Supplemental Materials

FAK activation promotes SMC dedifferentiation via increased DNA methylation in contractile genes by stabilizing DNMT3A

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Short title: FAK and DNMT3A in SMC plasticity

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Online Supplemental Materials and Methods

Generation of smooth muscle cell specific FAK-WT and -KD mice

SMC-specific Cre (Myh11-Cre-ER^{T2}) male mice were bred with female FAK flox/flox (FAK$^{FL/FL}$) mice to generate FAK$^{FL/FL}$;Myh11-Cre-ER^{T2} mice. Male FAK$^{FL/FL}$;Myh11-Cre-ER^{T2} mice were crossed with female FAK wild type/kinase-dead (FAK$^{WT/KD}$) mice to generate SMC-specific FAK$^{FL/WT}$;Myh11-Cre-ER^{T2} and FAK$^{FL/KD}$;Myh11-Cre-ER^{T2} mice. The flox FAK allele was excised by treating male mice (age, 6 week) with tamoxifen (2 mg, intraperitoneal, every other day) for 2 weeks, generating FAK$^{-/WT}$;Myh11-Cre-ER^{T2} (FAK-WT) and FAK$^{-/KD}$;Myh11-Cre-ER^{T2} (FAK-KD) mice as described previously.[31]

Animal experiments

Animal experiments were approved and performed according to the guidelines of the University of South Alabama Institutional Animal Care and Use Committee. Mice were kept in a room on a 12h:12h light:dark timer. Additionally, mice were fed normal rodent chow and kept in cages with normal paper pulp bedding. Femoral artery injury was induced by wire insertion in C57BL/6 male mice (8-week-old) as described previously.[31] Only male mice were used in this study since Myh11-Cre-ER^{T2} is expressed on the Y chromosome. Animals were randomly assigned to the experimental groups by block randomization. Power analyses were performed to determine mouse numbers per group (4-5 animals) for 2-way ANOVA (Standard deviation 0.5, power 0.8, and significance 0.05). For sham controls, the same procedure was performed except for transverse arteriotomy. Mice were anesthetized with intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg). A straight wire (diameter, 0.38 mm; No. C-SF-15-15, COOK, Bloomington, IN) was inserted into the femoral artery toward the iliac artery and left in place for 1 minute to denude and dilate the artery. Blood flow was restored through the femoral artery, and the skin was sutured. For FAK inhibitor experiments, mice were treated twice daily with vehicle (10 mM citrate, pH 6.0) or FAK inhibitor VS-4718 (50 mg/kg) by oral gavage. Nonblinding treatments were not performed as VS-4718 is gray and not fully soluble in vehicle. For FAK-WT and FAK-KD mice, wire injury was performed after tamoxifen (2 mg in 100 μl corn oil) was injected every other day for 2 weeks. For short hairpin RNA (shRNA) DNMT3A lentiviral infection, concentrated lentivirus (10^7 transducing units) was mixed with Pluronic F-127 (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS) as described previously.[31] The lentiviral mixture was pasted to the outside of the femoral artery and incubated for 15 minutes before closing the incision. The contralateral right femoral artery was used as an internal control for injured samples. Arteries were excised and embedded in optimal cutting temperature

compound (OCT, Fisher Scientific, Waltham, MA) 2 weeks post-injury. Formation of hyperplasia was evaluated by hematoxylin and eosin staining. Areas of the media, intima, or neointima were analyzed by software (Image J, National Institutes of Health, Bethesda, MD), and severity of injury was evaluated by the intima-to-media (I/M) ratio. To evaluate the interaction between FAK and DNMT3A *in vivo*, mice were treated with MG132 (0.3 mg/kg) by intraperitoneal injection at 24, 6, and 1 h before euthanasia. Blinding procedures were not employed in this study due to the following reasons: high reproducibility, noticeable differences between specimen, and data acquisition by quantification.

Reagents

FAK (#05-537), SM22 (TAGLN) (#MABT167), Calponin (CNN1) (#C2687), alpha smooth muscle actin (ACTA2) (#A5228 and # ABT1487), 5-mC (SAB2702243), Ubiquitin (#04-262), H4ac (#06-866), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (#MAB374) antibodies were from Millipore (Burlington, MA). β-actin (#SC47778), DNMT3A (#SC365769), DNMT1 (#SC20701), and SRF (#SC335) antibodies were obtained from Santa Cruz Biotech (Dallas, TX). pY397 FAK (#44-624G), DNMT3B (#PA1-32317) antibodies were from Thermo Fisher (Waltham, MA). Caldesmon (CALD1) (#ab32330), H3K27ac (#ab4729), H3K9me3 (#ab8898), H3 (#ab1791) and H4 (#ab10158) antibodies were purchased from Abcam (Cambridge, UK). Smooth muscle myosin heavy chain (MYH11) (#BT562) antibody was from Biomedical Technologies (Stoughton, MA). vWF (#A0082) antibody was obtained from Agilent Technologies (Santa Clara, CA). MG132, cycloheximide, and actinomycin D were from Sigma Aldrich (St. Louis, MO). Small molecule FAK inhibitor VS-4718 was from MedKoo (Morrisville, NC). Fibronectin and Boyden chambers were obtained from Sigma-Millipore. Recombinant PDGF and Rat Collagen I (#344702001) were from R&D system.

Isolation of mouse smooth muscle cells

Mouse aortic SMCs were isolated from $FAK^{FL/WT}$ and $FAK^{FL/KD}$ mice on C57BL/6 background as described previously.[53, 54] Mice were anesthetized with intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg). The aorta was isolated and surrounding tissue excised under a surgical microscope. A 24-gauge syringe was inserted into the aorta, and the lumen was washed with PBS. The distal end of the aorta was ligated, the lumen was filled with collagenase II (2 mg/ml) for 30 min, and detached endothelial cells were flushed out 3 times with PBS. The aorta was dissected into pieces and embedded in Matrigel (Corning Inc., Corning, NY). After the SMCs migrated out of the aorta in Dulbecco Modified Eagle Medium

(DMEM) containing 20% fetal bovine serum (FBS), SMCs were then pooled together from multiple mice to be expanded and maintained in DMEM containing 10% FBS. Isolated SMCs were checked for expression of SMC markers such as Myh11 by immunostaining and immunoblotting. Isolated $FAK^{FL/WT}$ and $FAK^{FL/KD}$ SMCs were treated with Cre adenovirus (Ad-Cre) to generate FAK $^{-/WT}$ (FAK-WT) and FAK $^{-/KD}$ (FAK-KD) SMCs. Successful deletion of FAK flox allele was confirmed with polymerase chain reaction (PCR) and immunoblot analysis.

Cell culture

Mouse SMCs and HEK293FT cells were maintained in DMEM containing 10% FBS at 37°C under 5% carbon dioxide. All cell culture experiments were performed with subconfluent cells in complete. Human coronary artery smooth muscle cells (hCASMCs; #CC2583, Lonza) grown in SMC growth basal medium (SmBM) and additional growth medium (SmGM-2 SingleQuots) were purchased from Lonza (Basel, Switzerland). Mouse and human primary SMCs (passage 4-8) were used for experiments.

Immunoblotting

Cells and femoral arteries were lysed in RIPA buffer (pH 7.4) that included 50 mM HIPES, 150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10% glycerol, and Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany). Lysates were cleared by centrifugation at 13 500 × g for 20 min at 4°C. Equal amounts of proteins were boiled in SDS loading buffer for 5 min. Samples were separated by 10% or 4-12% SDS polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membrane. Transfers were blocked 1 h at RT with 2% (v/v) BSA in TBST (0.1% Tween-20) and incubated for overnight at 4°C in primary antibody. The membranes were washed with TBST and incubated for 1 h at RT in HRP-conjugated secondary antibody in 2% BSA in TBST and then washed 3 times with TBST. The immunoblot was developed by using an ECL, and the signal was detected using Bio-Rad imager. All immunoblots were repeated at least three times. Blots were quantitated using ImageJ densitometry plugin and normalized using loading control.

Immunoprecipitation

Cells or aorta were lysed with Triton buffer that includes 1% Triton X-100, 50 mM HEPES, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM sodium orthovanadate, and protease inhibitors. The lysates were centrifuged at 13 500 × g for 20 min at 4°C and the supernatants were subjected to

immunoprecipitation with anti-FAK, ant-DNMT3A or anti-Myc antibody. The lysates were rotated overnight at 4°C, then 30 µl of protein G or A Agarose was added and the mixture was rotated for 2 h at 4°C. After incubation, beads were washed three times in ice-cold Triton buffer. After the final wash the pellet was re-suspended in 30 µL of 2× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. The immunoprecipitates were washed three times with Triton buffer and suspended with 2X SDS-loading buffer. Samples were separated by SDS-PAGE and immunoblotted.

Boyden chamber and wound healing migration assay

Migration of SMCs (5×10^4 cells) through a Boyden chamber (pore size, 8 µm) coated with fibronectin (10 µg/ml) with or without the presence of FAK-I (VS-4718, 2.5 µM) for 6 h was evaluated as described previously.[55] Migrated SMCs on the bottom chamber were stained with crystal violet and counted under an inverted microscope. For wound healing migration assay, after SMCs reached 90% confluence, they were serum starved for 12 hours. A scratch was made using a 10-µL pipette tip, and cells were treated with DMSO or FAK-I (2.5 µM) for 1 hour before PDGF-BB (20 ng/ml) stimulation. After 24 hours, percent of wound closure was calculated and plotted (ImageJ).

Collagen gel contraction assays

A collagen gel contraction assay was used to examine the contractile capacity of SMCs as described previously.[56] A collagen suspension (1 mg/ml) containing the SMCs (3×10^5 cells) was cast in each well of a 24-well well culture plate and allowed to polymerize for 30 min at 37°C. Once polymerized, the gel was carefully detached from the 24 well bottom and side wall and added 0.5 ml culture medium. 10 hours after the detachment of gel from the dish, the gel surface area was measured and demonstrated as percentage of the initial surface area.

