Supplementary Information

Supplementary figure legends

Figure S1. FoxO3A is activated by hypoxia in a manner dependent on HIF-1α and HIF-2α.

(A) Status of FoxO transcription factors in hypoxia. HeLa, CaKi, MCF-7 and MEF cells were incubated in normoxia, hypoxia (0.5% O_2) or in the presence of DFO (100 μM) for 16 hours. Cell lysates were analyzed by immunoblotting. HeLa cells were transfected with control siRNA, siRNA against HIF-1α, or siRNA against HIF-2α. 32 hours following transfection, the cells were incubated in normoxia or hypoxia (0.5% O_2) for 16 hours. Cell lysates were analyzed by immunoblotting (B) and by qPCR analysis (C). The expression levels were normalized to actin and are shown as fold of control (Ctrl siRNA in normoxia). Error bars indicate mean ± SD of three experiments. (D) HeLa cells were transfected with control siRNA or siRNA against HIF-1α and HIF-2α in combination. 32 hours following transfection, the cells were incubated in normoxia or hypoxia (0.5% O_2) for 16 hours. Cell lysates were analyzed by immunoblotting. (E) Cell fractionation was performed on HeLa cells incubated in normoxia or hypoxia (0.5% O_2) for 16 hours. Total lysate, cytosolic- and nuclear fractions were analyzed by immunoblotting. GAPDH and Histone H3 were used as controls for the cytosolic and nuclear fractions, respectively.

Figure S2. The *FOXO3A* promoter region can be transactivated by HIF-1 and HIF-2 and is bound directly by HIF-1α in hypoxia.

(A) The -167 to +14 region of the human *FOXO3A* locus containing three potential HREs was cloned into the pGL2 luciferase reporter vector. Point mutations in the HREs were introduced in order to assess their relative contributions. (B) Reporter gene assays were performed in HeLa cells co-transfected with reporter and HIF-1α or HIF-2α expression plasmid before subsequent incubation for 16 hours in normoxia or hypoxia (0.5% O_2). Values are presented as relative luciferase expression (RLE) and errorbars indicate mean ± SD of three experiments.

(C) ChIP assay. HeLa cells were incubated in normoxia or hypoxia (0.5% O_2) for 6 hours before cross-linking. ChIP was performed using antibodies against HIF-1α and HA (negative control). Primers were designed for the *FOXO3A* promoter region as well as for a known HRE (*VEGF*). Results are presented as percentage bound over input. Error bars indicate mean ± SD.

Figure S3. Knockdown of HIF-1α impairs hypoxic repression of FoxO3A-dependent mitochondrial genes.

HeLa cells were transfected with control siRNA or siRNA against HIF-1α. 32 hours following transfection, the cells were incubated in normoxia or hypoxia (0.5% O_2) for 20 hours. RNA was extracted for qPCR analysis. The results are normalized to 18S rRNA and are shown as fold of normoxic control. Error bars indicate mean ± SD of three experiments, **p<0.01, ***p<0.001 using Student's t-test.

Figure S4. Verification of FoxO3A- and Myc-dependency of hypoxia-repressed mitochondrial genes in WI-38 cells. (A) Test of FoxO3A and Myc knockdown. WI-38 cells were reverse transfected with Ctrl, Myc, or FoxO3A siRNA or left untransfected. Cells were lysed 32 hours after transfection and analyzed by

immunoblotting. (B) WI-38 cells were reverse transfected with control, Myc, or FoxO3A siRNA or FoxO3A and Myc siRNA in combination. 32 hours post transfection, the cells were incubated in normoxia or hypoxia for 20 hours. RNA was extracted for qPCR analysis. The results are normalized to 18S rRNA and are shown as fold of normoxic control. Error bars indicate mean ± SD of three experiments, **p<0.01, ***p<0.001 using Student's t-test.

Figure S5. Verification of FoxO3A- and Myc-dependency of hypoxia-repressed mitochondrial genes in TIG-3 cells. (A) Test of FoxO3A and Myc knockdown. TIG-3 cells were reverse transfected with Ctrl, Myc, or FoxO3A siRNA or left untransfected. Cells were lysed 32 hours after transfection and analyzed by immunoblotting. (B) TIG-3 cells were reverse transfected with control, Myc, or FoxO3A siRNA or FoxO3A and Myc siRNA in combination. 32 hours post transfection, the cells were incubated in normoxia or hypoxia for 20 hours. RNA was extracted for qPCR analysis. The results are normalized to 18S rRNA and are shown as fold of normoxic control. Error bars indicate mean ± SD of three experiments, *p<0.05, **p<0.01, ***p<0.001 using Student's t-test.

Figure S6. Hypoxic mRNA levels of Myc, Max, Mad1, and Mxi1-SRα are unaffected by knockdown of FoxO3A.

Hela Ctrl and FoxO3A knockdown cell clones were incubated for 20 hours in normoxia or hypoxia (0.5% O_2). RNA was extracted and analyzed by qPCR. The qPCR results are normalized to 18S rRNA and are shown as fold of normoxic Ctrl. Error bars indicate mean ± SD of three experiments.

Figure S7. Knockdown of Mxi1-SRα does not affect the hypoxic repression of FoxO3A-dependent mitochondrial genes.

(A) Test of Mxi1-SRα knockdown. HeLa cells were transfected with Ctrl or Mxi1-SRα siRNA or left untransfected. Cells were lysed 32 hours after transfection and analyzed by immunoblotting. (B) HeLa cells were transfected with control siRNA or siRNA against Mxi1-SRα. 32 hours following transfection, the cells were incubated in normoxia or hypoxia (0.5% O_2) for 20 hours. RNA was extracted for qPCR analysis. The results are normalized to 18S rRNA and are shown as fold of normoxic control. Error bars indicate mean ± SD of three experiments.

Figure S8. De novo protein synthesis is not required for FoxO3A(A3)-ER-mediated repression of mitochondrial genes.

U2OS cells stably expressing FoxO3A(A3)–ER were induced with 250 nM 4-OHT 30 minutes after pretreatment with 20 μg/mL cycloheximide or solvent. RNA was extracted after 10 hours and qPCR analysis was performed. Results are normalized to 18S rRNA and are shown as fold of untreated control. Error bars indicate mean ± SD of three independent experiments **p<0.01, ***p<0.001 using Student's t-test.

Figure S9. FoxO3A also antagonizes Myc on the promoters of *OXNAD1* and *LARS2*.

(A and B) HeLa ctrl, FoxO3A-KD#1, and FoxO3A-KD#2 cell clones were incubated in normoxia or hypoxia (0.5% O_2) for 16 hours before cross-linking. ChIP was performed using anti-FoxO3A (upper panel), anti-Myc (lower panel), and IgG (negative control) antibodies. Primers scanning the *OXNAD1* (A) and *LARS2* (B) loci

were designed. Results were generated by qPCR and are presented as percentage bound/input. Error bars indicate mean ± SD.

Figure S10. Hypoxic repression of canonical Myc target genes is independent of FoxO3A.

(A) Hela Ctrl and FoxO3A knockdown cell clones were incubated for 20 hours in normoxia or hypoxia (0.5% O_2). RNA was extracted and analyzed by qPCR. The qPCR results are normalized to 18S rRNA and are shown as fold of normoxic Ctrl. Error bars indicate mean ± SD of three independent experiments. (B) HeLa ctrl and FoxO3A knockdown cell clones were incubated in normoxia or hypoxia (0.5% O_2) for 16 hours before cross-linking. ChIP was performed using anti-FoxO3A (upper panel), anti-Myc (lower panel), and IgG (negative control) antibodies. Primers targeting the E-boxes of *CAD* and *ODC* were designed. Results were generated by qPCR and are presented as percentage bound/input. Error bars indicate mean ± SD.

Figure S11. FoxO3A(A3)-ER and FoxO3A(A3)-H212R-ER antagonizes Myc at the *MRPL12* and *ACO2* loci.

FoxO3A(A3)-ER or FoxO3A(A3)-H212R-ER stably expressing U2OS cell clones were incubated in the absence or presence of 250 nM 4-OHT for 2 hours before cross-linking. ChIP was performed using anti-FoxO3A, anti-Myc, and IgG (negative control) antibodies. Results were generated by qPCR using primers scanning the *MRPL12* (A) and *ACO2* (B) loci and are presented as percentage bound/input. Error bars indicate mean ± SD.

Figure S12. FoxO3A(A3)-ER and FoxO3A(A3)-H212R-ER antagonizes Myc at the *OXNAD1* and *LARS2* loci.

FoxO3A(A3)-ER or FoxO3A(A3)-H212R-ER stably expressing U2OS cell clones were incubated in the absence or presence of 250 nM 4-OHT for 2 hours before cross-linking. ChIP was performed using anti-FoxO3A (upper panel), anti-Myc (lower panel), and IgG (negative control) antibodies. Results were generated by qPCR using primers scanning the *OXNAD1* (A) and *LARS2* (B) loci and are presented as percentage bound/input. Error bars indicate mean ± SD.

Figure S13. FoxO3A(A3) and Myc do not interact directly in normoxia or hypoxia.

