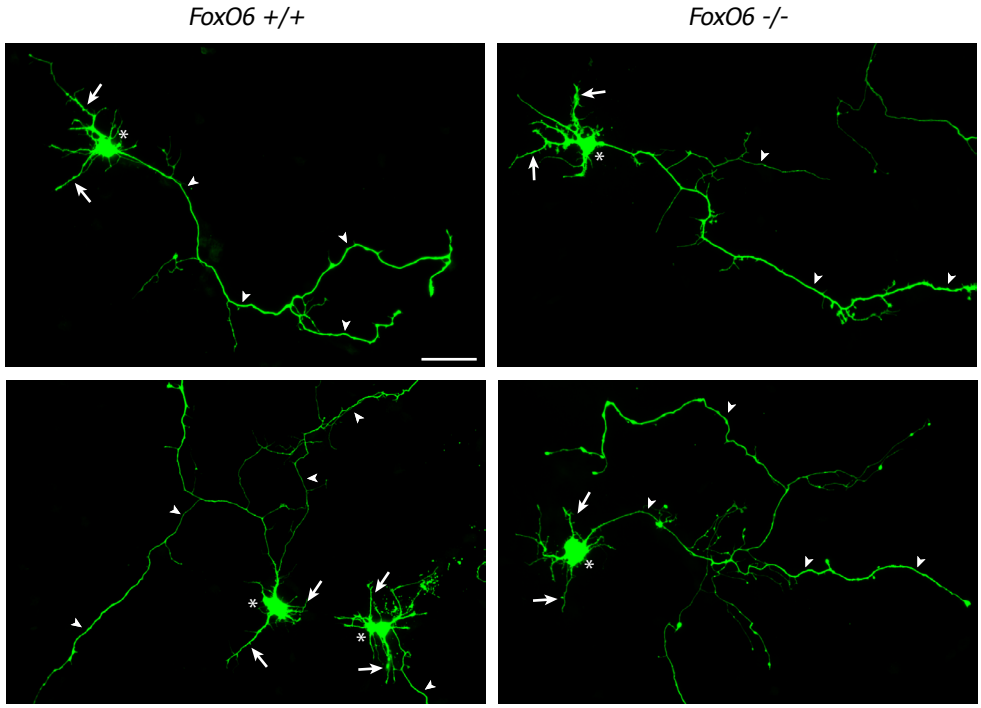
A


In vitro
FoxO6 +/+
FoxO6 -/-
Spine width (µm)
0.75
0.50
0.25
0

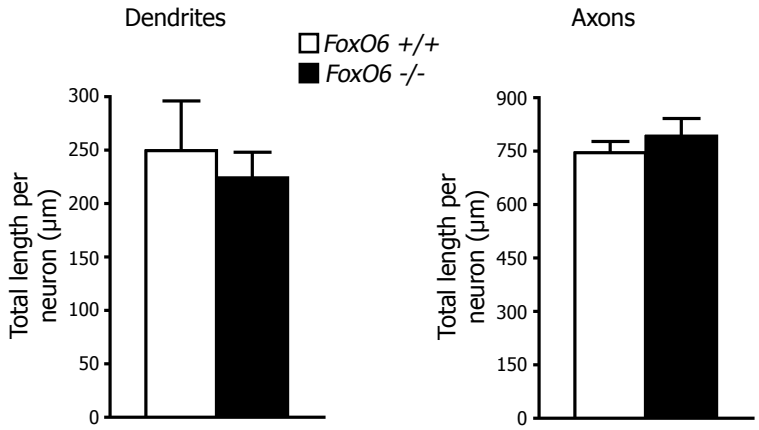
B


In vivo
FoxO6 +/+
FoxO6 -/-
Spine width (µm)
0.6
0.5
0.4
0.3
0.2
0.1
0

C


FoxO6 +/+
FoxO6 -/-

D


Dendrites
Axons
FoxO6 +/+
FoxO6 -/-
Total length per neuron (µm)
300
250
200
150
100
50
0
900
750
600
450
300
150
0