Analysis of FAK activation and DNMT3A expression on various matrix

SMCs were starved with 0.5% FBS DMEM for overnight. The cells were trypsinized, added soybean trypsin inhibitor (0.25 mg/ml), and centrifuged. The pellets were resuspended in DMEM containing 0.5% BSA at 37 °C for 1 h to eliminate basal signaling activity. Resuspended cells were plated at equal cell density on tissue culture plastic, poly-L-lysine and various ECM (laminin, gelatin, collagen I, or fibronectin) coated dishes (10 µg/ml) at 37 °C for 1 or 3 h. After incubation for the indicated times, cells were lysed and subjected to immunoblotting.

Genomic DNA dot blot

Genomic DNA (gDNA) was extracted from SMCs or femoral arteries using QIAamp DNA mini kits according to the manufacturer's instructions (Qiagen). gDNA samples were diluted with 2 N NaOH and 10 mM Tris-HCl, pH 8.5, and then loaded on Amersham Protran Nitrocellulose membranes 0.2 μm (GE Healthcare) using a 96-well dot-blot apparatus (Bio-Rad). After being dried at RT for 1 h and gDNA was crosslinked to the membrane using GS Gene Linker (Bio-Rad, Hercules, CA), then blocked with 5% nonfat milk in TBST for 1 h at RT. Membranes were incubated in primary antibodies against 5-mC at 4 °C overnight. 5-mC were visualized by using an ECL. To ensure equal loading, membranes were stained with methylene blue (0.02%) after immunoblotting.

Bisulfite conversion and sequencing

Bisulfite conversion of gDNA was performed using EpiTect Fast DNA bisulfite kit (Qiagen). Following PCR amplification, products were subcloned into pGEM T-Easy Vector (Promega). 9 individual clones were sequenced.

RNA extraction and quantitative real-time quantitative polymerase chain reaction

Total RNAs were isolated using RNeasy kit (Qiagen) and converted to cDNA using random hexamers and Superscript III (Life Technologies, Carlsbad, CA). Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using iTaq Universal SYBR Green SMX and CFX Connect real time system (Bio-Rad). All qPCR reactions were performed with the following steps: initial denaturation, 95°C for 10 min; 40 cycles of denaturation, 95°C for 15 sec and annealing/extension, 60°C for 60 sec. Quantification was performed using the $\Delta\Delta$CT method. All RT-qPCR was repeated at least three times and mean ±SEM was used as error bar. PCR primers were listed in Major Resources Table.

RNA sequencing and analysis

Mice were treated with or without FAK-I for 2 weeks after wire injury. Blood vessels were pressure-perfused with saline containing 5 mM EDTA via the left ventricle after severing the inferior vena cava. Femoral arteries were isolated, and the fat and adventitia layers were carefully removed under surgical microscope. To remove endothelial and immune cells, the artery lumen was quickly flushed with 150 μl TRIzol lysis reagent (Invitrogen) using a 29-gauge insulin syringe.[57] Media were frozen in liquid nitrogen and lysed in 500 μl TRIzol per artery, and total RNA was isolated using the RNeasy column (Qiagen). Purified RNA was analyzed for

integrity, quality and quantity using Agilent BioAnalyzer 2100. Three independent RNA samples per experimental group were prepared. cDNA library preparation and RNA sequencing were performed on IlluminaHiSeq platform with paired-end 150-bp reads by Genewiz (South Plainfield, NJ). Raw data quality was evaluated with FastQC, reads were trimmed using Trimmomatric v.0.36 to remove adapter sequences and poor-quality nucleotides. Reads were then mapped onto Mus musculus GRCm38 reference genome using STAR aligner v.2.5.2b. Generated BAM files were used to extract unique gene hit counts using FeatureCounts from Subread package v.1.5.2. Genes were identified on mapped reads followed by downstream differential expression analysis using R package DESeq2. Genes with less than 5 reads per sample were removed. Wald test was used to statistically identify genes with P value <0.05. Using regularized logarithm (rlog) function, count data was transformed for visualization on a $\log_2$ scale. Kyoto Encyclopedia of Genes and Genomes (KEGG) was analyzed using ShinyGO v0.60 (http://bioinformatics.sdstate.edu/go/). Heatmap was generated using GraphPad and normalized (smallest value and the largest value of each gene expression were set to 0 and 100%, respectively), to draw a heat map illustrating the pattern of differential gene expression in the samples. The sequencing data in this manuscript have been deposited in the Gene Expression Omnibus under accession number GSE183143.

Human atherosclerosis specimens using public data sets.

Human DNMTs expression were analyzed using NCBI Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo) data repository (database accession GSE40231) with human atherosclerotic aorta samples with matched comparisons to non-atherosclerotic internal mammary artery in coronary artery bypass patients,[37] and GEO database accession GSE43292 with human carotid endarterectomy samples comparing atheroma plaque (stage IV and over of the Stary classification) and adjacent areas free of macroscopic disease (stages I and II).[38]

Generation of plasmid constructs and lentivirus production

pcDNA-Myc-DNMT3A (#35521) was from Addgene (Cambridge, MA). N-term (1 to 70 a.a.) or N-term deletion (71 to 912 a.a.) mutants of DNMT3A were PCR amplified and ligated into the *EcoRI* and *BamHI* restriction sites in the pcDNA3.1-Myc vector. Human UHRF1 and UHRF2 were amplified from human cDNA and ligated into the *KpnI* and *EcoRV* restriction sites in the pcDNA3.1-HA vector. Scramble, DNMT3A and DNMT3B shRNA were ligated into pLentiLox 3.7-puro using *HpaI* and *XhoI* sites. Primer sequences are listed in Major Resources Table. Scramble and DNMT3A shRNA were then transferred to pSicoR-mCherry (#21907,

Addgene)[58] using *XbaI* and *NotI* sites for *in vivo* tracing. pCDH-FLAG-FAK-WT-puro and FAK-NLM-puro (nonnuclear localizing mutant) constructs were generated as previously described.[31] Lentivirus was produced using a third-generation packaging system in HEK 293FT cells. At 72 hours after transfection, lentivirus-containing medium was centrifuged to remove cell debris and passed through a 0.45-μm filter. Lentivirus was concentrated by ultracentrifugation at 25,000 rpm for 1.5 hours using SW40-Ti rotor (Beckman Coulter, Brea, CA). Supernatant was removed and lentivirus pellet was resuspended in sterile PBS. Lentivirus was divided into aliquots and stored at −80°C until used.

Generation of stable smooth muscle cell lines

SMCs were incubated with concentrated lentivirus expressing scramble DNMT3A or shDNMT3B shRNAs (20 MOI). For FLAG-FAK-WT and FAK-NLM SMCs, isolated $FAK^{FL/FL}$ cells were transduced with FLAG-FAK-WT or -NLM lentivirus (20 MOI), and then treated with Ad-Cre (50 MOI) to delete endogenous FAK.[31] Successful deletion of FAK flox allele was confirmed with immunoblot analysis. After 48 hours, SMCs were selected with puromycin (2 μg/mL).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assay was performed as described previously.[59] Cells were fixed by 1% formaldehyde for 10 min at RT and quenched by 125 mM glycine for 5 min. Whole cell extracts were sonicated into 200- to 500-bp fragments for 30×20 s at 20% amplitude (Branson Sonifier, SFX150) in lysis buffer (1% SDS, 10 mM EDTA, 50mM Tris–HCl, pH 8.1 and protease inhibitors), precleared using salmon sperm DNA, and then incubated with a specific antibody overnight at 4°C. After washing the immunoprecipitates, DNA/antibody complexes were eluted, crosslinks reversed, and DNA was purified by QIAquick PCR purification kit (Qiagen). Input DNA starting from aliquots of sonicated sample was purified using QIAquick PCR purification kit. The purified DNA and input genomic DNA were analyzed by iTaq Universal SYBR Green SMX and CFX Connect real time system (Bio-Rad) with PCR primers listed in Major Resources Table.

Human atherosclerotic tissues

Experiments using human tissue were considered nonhuman research by the University of South Alabama Institutional Review Board due to the exclusive use of postmortem samples. Human tissue was collected during routine autopsy at the University of South Alabama University Hospital and frozen in OCT (Fisher Scientific).

Immunofluorescence staining

Frozen sections were fixed with cold acetone for 15 minutes and washed thrice with PBS. Samples were permeabilized with 0.1% Triton X-100 for 10 minutes, washed with PBS, and incubated with blocking solution containing 3% bovine serum albumin (BSA) and 1% goat serum for 1 hour at RT. Sections were incubated with anti-FAK (1:200), anti-pY397 FAK (1:100), anti-ACTA2 (1:500), anti-DNMT3A (1:100), anti-DNMT3B (1:100), anti-DNMT1 (1:100), or anti-vWF (1:500) overnight at 4°C. For staining of 5-mC, DNA was denatured with 2N HCL for 30 min, and then neutralized in Tris-HCl (PH 7.4) for 10 min before blocking. Sections were washed with PBS and incubated with conjugated goat anti-rabbit or mouse secondary antibodies (1:1000) (Alexa Fluor 594 or 488, Thermo Fisher) for 1 hour at RT. Species-specific IgG or secondary antibodies were used as a negative control. Slides were mounted (Fluoromount-G, SouthernBiotech, Birmingham, AL), and images were acquired with a confocal microscope at 60-fold magnification using Nikon A1R confocal microscope (Nikon, Tokyo, Japan). Cell aspect ratio was calculated by measuring the longest and shortest length of each cell from differential interference contrast (DIC) images using ImageJ.[32] Fluorescence intensity of the anti-TAGLN staining was measured for each cell and normalized to the cell area using ImageJ.