HeLa cells were co-transfected with expression constructs for HA-FoxO3A(A3) and Myc. Eight hours post transfection, cells were incubated in normoxia or hypoxia (0.5% O_2) for a further 16 hours before lysis. Immunoprecipiation was performed using anti-HA, anti-Myc and IgG (negative control) antibodies followed by immunoblotting.

Figure S14. Examples of classification of FoxO3A staining in DCIS.

Representative images are shown to exemplify the scoring of FoxO3A staining in DCIS as ‘nuclear FoxO3A signal increasing towards the necrotic core’ (A) or ‘no increase in nuclear FoxO3A signal towards the necrotic core’ (B). The increased nuclear FoxO3A staining towards the necrotic core is highlighted by red color (A).

The evenly distributed, predominantly cytoplasmic staining is highlighted by green color (B). This type of analysis was the basis for the statistical analysis presented in supplementary Table S7. Scale bars indicate 100 μm (A) and 50 μm (B)

Figure S15. FoxO3A knockdown xenograft tumors display increased levels of caspase 3 cleavage.

Four pairs of Ctrl and FoxO3A-KD#1 tumors were stained by immunefluorescence for cleaved caspase-3 positive cells (red channel, see arrows). Representative confocal images of a control tumor (A) and a FoxO3A-KD#1 tumor (B) are shown. The tumors were counterstained with DAPI (blue channel). Scale bar indicates 50μm.

Supplementary materials and methods

Microarray analysis

RNA was extracted using an RNeasy mini kit (Qiagen). Samples were labeled according to the manufactures guidelines. In short, 2 μg of total RNA was transcribed into cDNA using an oligo-dT primer containing a T7 RNA polymerase promoter. cDNA was used as a template in the in-vitro-transcription reaction driven by the T7 promoter under which, Biotin labeled oligo-nucleotides were incorporated into the synthesized cRNA. The labeled samples were hybridized to the HG-U133plus2 GeneChip array (Affymetrix). The arrays were washed and stained with phycoerytrin conjugated streptavidin (SAPE) using the Affymetrix Fluidics Station® 450, and the arrays were scanned in the Affymetrix GeneArray® 2500 scanner to generate fluorescent images, as described in the Affymetrix GeneChip® protocol.

The Cel files were imported into the statistical software package R v. 2.7.2 using BioConductor v. 2.8 and RMA modeled using quantiles normalization and "Lowess" summarization. The modeled log-intensity of 56,400 probe sets was used for high-level analysis of selecting differentially expressed genes.

The gene expression values were imported into the software package dChip for class comparison analysis. Genes were defined as being differentially expressed in a class comparison if they were selected in the uni-variate two-sample t-test. A probe set is defined as being differentially expressed if the p-value is below 0.02 applying and have a fold change larger than 1.3 between the groups compared.

Immunoblotting

For Immunoblotting, cells were lysed in TNN buffer (50 mM Tris-HCl, pH 7.5; 250 mM NaCl; 5 mM EDTA; 0.5% NP-40 supplemented with protease and phosphatase inhibitors). Primary antibodies used were anti-HIF-1α (BD Bioscience 610959), anti-β-actin (Chemicon MAB1501), anti-Myc (9E10 hybridoma clone culture supernatant), anti-cleaved caspase 3 (Cell signalling #9661), anti-FoxO3A (Upstate), anti-FoxO1 (Cell signalling #9462), anti-FoxO4 (Cell signalling #9472), anti-GAPDH (Abcam ab9484), anti-histone H3 (Abcam ab1791), anti-MRPL12 (abcam ab58334) anti-Mxi1 (Santa Cruz sc-1042) The antibodies were used in the concentrations recommended by the suppliers.

Immunoprecipitation

Cells were lysed in TNN buffer as described above. The lysate was precleared for 30 minutes using protein G sepharose beads. 800 μg of protein was used per IP. For HA, monoclonal anti-HA agarose (Sigma A2095) was used. For Myc, 5 μg of purified anti-Myc (9E10) antibody was used. 5 μg of mouse IgG (Sigma) was used as negative control. The IPs were incubated with gentle rotation at 4 °C for 4 hours, followed by 3 washes with cold TNN and finally heated at 95 °C for 5 minutes in SDS sample buffer.

Cell fractionation

Cells were lysed/swelled in hypotonic Dignam A Buffer (10 mM Hepes-KOH pH 7.9; 1.5 mM $MgCl_2$; 1 mM EDTA; 0.5% NP-40 supplemented with protease and phosphatase inhibitors) for 20 minutes on ice. Cytosolic and nuclear fractions were subsequently separated by centrifugation at 2000g for 5 min at 4^oC. The nuclear pellet

was Lysed in Dignam C (20 mM Hepes-KOH, pH 7.9; 400 mM NaCl; 1.5 mM $MgCl_2$; 1 mM EDTA; 20% glycerol supplemented with protease and phosphatase inhibitors) and homogenized by passing through a syringe.

NAO staining

Cells were incubated in NAO-containing media (10 nM) for 30 minutes in the incubator, harvested, resuspended in PBS + 5% FCS (4 °C) and analyzed by flow cytometry on a FACS-Calibur (BD Biosciences). A minimum of 10,000 events were analyzed using FlowJo software. Treatment and staining of hypoxia-treated cells was performed with pre-equilibrated, hypoxic solutions.

Oxygen consumption measurements

Cellular oxygen consumption was determined by polarographic respirometry in an Oxygraph-2k (Oroboros) in full DMEM, glucose-free, or glutamine free medium +10% FCS at 37 °C. Prior to collection of the cells by trypsinization, the cells were equilibrated for 1 hour in the appropriate media type using hypoxic solutions for the hypoxic samples. Measurements were done in triplicates and the rate of oxygen consumption was normalized to cell numbers.

DCFDA staining for ROS

HeLa stable ctrl and FoxO3A knock-down cell clones were incubated in normoxia or hypoxia (0.5% O_2) for a further 24 hours. Cells treated with 100 μM tert-butylperoxide for 15 minutes served as control. Cells were equilibrated in 2μM carboxy-H_2DCFDA in PBS/50mM HEPES for 30 minutes, followed by a recovery in full DMEM + 10% FCS for 30 minutes, washed in PBS, trypsinized and resuspended

in ice cold PBS with 10% FCS. Analysis was performed by flow cytometry on a FACS-Calibur (BD Biosciences). A minimum of 10,000 events were analyzed using FlowJo software. Treatment and staining of hypoxia-treated cells was performed with pre-equilibrated, hypoxic solutions.

Growth curves and cell death assays

HeLa control and stable FoxO3A knock-down cell clones were plated in triplicate in 6-well plates and left to settle for 24 hours before incubation in normoxia or hypoxia. At each 24 hour time interval, the cells were trypsinized, stained with trypan blue and viable cells were counted. For transiently siRNA transfected U2OS cells, the cells were transfected in 10 cm dishes as detailed above and replated into 6-well dishes 24 hours after transfection.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tumor material including ductal carcinoma in situ (DCIS) was identified from archive materials and samples with central necrotic areas were selected. From this test series of DCIS samples, 4 μm sections were de-paraffinised with xylene and rehydrated in graded ethanol series. Slides were then processed in a PT Link system (Dako, Glostrup, Denmark) using the Envision Flex Target retrieval solution (Dako) and the High pH-kit (K8004, Dako) for 20 minutes at 98 °C before processing in an automated immunostainer (Dako Autostainer Plus, Dako). Antibodies used were anti-CAIX (M75, kindly provided by S. Pastorekova), anti-p27 (BD biosciences), and anti-Foxo3A (Cell Signaling). For the M75 antibody, additional post staining EnVision Flex Mouse (Linker) treatment was applied for 15

minutes (K8021, Dako). The study was approved by the ethical committee at Lund University.

In vivo xenograft tumor experiments

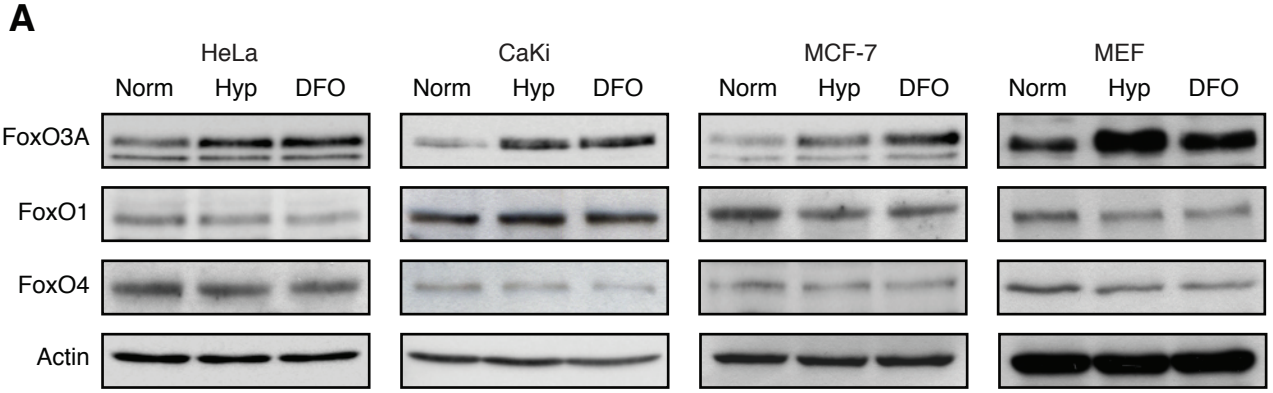
Cells were collected using Cell Disassociation Solution (Sigma) and resuspended in a 50/50% slurry of ice-cold matrigel and DMEM (+10% FCS, +5% P/S) at a concentration of $4x10^6$ cells per 100 µl.

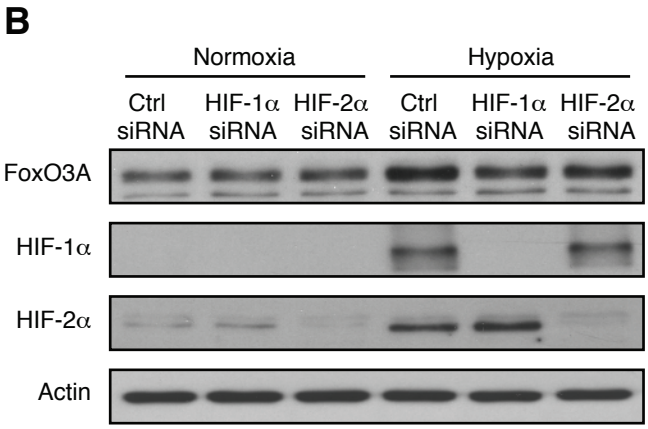
Twenty-four Nude-NMRI mice (Taconic, Borup, Denmark) were split in two groups of 12 (A and B) and injected subcutaneously with either $8x10^6$ HeLa Ctrl and FoxO3A-KD#1 cells or $8x10^6$ HeLa Ctrl and FoxO3A-KD#2 cells in either flank in a 200 µl volume with a 1:1 mixture of suspended cells and Matrixgel™ (BD Biosciences, San Jose, CA, USA). Tumor growth was monitored using a caliper. All experiments involving animals were approved by the national authority.

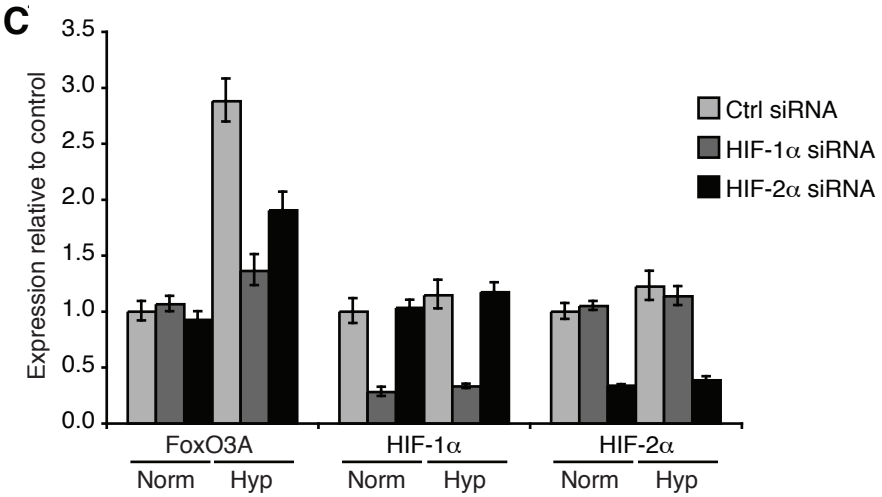
At the final day of the experiment, F-18-fluorodeoxyglucose (F-18-FDG) was injected intravenously in a tale vein of anesthetized mice fasted for at least 4 hours. The mice were kept warm after F-18-FDG injection. One hour post-injection PET scans were acquired on a MicroPET Focus 120 (Siemens). General anesthesia was maintained using 3% sevofluran (Abbott) mixed with 35% O_2 in N_2. Data were reconstructed with the maximum a posterior (MAP) reconstruction algorithm. A computer tomography (CT) scan was acquired prior to the PET scan for anatomical localization of foci on a MicroCAT® II system (Siemens). PET and CT images were fused using the Inveon software (Siemens).

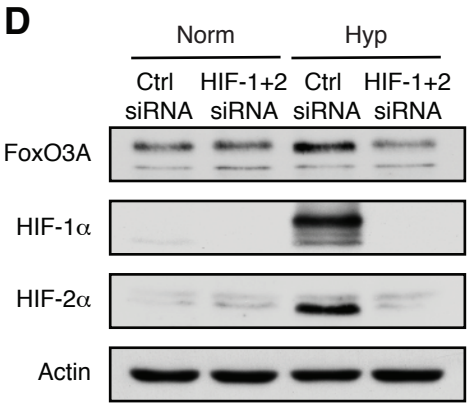
Immunofluorescence detection of apoptosis in experimental tumours

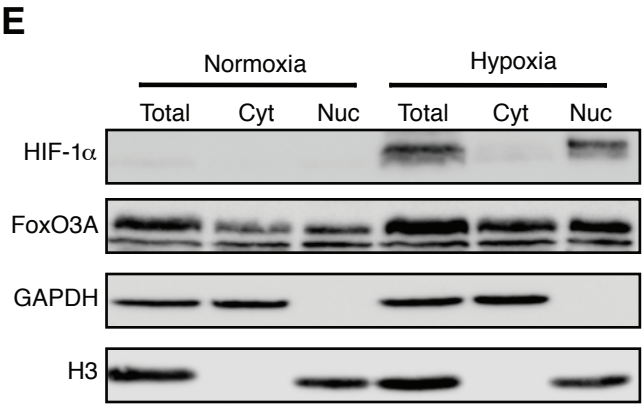
The xenograft tumour tissues were snap-frozen and sections at a thickness of 5-7 mm were prepared. The cryosections were immediately fixed with 4% freshly prepared paraformaldehyde in PBS pH 7.4 at room temperature for 30 min, followed by 3 washes in PBS. Free aldehydes were quenched with 25mM glycine in PBS for 30 min and rinsed 3 times in PBS. Apoptosis in tumour cells was detected using a rabbit monoclonal antibody against human cleaved caspase-3 (Asp175, clone 5A1E, diluted 1:100; Cell Signaling Technology) followed by immunofluorescence detection with a goat anti-rabbit Alexa Fluor 647-conjugated secondary antibody (diluted 1:1000; Molecular Probes, Invitrogen). Sections were washed in PBS, counterstained with DAPI (300nM for 3 min; Molecular Probes) and mounted with ProLong Gold antifade reagent (Molecular Probes). Confocal images were taken with a Zeiss LSM510 Laser Scanning Microscope, equipped with a diode (405nm) and a HeNe2 (633nm) laser and using a C-Apochromat 40x/1.2 Water Corr lens with a pinhole set at 1.0 Airy Units. Images were acquired using the ZEN 2009 Sp2 software and exported as Tiff files to Adobe Photoshop CS4 (Adobe Systems Inc.).