Statistical analysis

All data were presented as mean ± standard error of the mean (SEM). For western blots, RNA-seq, qPCR, ChIP, migration assay, and gel contraction experiments, as each data set is an average from a large number of cells, we assumed the data was normally distributed by the central limit theorem. Statistical analysis was performed using t-test (2-group studies). For >2 group comparisons we performed one-way ANOVA with Bonferroni multiple comparisons test. For data with more than 2 variables, analysis was performed using two-way ANOVA with Bonferroni multiple comparisons test. For in vivo sample sizes >6 (vessel parameters), each data was evaluated for normal distribution using Shaprio-Wilk. *P* were >0.05 on test indicated that the data were approximately normally distributed for each group. Bonferroni's correction to adjust for inflation of the type I error was used for multiple testing. Unless otherwise stated, a $P<0.05$ was considered statistically significant. Tests used to assess significance are detailed in each Figure legend and precise p-values are indicated on the graph. All statistical analyses were performed using Prism software v9.0d (GraphPad Software, La Jolla, CA). Power analyses were performed to determine sample size for 2-way ANOVA. Blinding procedures

were not employed in this study due to the following reasons: high reproducibility, noticeable differences between specimen, and data acquisition by quantification.

Online Figure Legends

Online Figure I. FAK inhibition reduces neointima formation following wire injury. **(A)** Mice were treated with vehicle or FAK-I (VS-4718, 50 mg/kg, twice daily) following wire injury. Representative H&E staining of femoral artery cross sections 2 weeks after wire injury for vehicle or FAK-I treated (n=6). **(B)** Representative H&E staining of FAK-WT and FAK-KD femoral artery cross sections 2 weeks after wire injury were shown (n=6). Scale bars, 50 μm. (**A and B**) The numbers above enlarged figures indicates quantification of nucleus in the intima (n=6). Vessel parameters including arterial perimeter, medial area, lumen area, neointimal area, and Intima/media ratio are measured and plotted. Data are mean±SD. Data passed Shapiro-Wilk test of normality. *P* values were determined using 2-way ANOVA with Bonferroni multiple comparisons test (**A, B**; *P* values adjusted for 6 comparisons).

Online Figure II. FAK inhibition induces SMC specific contractile gene expression. (**A**) FAK-I (VS-4718, 2.5 μM) was treated in SMCs for 2 days. Shown are replicates (#2 and #3) of immunoblots of lysates for active FAK (pY397 FAK), total FAK, DNMT3A, DNMT3B, DNMT1, MYH11, CALD1, ACTA2, CNN1, TAGLN, and GAPDH as loading control. Blots were quantified and plotted relative to GAPDH (n=3) and relative blot intensity were depicted in **Figure 1B** (replicate #1). (**B**) Shown are replicate immunoblots (#2 and #3) of lysates of FAK-WT or FAK-KD SMCs for active FAK (pY397 FAK), total FAK, DNMT3A, DNMT3B, DNMT1, MYH11, ACTA2, CNN1, TAGLN, and GAPDH as loading control. Blots were quantified and plotted relative to GAPDH (n=3) and relative blot intensity were depicted in **Figure 1G** (replicate #1). Data are mean±SEM. *P* values were determined using 2-way ANOVA with Bonferroni multiple comparisons test (**A**; *P* values adjusted for 2 comparisons), parametric t-test (**B**).

Online Figure III. FAK inhibition promotes spindle-like SMC morphology and TALGN expression. SMCs were treated with vehicle or FAK-I (VS-4718, 2.5 μM) for 2 days. Representative immunofluorescence staining for TAGLN and DIC (differential interference contrast) are shown (n=4). Dotted line indicates cuboidal-shaped SMCs. Merge: TAGLN and DIC. Scale bars, 50 μm. Measurement of Tagln intensity in spindle- or cuboidal-shaped SMCs were shown in **Figure 1D and E**.

Online Figure IV. FAK inhibition reduces SMC migration. Number of mouse SMCs (n=4) **(A)** or hCASMCs (n=3) **(B)** that migrated toward fibronectin (10 ng/μl) with or without FAK-I (VS-

4718, 2.5 μM) for 6 h were enumerated. **(C)** Shown is percent wound closure after 24 h of mouse SMCs stimulated with PDGF-BB (20 ng/ml) with or without FAK-I (n=3). Data are mean±SEM. *P* values were determined using parametric t-test (**A**, **B**), 1-way ANOVA with Bonferroni multiple comparisons test (**C**; *P* values adjusted for 6 comparisons).

Online Figure V. Changes in GATA4 levels do not alter contractile gene expression in SMCs. SMCs were infected either lentiviral shRNA GATA4 or GATA4 overexpressing plasmid and the cell lysates were subjected to immunoblotting for GATA4, MYH11, CALD1, ACTA2, CNN1, pY397 FAK, DNMT3A, DNMT3B, DNMT1, and GAPDH as loading control indicated antibodies (n=3).

Online Figure VI. Loss of FAK activity induces collagen gel contraction. FAK-WT or FAK-KD SMCs were embedded in collagen gel for 10 h. Representative images (left panel) from the collagen gel contraction are shown. Percent gel contraction was calculated by at 10h relative to initial area at 0 h and plotted (n=3). Data are mean±SD. *P* values were determined using parametric t-test.

Online Figure VII. RNA-sequencing analyses reveal that FAK activity primarily correlates with gene sets of vascular smooth muscle contraction following wire injury. Mice were treated with vehicle or FAK-I (VS-4718, 50 mg/kg) twice daily following wire injury for 2 weeks. **(A** and **B)** The top five most enriched KEGG terms in each compared group. The red or the blue bars represent the upregulated or downregulated KEGG pathways, respectively (n=3). **(C** and **D)** Heatmap analyses of differentially expressed genes (DEGs) associated with SMC differentiation and de-differentiation between sham vs. injured (n=3) or injured vs. injured+FAK-I (n=3). **(E)** Normalized read values for various cell lineage markers from RNA sequencing (n=3). *Ly6a* (mesenchymal-stem cell-like cells), *Lgals3* (macrophage-like cells), *Sox9* (chondrocyte-like cells). Data are mean±SEM. *P* values were determined using 1-way ANOVA Bonferroni multiple comparisons test (**E**; *P* values adjusted for 3 comparisons).

Online Figure VIII. RNA-Sequencing data reveal no significant difference in expression of SMC-specific contractile genes between sham and injured group treated with FAK-I. Mice were treated with vehicle or FAK-I (VS-4718, 50 mg/kg) twice daily following wire injury for 2 weeks. Total RNA from artery samples were analyzed. Normalized read values for SMC contractile genes from RNA sequencing (sham vs. injured+FAK-I, n=3) are plotted. *Myh11* **(A),** *Acta2* **(B),** *Cnn1* **(C),** *Tagln* **(D),** and *Cald1* **(E).** Data are mean±SEM.

Online Figure IX. FAK inhibition decreases DNA methylation. SMCs were treated with FAK-I for 24 h. Immunofluorescence staining for 5-mC is shown (n=4). The images on the right show

magnifications of the areas framed in the merge. DAPI was used for nuclei staining. Scale bars, 20 μm. Shown are replicate immunostaining (#2 and #3) in **Figure 2B**.

Online Figure X. Schematic of CpG island methylation within the CArG-containing regions of *Tagln* and *Myh11* promoters. CpG island and promoter regions of *Tagln* **(A)** and *Myh11* **(B)**. CpG dinucleotides are indicated in red. CArG box: SRF biding sites. Refer to main **Figure 2C** for bisulfite sequencing data with or without FAK-I treatment and FAK-WT or FAK-KD. % methylation levels are indicated as to differentially filled circles.

Online Figure XI. High DNMT3A expression is observed in human atherosclerotic specimens from different vessel beds compared to healthy samples. Human brachiocephalic artery **(A)**, renal artery **(B)**, and aortic arch **(C)** were excised postmortem, and frozen sections were made for immunostaining. Representative immunostainings for DNMT3A, ACTA2, and DAPI are shown (n=5). Merge: DNTM3A, ACTA2, and DAPI (blue). Dashed line, boundary between media and diffuse intima. White line indicates endothelial layer. L, lumen; LC, lipid core. Scale bars: x4, 100 μm; or x60, 50 μm. Boxed areas are shown in **Figure 2H**.

Online Figure XII. High 5-mC levels are observed in human atherosclerotic specimens from different vessel beds. Human brachiocephalic artery **(A)**, renal artery **(B)**, aortic arch **(C)** were excised postmortem, and frozen sections were made for immunostaining. Representative immunostainings for 5-mC, ACTA2, and DAPI (blue) are shown (n=5). Merge: 5-mC, ACTA2, and DAPI (blue). Dashed line, boundary between media and diffuse intima. White line indicates endothelial layer. L, lumen; LC, lipid core. Scale bars: x4, 100 μm; or x60, 50 μm. Boxed areas are shown in **Figure 3A**.

Online Figure XIII. DNMT3B and DNMT1 expression was not changed in both normal and diseased human arteries. Human brachiocephalic arteries were excised postmortem, and frozen sections were prepared. **(A)** Representative immunostainings for DNMT3B, ACTA2, and DAPI (blue) are shown. Merge: DNMT3B, ACTA2, and DAPI (n=3). **(B)** Representative immunostainings for DNMT1, ACTA2, and DAPI (blue) are shown. Merge: DNMT1, ACTA2, and DAPI (n=3). Dashed line: boundary between media and diffuse intima. L: lumen. LC: lipid core. Scale bars: x4, 100 μm; or x60, 50 μm.

Online Figure XIV. Analyses of DNMT expression in human atherosclerosis specimens using public data sets. (**A**) *DNMT* expression in biopsies of non-atherosclerotic arteries and atherosclerotic arterial wall (raw data, GSE40231) (n=20 internal mammary artery, n=20 atherosclerotic arterial wall, $\log_2$ scale). (**B**) The expression of *DNMTs* in early disease lesion or severe plaque (raw data, GSE43292) (n=32 early lesion, n=32 advanced plaque, $\log_2$ scale).