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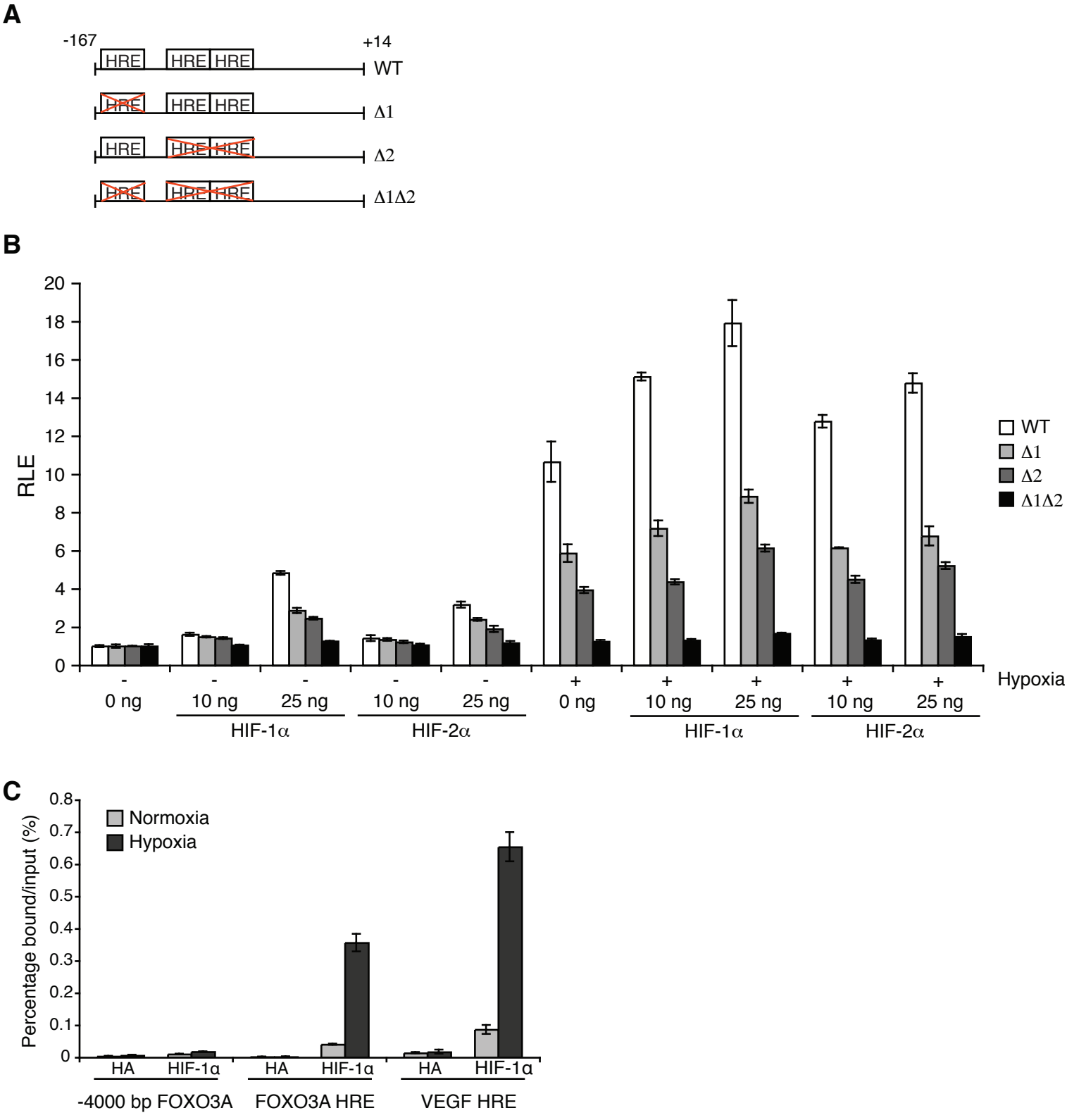

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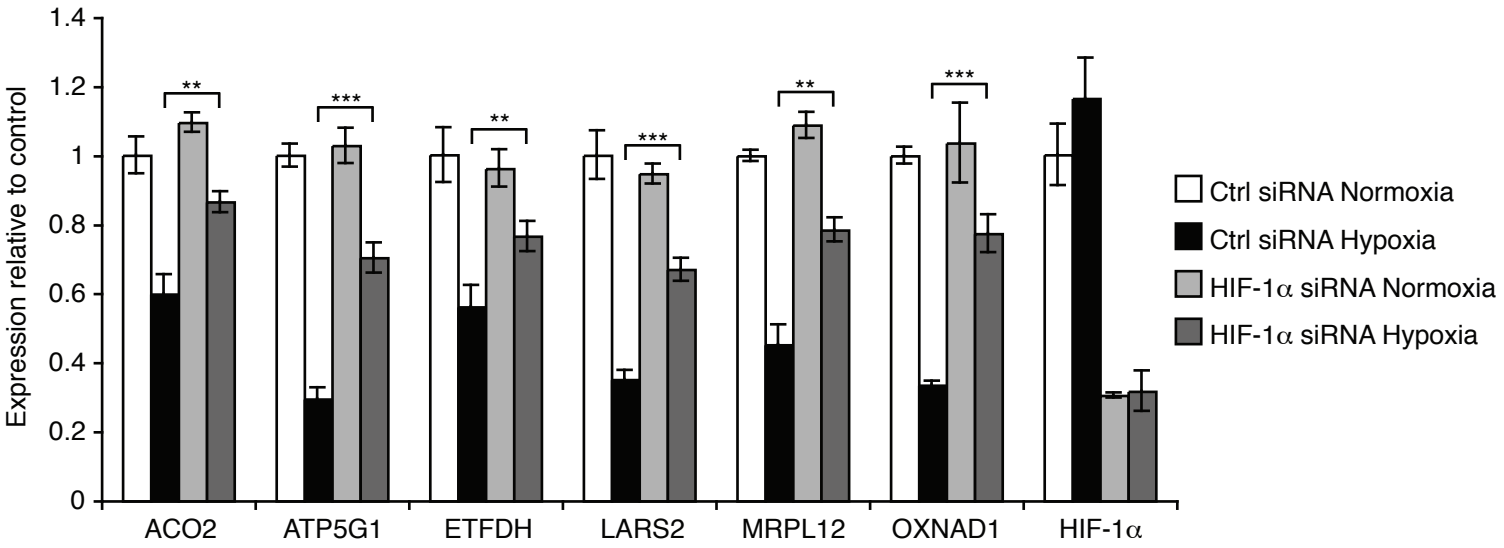

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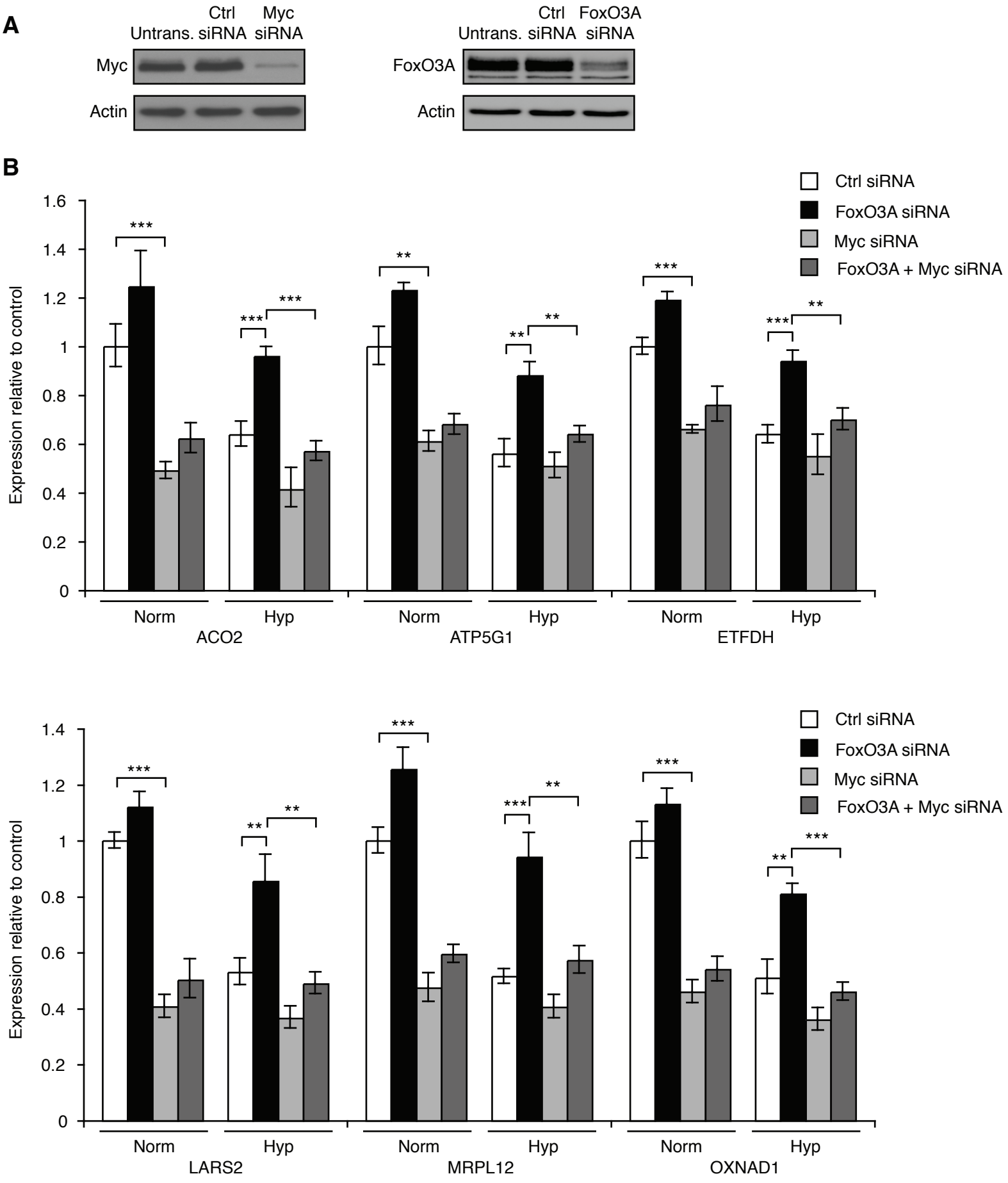

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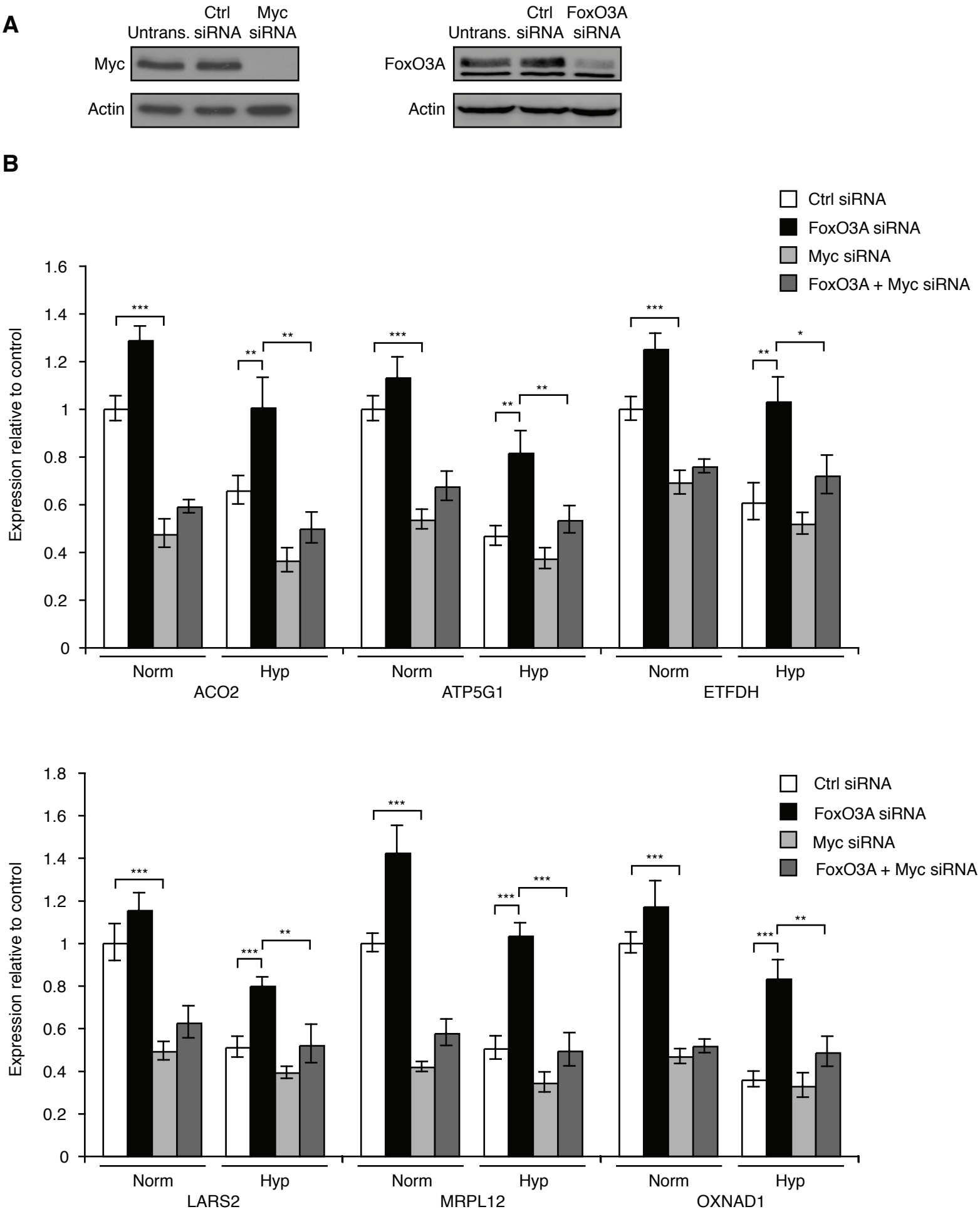

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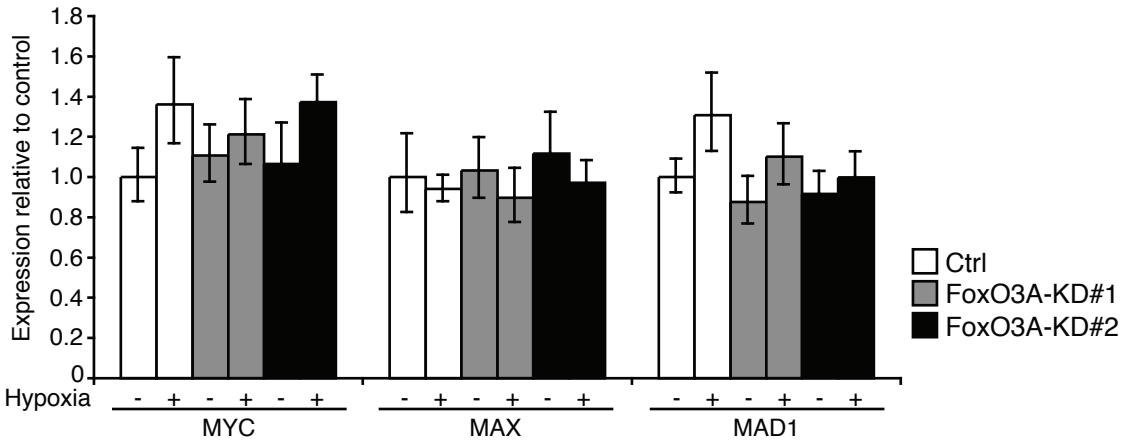

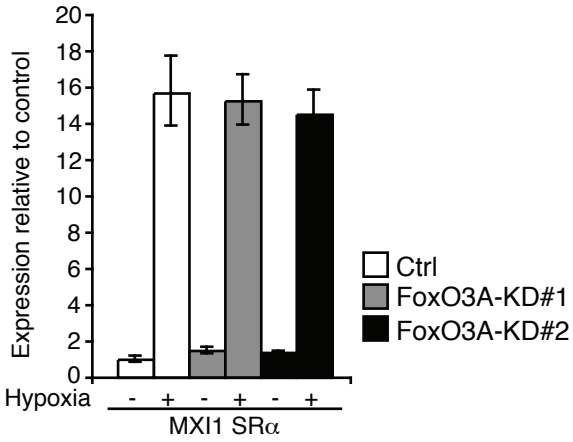

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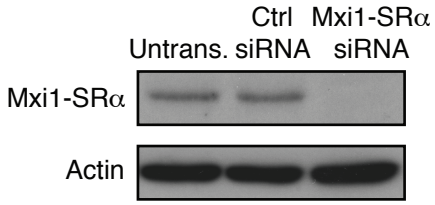

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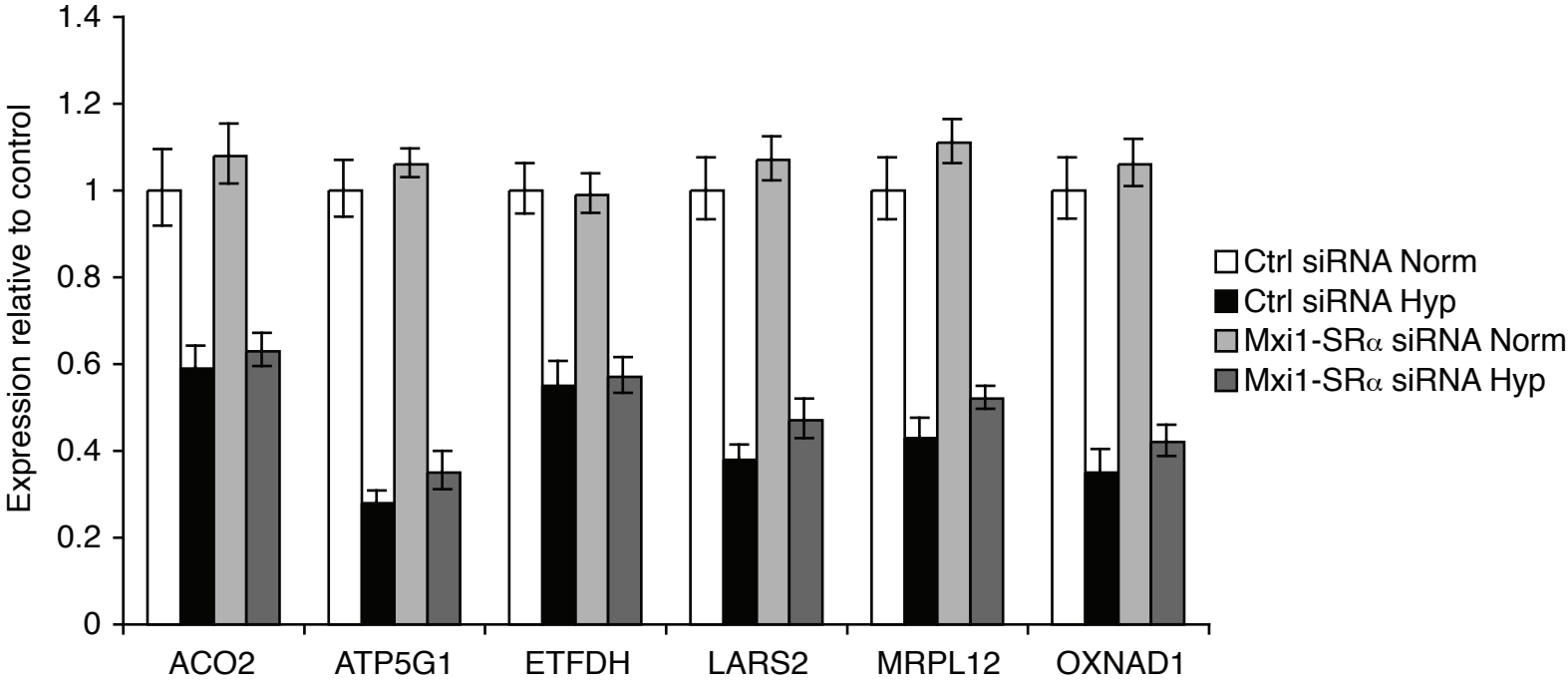