Individual data points are shown with mean. *P* values were determined by paired parametric t-test (**A**), unpaired parametric t-test (**B**)

Online Figure XV. Nuclear FAK reduces DNMT3A, but not DNMT3B. SMCs were treated with FAK-I (VS-4718, 2.5 μM). Immunostaining for FAK and DNMT3A or FAK and DNMT3B in SMCs treated with/without FAK-I for 12 h is shown. Merge: FAK and DNMT3A. Scale bars, 20 μm. Shown are replicate immunostaining (#2 and #3) in **Figure 3B**.

Online Figure XVI. FAK activity regulates DNMT3A expression.
SMCs were treated with FAK-I (VS-4718, 2.5 μM) for 12 h. Representative immunofluorescence staining for pY397 FAK and DNMT3A is shown (n=4). Merge: pY397FAK and DNMT3A. Scale bars, 20 μm.

Online Figure XVII. Nuclear FAK regulates DNMT3A turnover and promotes SMC contractile gene expression. FAK -/- SMCs stably expressing either FLAG-FAK-WT or –NLM (nonnuclear localizing mutant) were treated with/without FAK-I (VS-4718, 2.5 μM) for 24 h. Replicate immunoblots (#2 and #3) are shown for pY397 FAK, FAK, DNMT3A, DNMT3B, DNMT1, MYH11, CALD1, ACTA2, CNN1, TAGLN, and GAPDH as loading control. Blots were quantified and plotted relative to GAPDH and relative blot intensity were depicted in **Figure 3C** (replicate #1) (n=3). Data are mean±SEM. *P* values were determined using 2-way ANOVA Bonferroni multiple comparisons test (*P* values adjusted for 2 comparisons).

Online Figure XVIII. Cytoplasmic and active FAK is observed in human atherosclerotic specimens from different vessel beds compared to healthy samples. Human brachiocephalic artery **(A)**, renal artery **(B)**, aortic arch **(C)** were excised postmortem, and frozen sections were made for immunostaining. Representative immunostainings for FAK, pY397 FAK, and DAPI (blue) are shown (n=5). White arrows: nuclear FAK. Red arrows: cytoplasmic FAK. Merge: FAK, pY397 FAK, and DAPI. Dashed line, boundary between media and diffuse intima. White line indicates endothelial layer. L, lumen; LC, lipid core. Scale bars: x4, 100 μm; or x60, 50 μm. Boxed areas are shown in **Figure 3D**.

Online Figure XIX. Active FAK cytoplasmic localization is correlated with DNMT3A expression in human atherosclerotic lesions. Human brachiocephalic arteries were excised postmortem, and frozen sections were made for immunostaining. **(A)** Representative immunostainings for FAK, ACTA2, and DAPI (blue) are shown (n=3). White arrows: nuclear FAK. Red arrows: cytoplasmic FAK. It is noted that abundant cytoplasmic FAK is observed in SMCs of atherosclerotic lesions compared to no lesion area. Merge: FAK, ACTA2, and DAPI.

(B) Immunostaining for DNMT3A, vWF, and DAPI (blue) are shown (n=3). Merge: DNMT3A, vWF, and DAPI. DNMT3A is significantly upregulated in SMCs of atherosclerotic lesions. Dashed line: boundary between media and diffuse intima. L: lumen. LC: lipid core. Scale bars: x4, 100 μm; or x60, 50 μm.

Online Figure XX. Pharmacological FAK inhibition increases contractile gene expression in human coronary artery smooth muscle cells (hCASMCs) by decreasing DNMT3A expression. Three replicate immunoblots of hCASMCs treated with or without FAK-I (VS-4718, 2.5 μM) for pY397 FAK, FAK, DNMT3A, DNMT3B, DNMT1, MYH11, CALD1, ACTA2, CNN1, TAGLN, and GAPDH as loading control. Blots were quantified and relative blot intensity were depicted in **Figure 3E** (replicate #1) (n=3). Data are mean±SEM. *P* values were determined using 2-way ANOVA Bonferroni multiple comparisons test (*P* values adjusted for 2 comparisons).

Online Figure XXI. SMCs on different matrix alters FAK activity and DNMT3A expression. SMCs were plated on tissue culture dishes, poly-L-lysine or various ECM coated dishes (10 μg/ml) and incubated for the indicated time periods. The lysates were analyzed by immunoblotting for pY397 FAK, FAK, DNMT3A, and GAPDH as loading control (n=3). Three replicates are shown.

Online Figure XXII. FAK inhibition downregulates DNMT3A protein expression by posttranslational modification. (A) Replicate lysate immunoblots (#2 and #3) of DNMT3A, DNMT3B, DNMT1, pY397 FAK, FAK, and GAPDH as loading control. Also replicate immunoblots of FAK, TRAF6, and DNMT3A immunoprecipitates are shown. **(B)** SMCs were treated with cycloheximide (CHX, 50 μg/ml) with/without FAK-I (VS-4718, 2.5 μM) for indicated times. Shown are immunoblots for DNMT3A, DNMT3B, DNMT1, pY397 FAK, FAK, and GAPDH as loading control. pY397 FAK blots were quantified relative to total FAK and other blots were quantified relative to GAPDH. Changes in DNMT3A levels over time are plotted in **Figure 4C**. **(C)** SMCs were treated with actinomycin D (ActD, 5 μg/ml) with/without FAK-I (VS-4718, 2.5 μM) for 6 h. Immunoblotting was performed for DNMT3A, DNMT3B, DNMT1, pY397FAK, FAK and GAPDH as loading control. pY397 FAK blots were quantified relative to total FAK and other blots to GAPDH. Representative blots (n=3).

Online Figure XXIII. E3 ligase TRAF6 regulates DNMT3A protein expression. (A) 293T cells were transfected Myc-tagged TRAF6, and HA-tagged UHRF1 or UHRF2. The cell lysates were subjected to immunoblotting for DNMT3A, Myc, HA, and GAPDH as loading control (n=3). **(B)** Schematic representation of WT DNMT3A and its different deletion mutants. **(C)** Mice were

treated with MG132 (0.3 mg/kg) by intraperitoneal injection at 24, 6, and 1 h before euthanasia. Isolated aorta lysates were immunoprecipitated with anti-FAK antibody and FAK-IPs were subjected to immunoblotting for DNMT3A, DNMT3B, DNTM1, FAK, and GAPDH as loading control (n=6). Boxed areas are shown in **Figure 4F**.

Online Figure XXIV. Knockdown of DNMT3A, but not DNMT3B, promotes SMC contractile gene expression. Replicate immunoblots of shDNMT3A and shDNMT3B RNA expressing SMCs for DNMT3A, DNMT3B, DNMT1, pY397 FAK, FAK, CALD1, ACTA2, CNN1, and GAPDH as loading control. Blots were quantified and relative blot intensity were depicted in **Figure 3G** (replicate #1) (n=3). Data are mean±SEM. *P* values were determined using 1-way ANOVA Bonferroni multiple comparisons test (*P* values adjusted for 3 comparisons).

Online Figure XXV. Knockdown of DNMT3A slows SMC proliferation. SMCs were infected lentiviral shRNA DNMT3A for 2 days. Representative immunostaining of Ki-67 **(A)** and cell proliferation assays **(B)** were performed using two shRNA-mediated knockdown of DNMT3A SMCs (n=3). Scale bars: 100 μm

Online Figure XXVI and VII. Pharmacological FAK inhibition reduced injury-induced upregulation of DNMT3A and 5-mC preventing neointima formation in mice. Mice were treated with vehicle or FAK-I (VS-4718, 50 mg/kg) twice daily following wire injury for 2 weeks. Shown are representative immunofluorescence staining of frozen section from vehicle or FAK-I-treated femoral arteries for FAK, pY397 FAK, DNMT3A, 5-mC, and ACTA2. Red, green, and blue (DAPI) were merged. Scale bars: 20 μm. Shown are replicate immunostaining (#2 and #3) in **Figure 5**.

Online Figure XXVIII. Wire injury did not change DNMT mRNA expression. Mice were treated with vehicle or FAK-I (VS-4718, 50 mg/kg) twice daily following wire injury for 2 weeks. Total RNA from artery samples were analyzed. Volcano plot of transcriptome profiles sham vs. injured **(A)** and injured vs. injured+FAK-I **(B)**. Red dots: DNMT genes. Blue dots: genes significantly upregulated and downregulated (adjusted p value<0.05). Gray dots: genes with no significant changes. **(C)** Normalized read values for *Dnmt3a* and *Dnmt3b* from RNA sequencing (sham, injured, and injured+FAK-I, n=3 per group) are plotted.

Online Figure XXIX and XXX. SMC-specific genetic FAK-KD mice did not upregulate injury-induced DNMT3A and 5-mC expression protecting from neointima formation. Representative immunofluorescence staining of FAK-WT and FAK-KD femoral arteries 2 weeks postinjury for FAK, pY397 FAK, DNMT3A, DNMT3B, 5-mC and ACTA2. Green, red, and blue

(DAPI) were merged. Scale bars: 20 μm. Shown are replicate immunostaining (#2 and #3) in **Figure 6**.

Online Figure XXXI. Upregulated DNMT3A upon wire injury reduced contractile gene expression. (A) Mice were treated with vehicle or FAK-I (VS-4718, 50 mg/kg) twice daily for 2 weeks following wire injury. Replicate immunoblots (#2) of femoral artery lysates from FAK-I treated **(A)** or FAK-WT and -KD **(B)** mice 2 weeks following injury for pY397 FAK, FAK, DNMT3A, DNMT3B, DNMT1, MYH11, CALD1, ACTA2, CNN1, TAGLN and GAPDH as loading control (n=4). The blots were quantified and plotted relative to GAPDH. Blots were quantified and relative blot intensity were depicted in **Figure 7A** and **B** (n=4). Data are mean±SD. *P* values were determined using 2-way ANOVA Bonferroni multiple comparisons test (*P* values adjusted for 6 comparisons).