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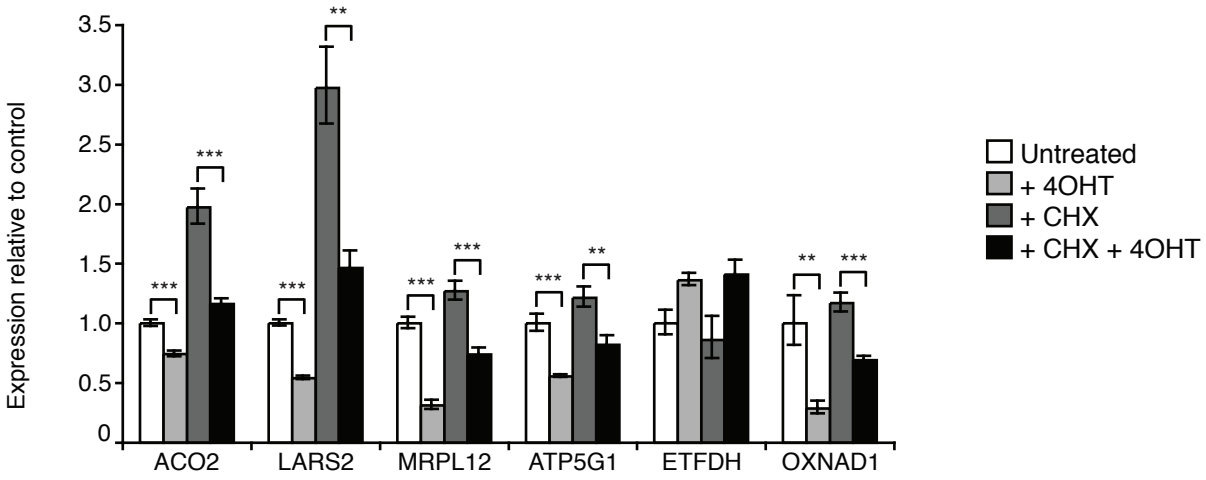

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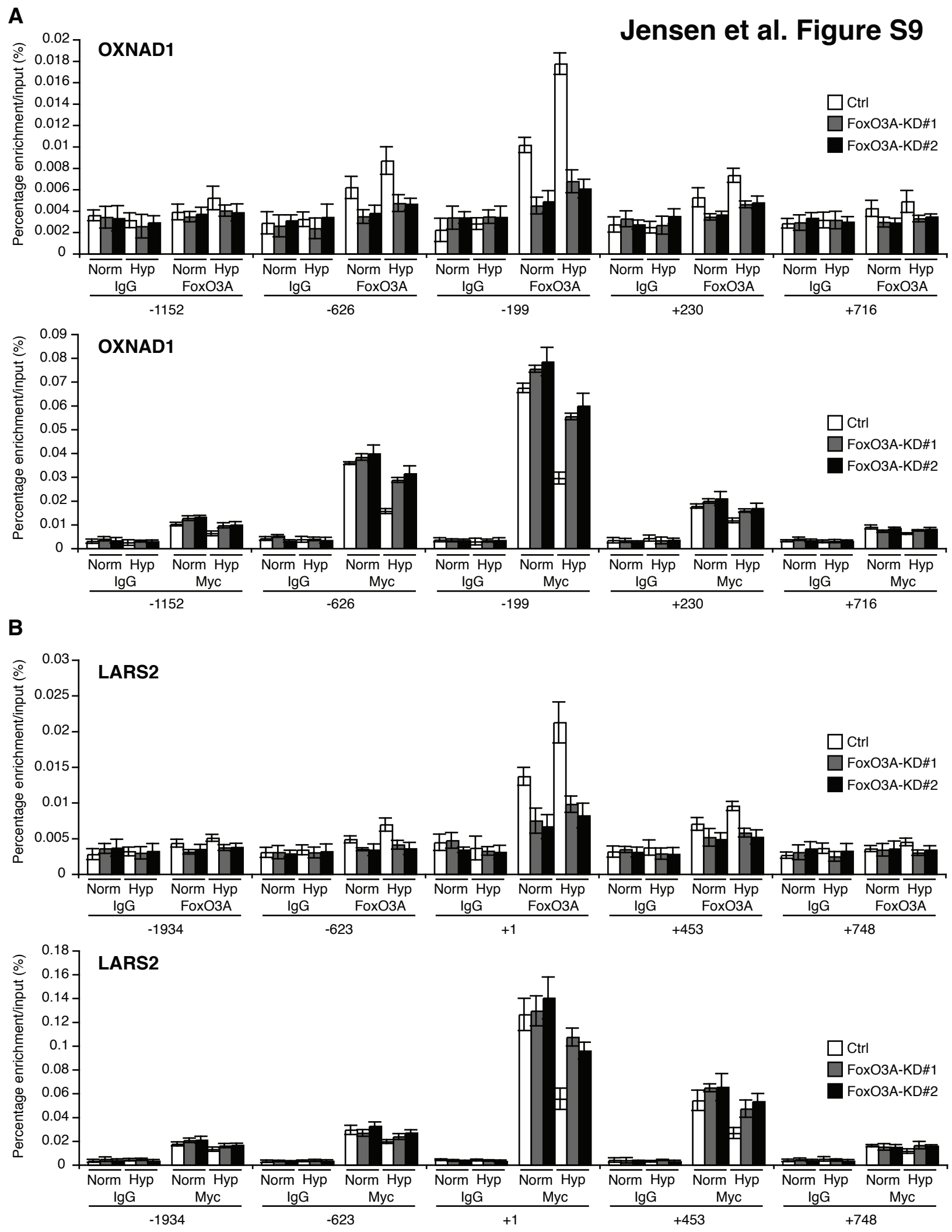
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FoxO3A-KD#2
Norm Hyp
IgG
FoxO3A
-1152
-626
-199
+230
+716
OXNAD1
Percentage enrichment/input (%)
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FoxO3A-KD#2
Norm Hyp
IgG
Myc
-1152
-626
-199
+230
+716
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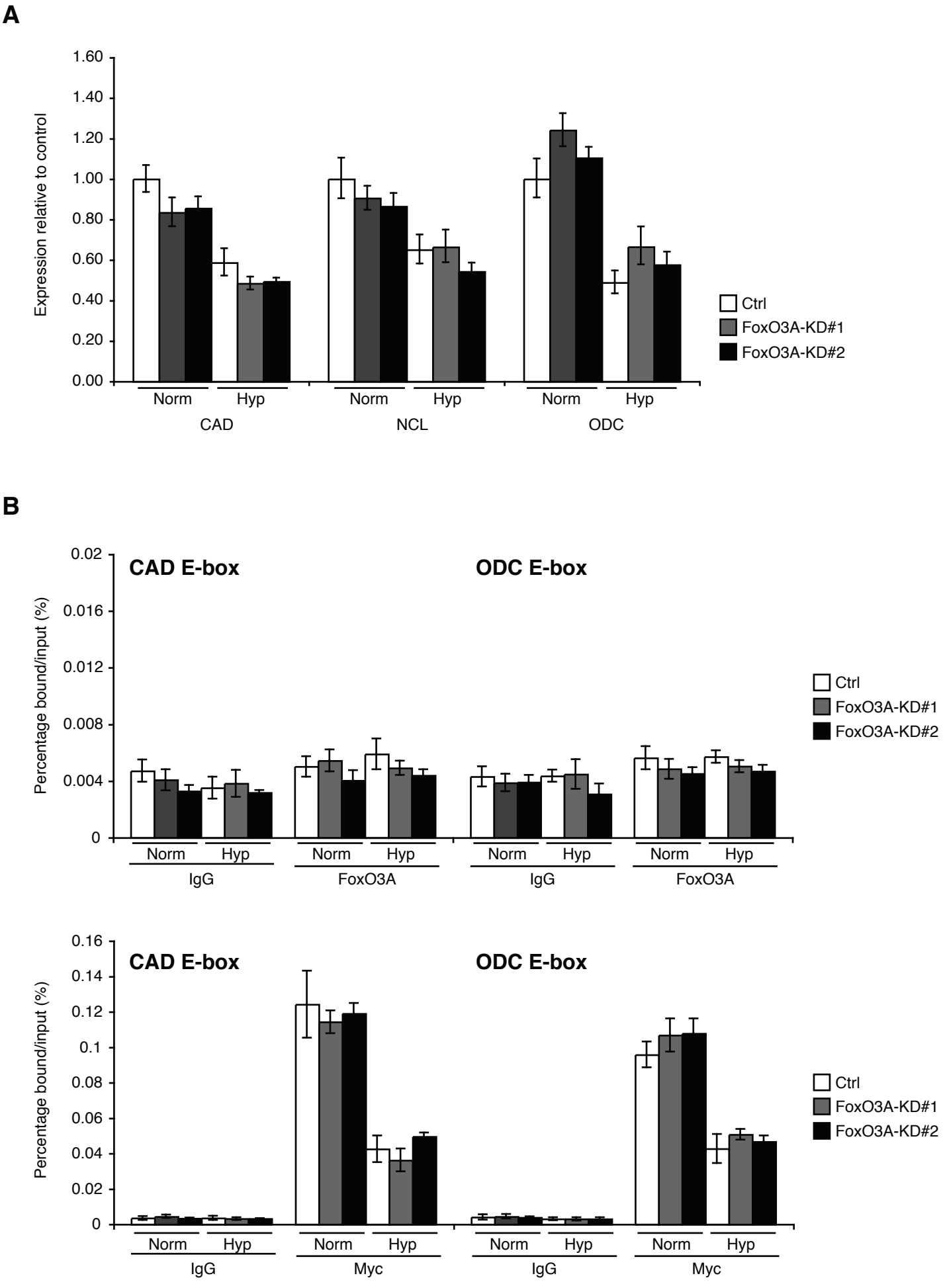

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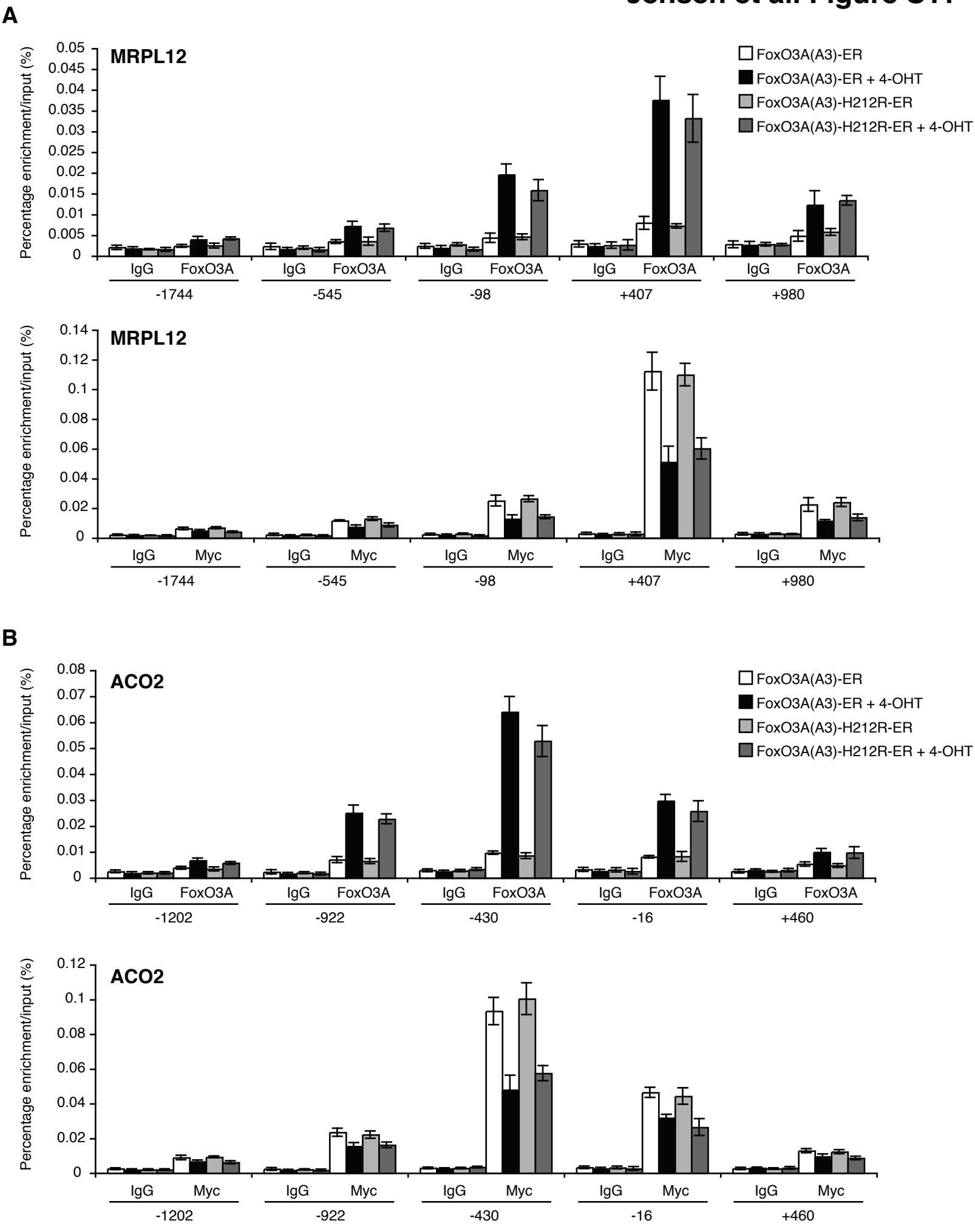

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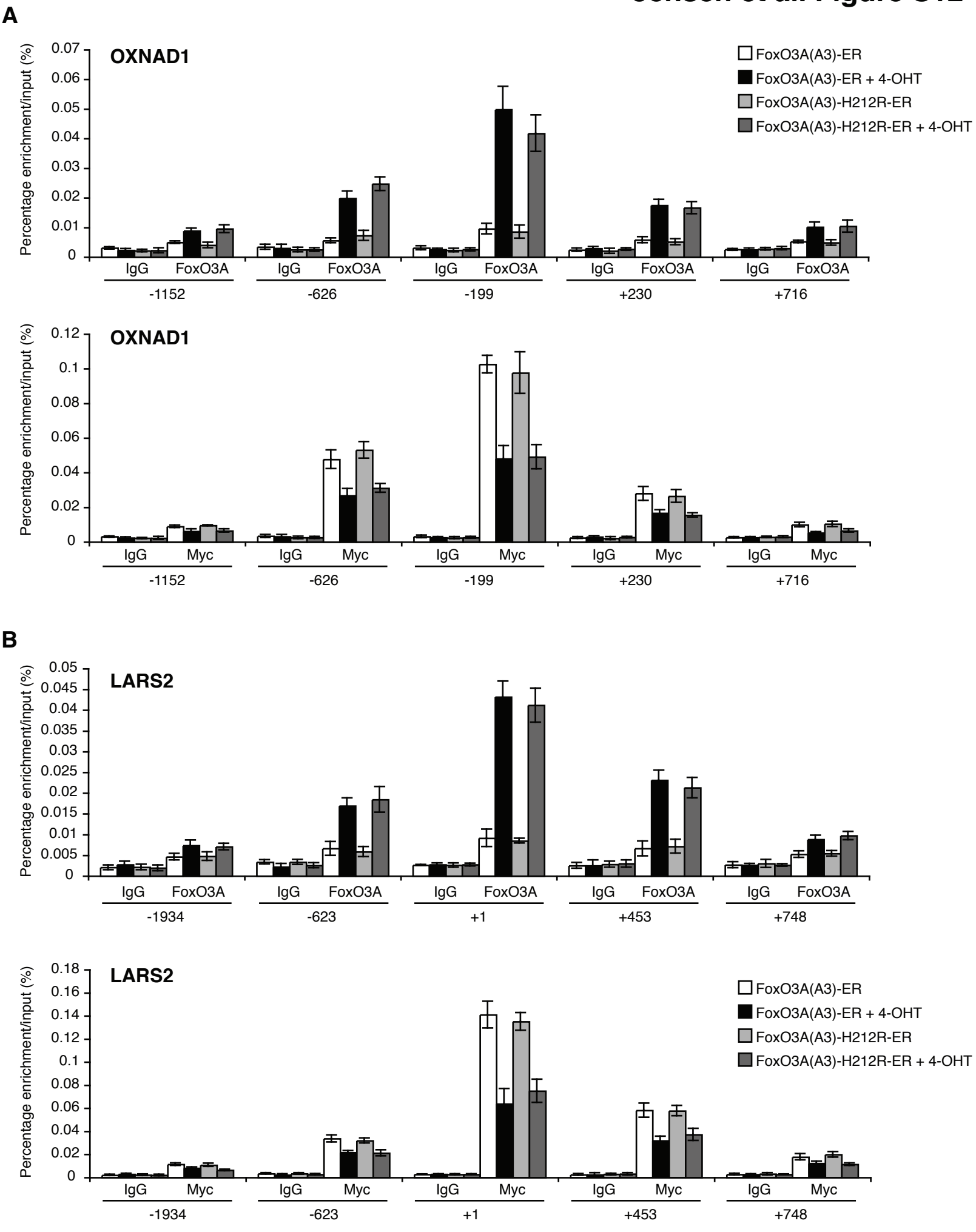