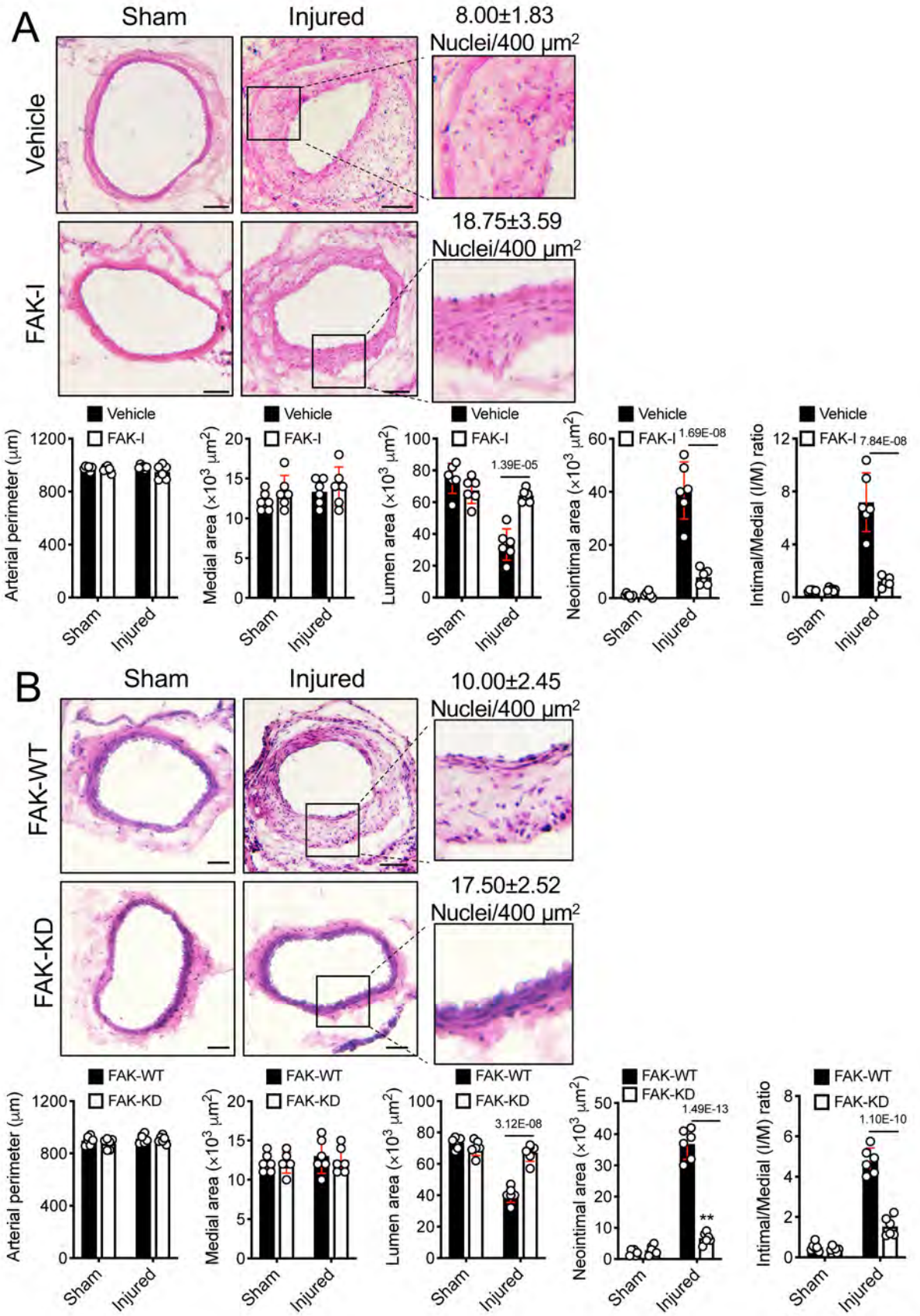
Online Figure XXXII. Schematic diagram of ChIP primers for SMC contractile gene promoters. Shown are primer locations (green arrows) surrounding CArG boxes in SMC contractile genes *Acta2, Myh11,* and *Tagln*. CArG box: SRF binding sites. TSS: transcription start site. (**B**) Histone H3 and H4 ChIP-qPCR were used as a control (n=3).

Online Figure XXXIII. In vivo knockdown of DNMT3A causes DNA hypomethylation and promotes SMC contractile gene expression. Femoral arteries were coated with shDNMT3A lentivirus immediately following wire injury. (**A**) Representative H&E staining of femoral artery cross sections 2 weeks after wire injury. Intima/media (I/M) ratios were quantified (n=5). Scale bar, 50 μm. (**B**) Relative DNMT3A mRNA expression in femoral arteries harvested 2 weeks postinjury were measured by RT-qPCR (n=4). (**C**) Representative dot blots for 5-mC expression using genomic DNA (gDNA, 150ng per dots) isolated from femoral arteries 2 weeks postinjury (n=3). The methylene blue staining was used to verify equal loadings. (**D**) The top five most upregulated or downregulated KEGG terms in injured vs. injured+shDNMT3A (n=3). The red bars: upregulated KEGG pathways. The blue bars: downregulated KEGG pathways. (**E**) Volcano plot of transcriptome profiles in injured vs. injured+shDNMT3A (n=3). Red dots: SMC-specific contractile genes. Blue dots: genes that are significantly upregulated and downregulated (adjusted p value<0.05). Gray dots: genes with no significant changes. Data are mean±SEM. *P* values were determined using 2-way ANOVA Bonferroni multiple comparisons test (*P* values adjusted for 6 comparisons).

Online Figure XXXIV and XXXV. In vivo knock-down of DNMT3A in femoral artery reduces wire injury-induced neointima formation. Femoral arteries were coated with either scramble shRNA (shScr) or DNMT3A shRNA (shDNMT3A) lentivirus coexpressing mCherry immediately following wire injury. Representative immunostainings of 2-week post-injury samples for ACTA2,

DNMT3A and DNMT3B are shown (n=4). Green, mCherry (red) and DAPI (blue) were merged. mCherry was used to verify lentiviral infection. Scale bars: 20 μm. Shown are replicate immunostaining (#2 and #3) in **Figure 8**.

Online Figure XXXVI. DNMT3A shRNA knockdown in vivo did not affect wire injury-induced FAK localization and activation. Femoral arteries were coated with either scramble shRNA (shScr) or DNMT3A shRNA (shDNMT3A) encoding mCherry cassette immediately following wire injury. Artery samples were collected and prepared for frozen sections after 2-week post injury. Immunostaining of artery sections for FAK, pY397 FAK and 5-mC. Green, mCherry (red) and DAPI (blue) were merged (n=4). mCherry was used to verify lentiviral infection. Dotted line in image marks the elastic lamina. White line indicates endothelial layer. Scale bars: x20, 100 μm; or x60, 20 μm.



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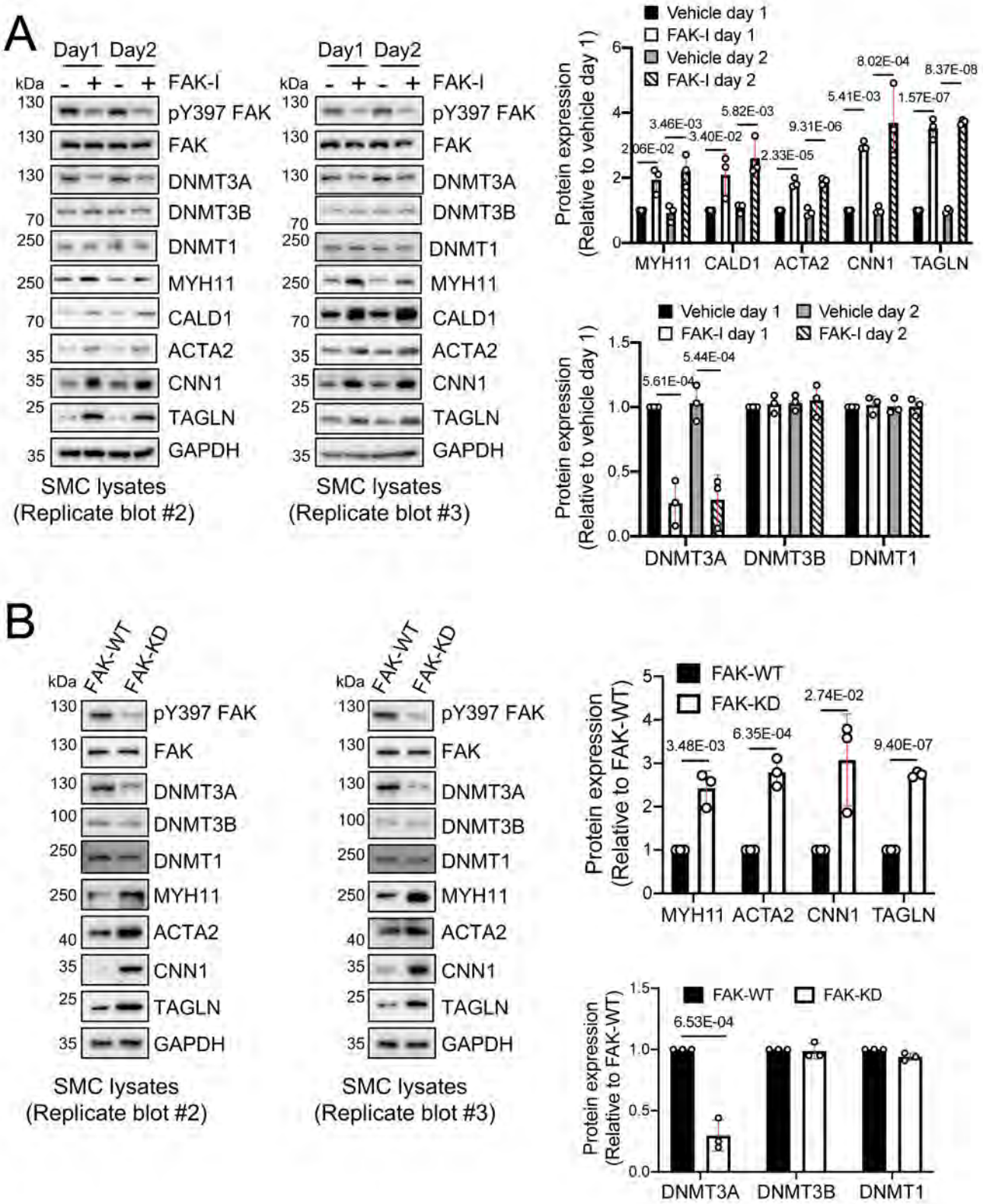

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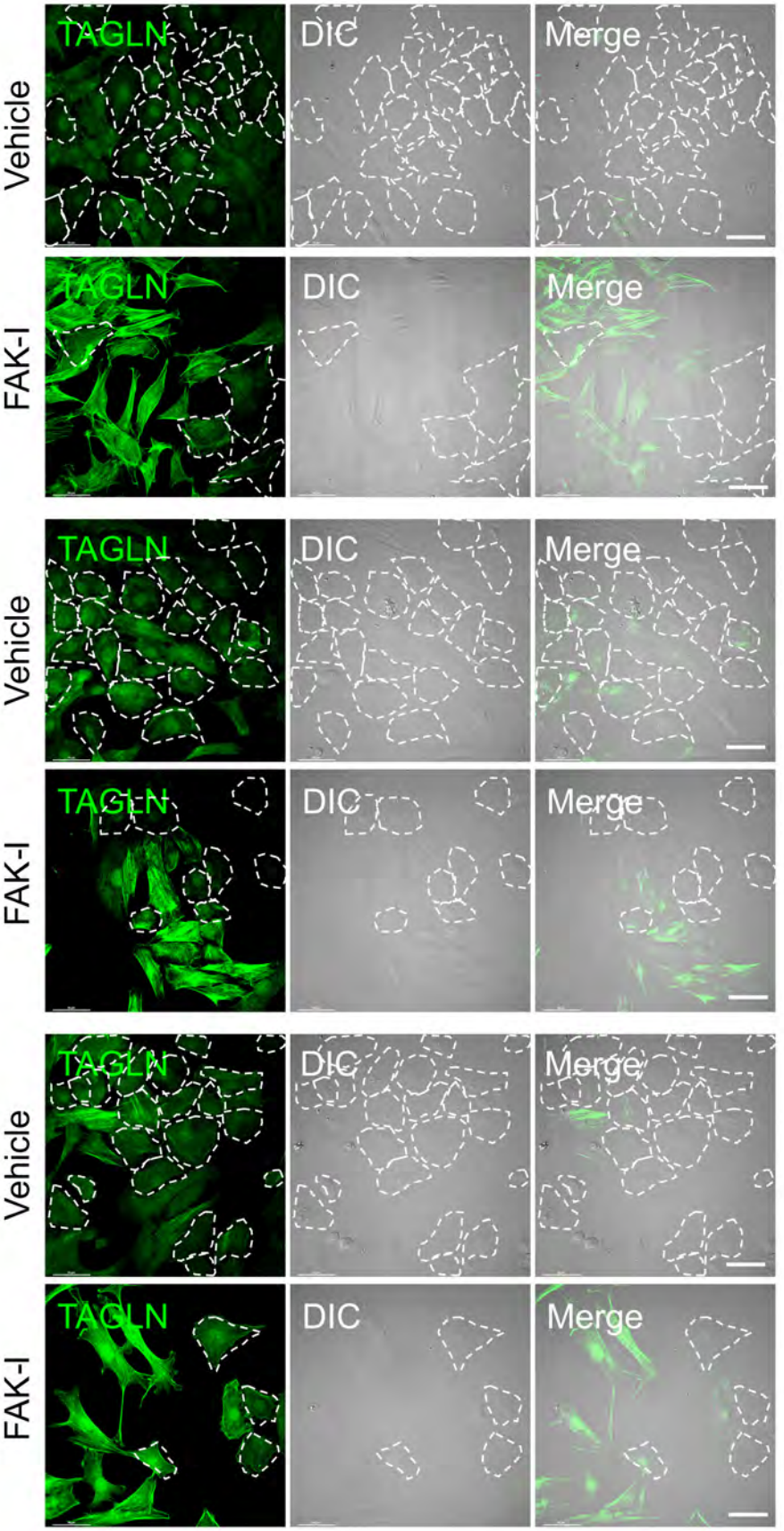

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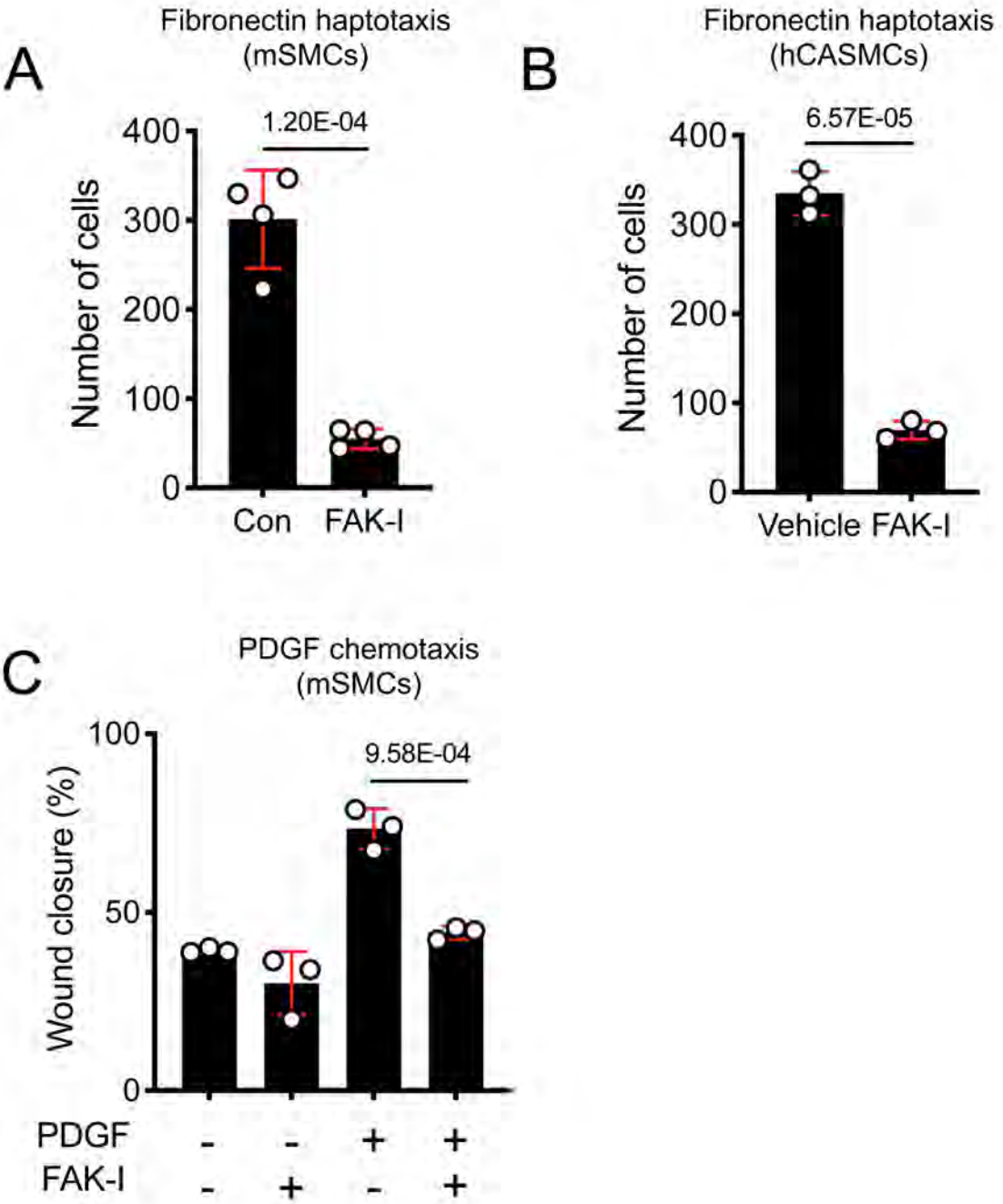

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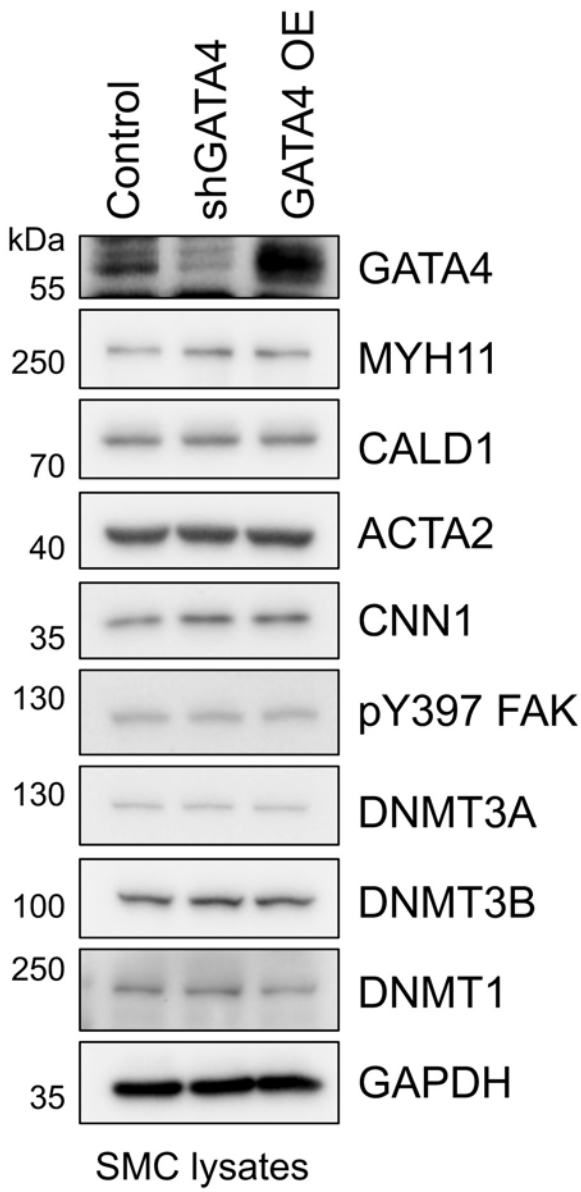