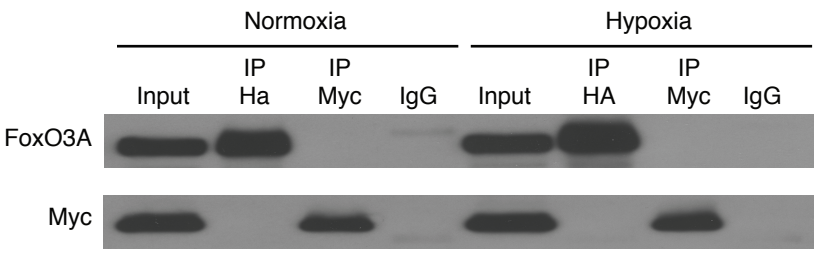

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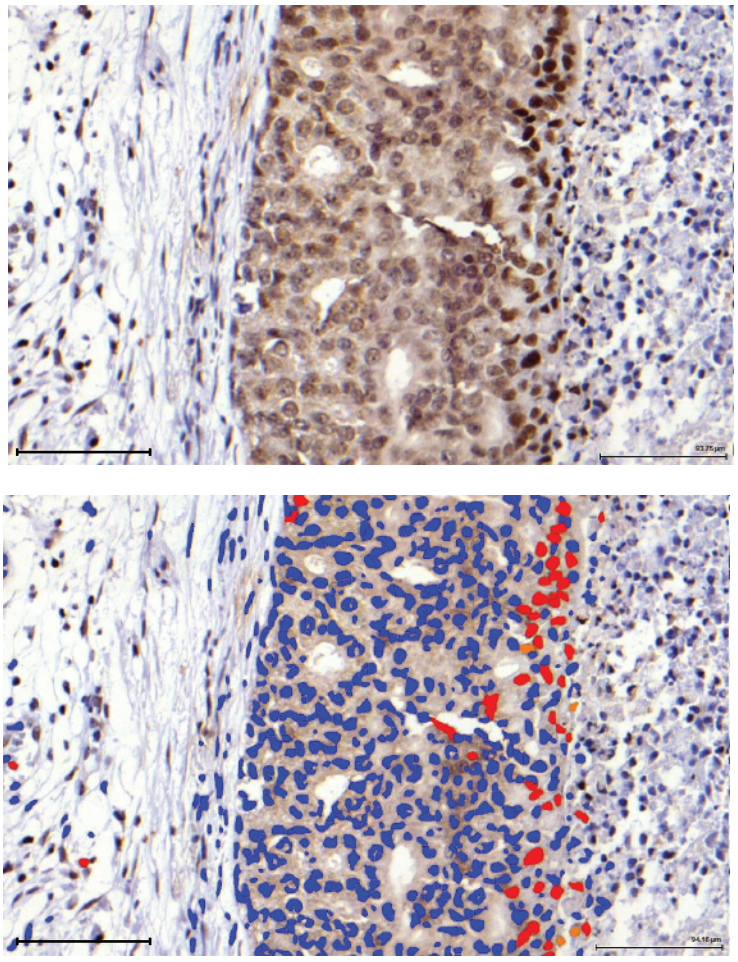

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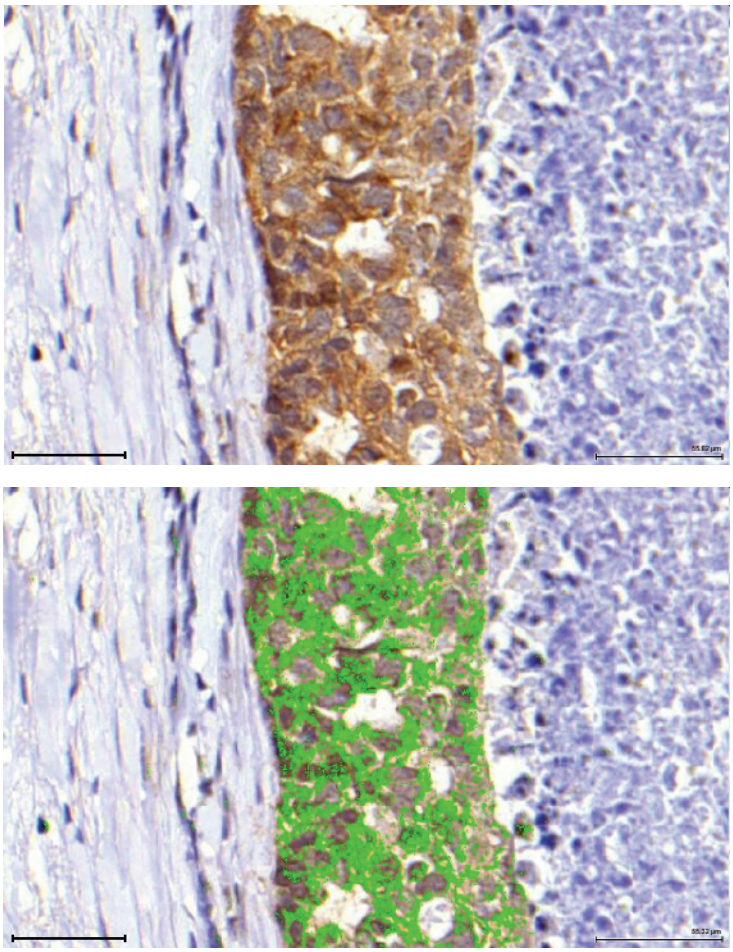

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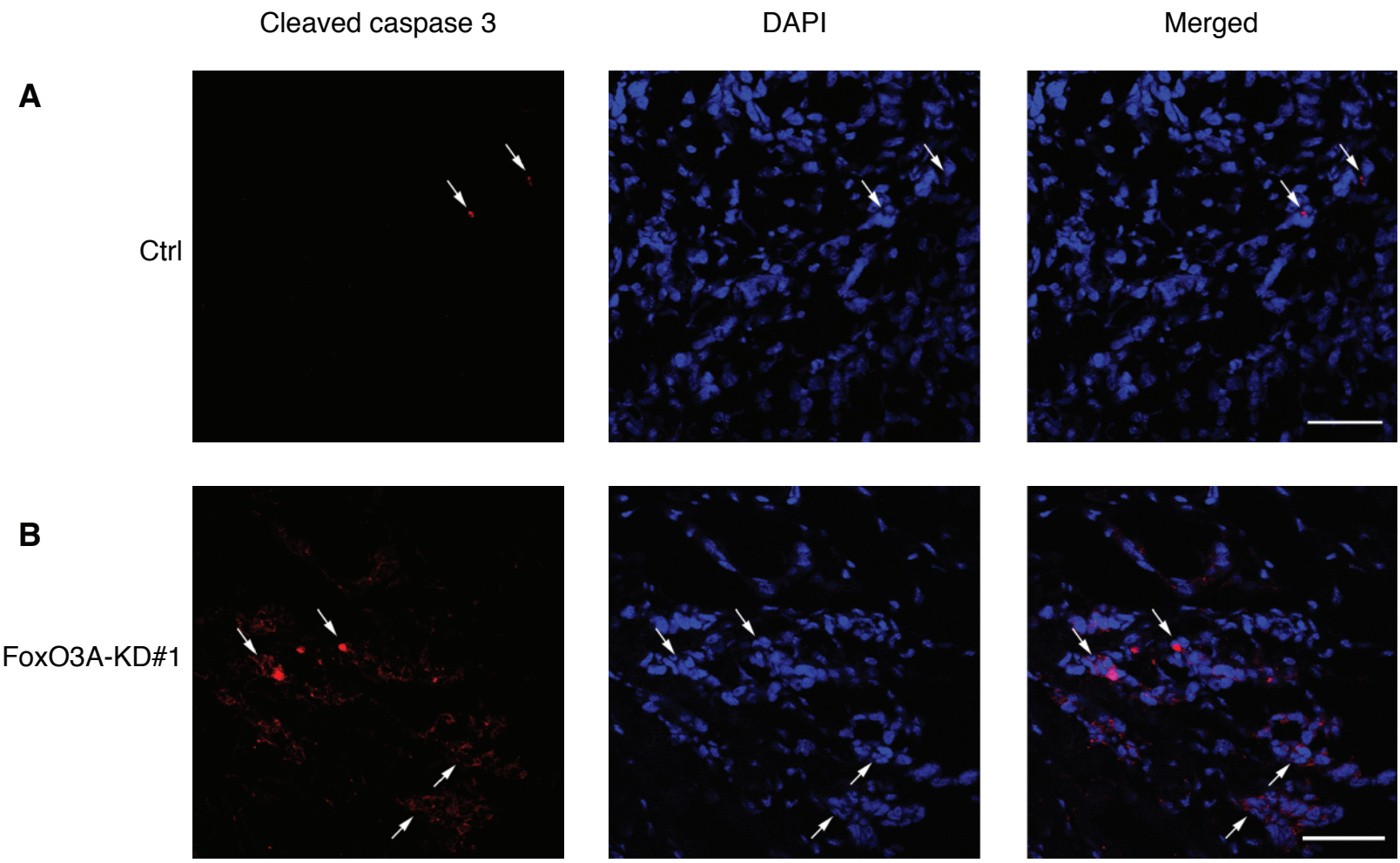

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