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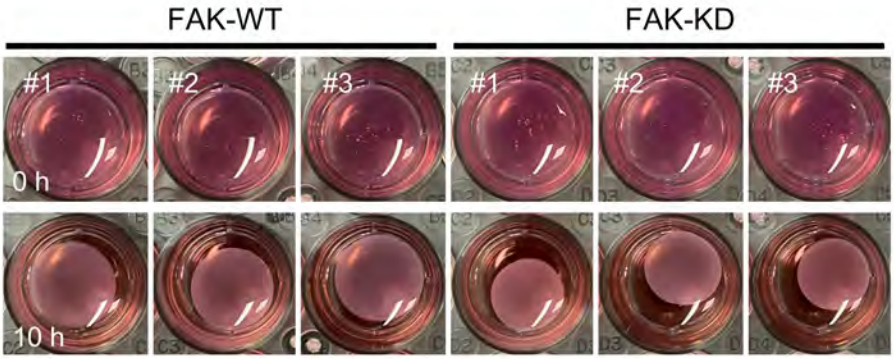

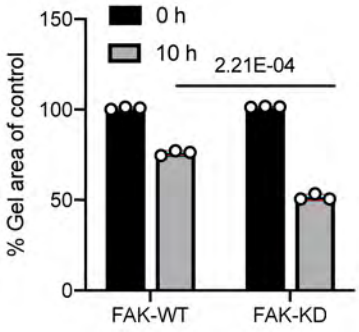

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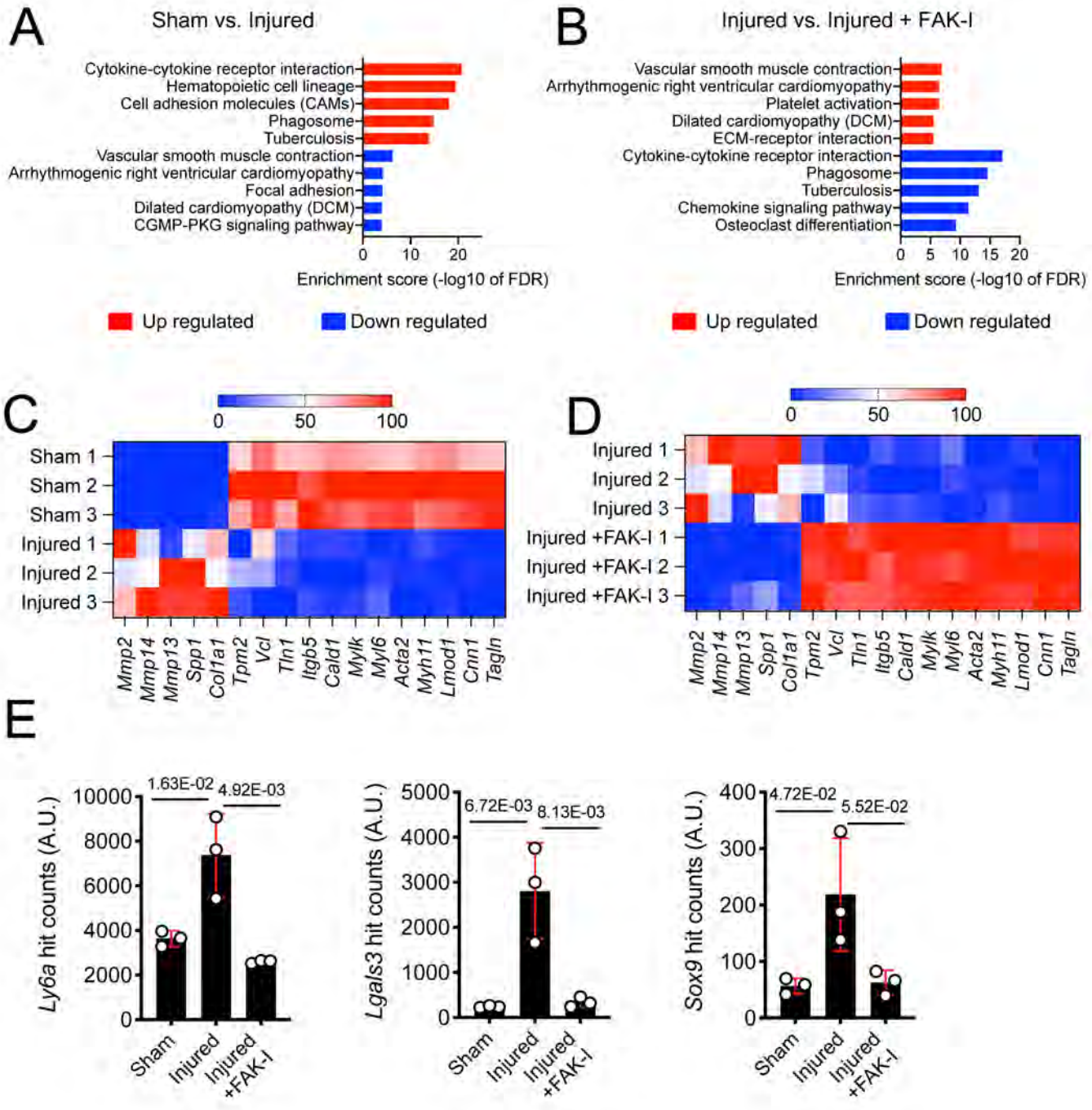

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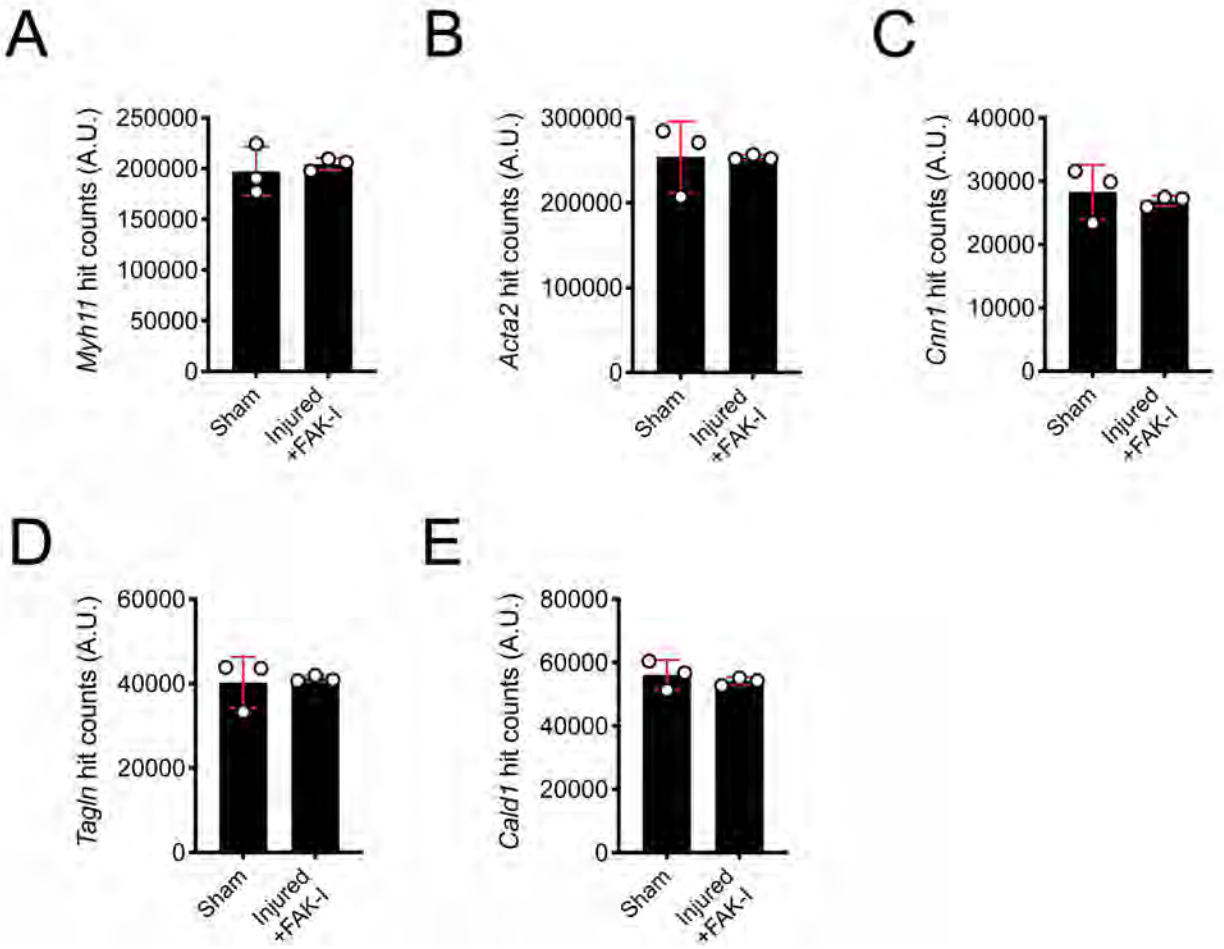

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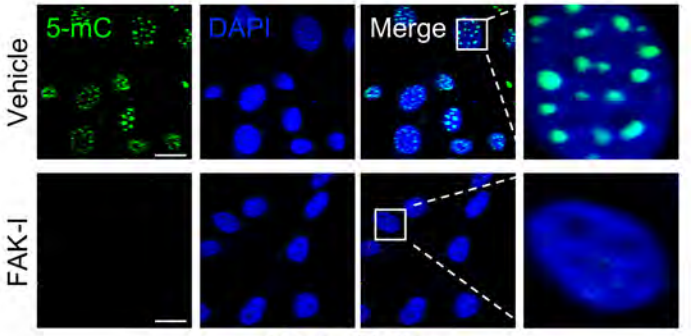

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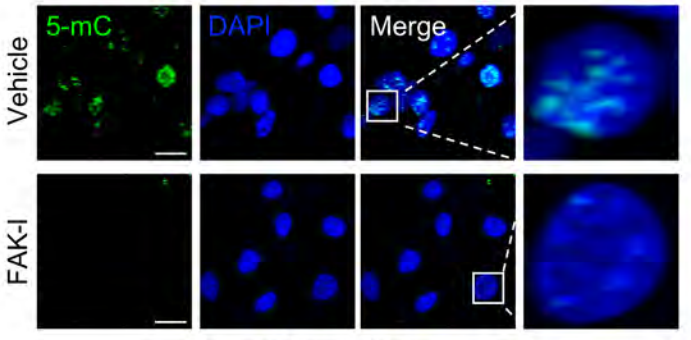

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Online Figure IX

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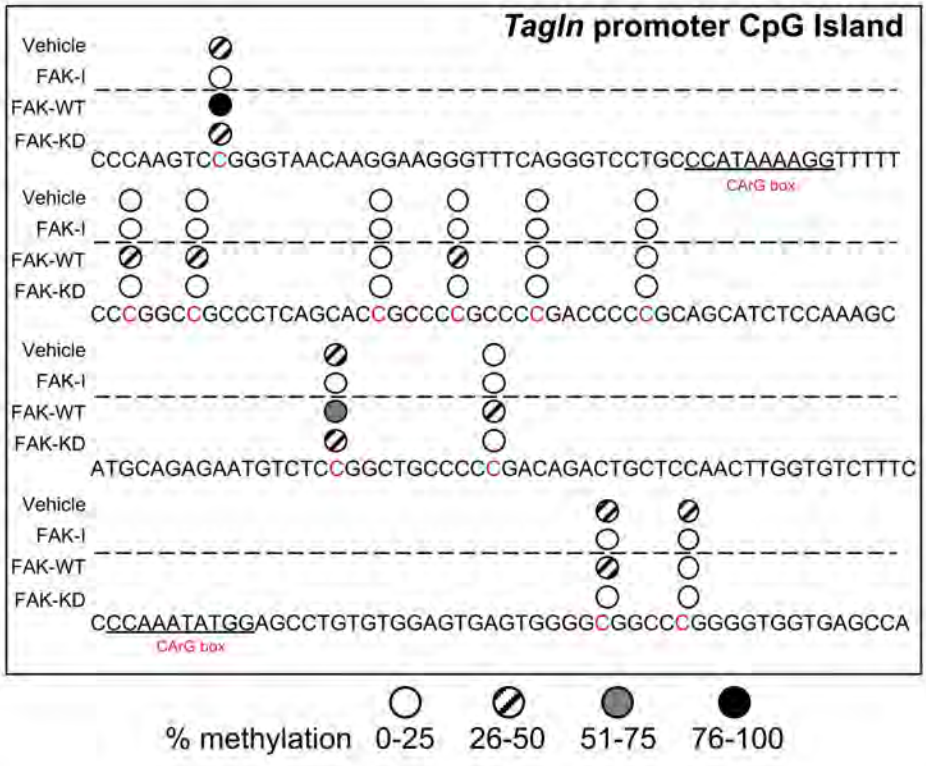

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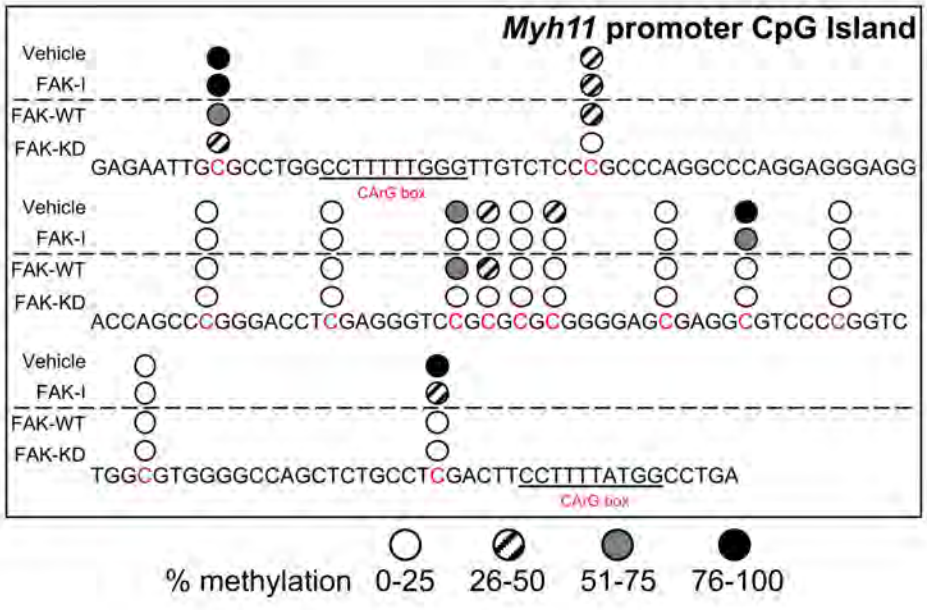

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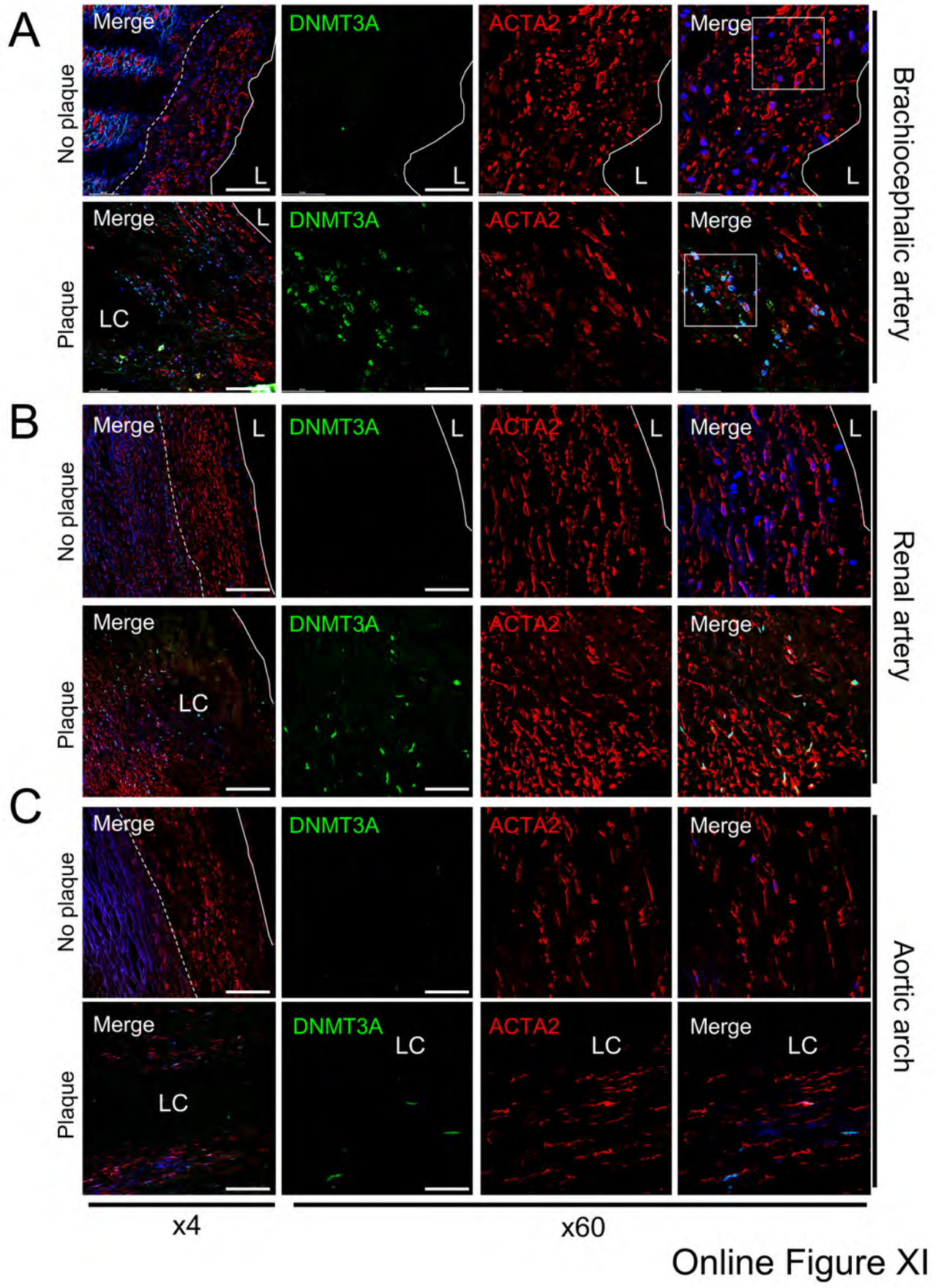

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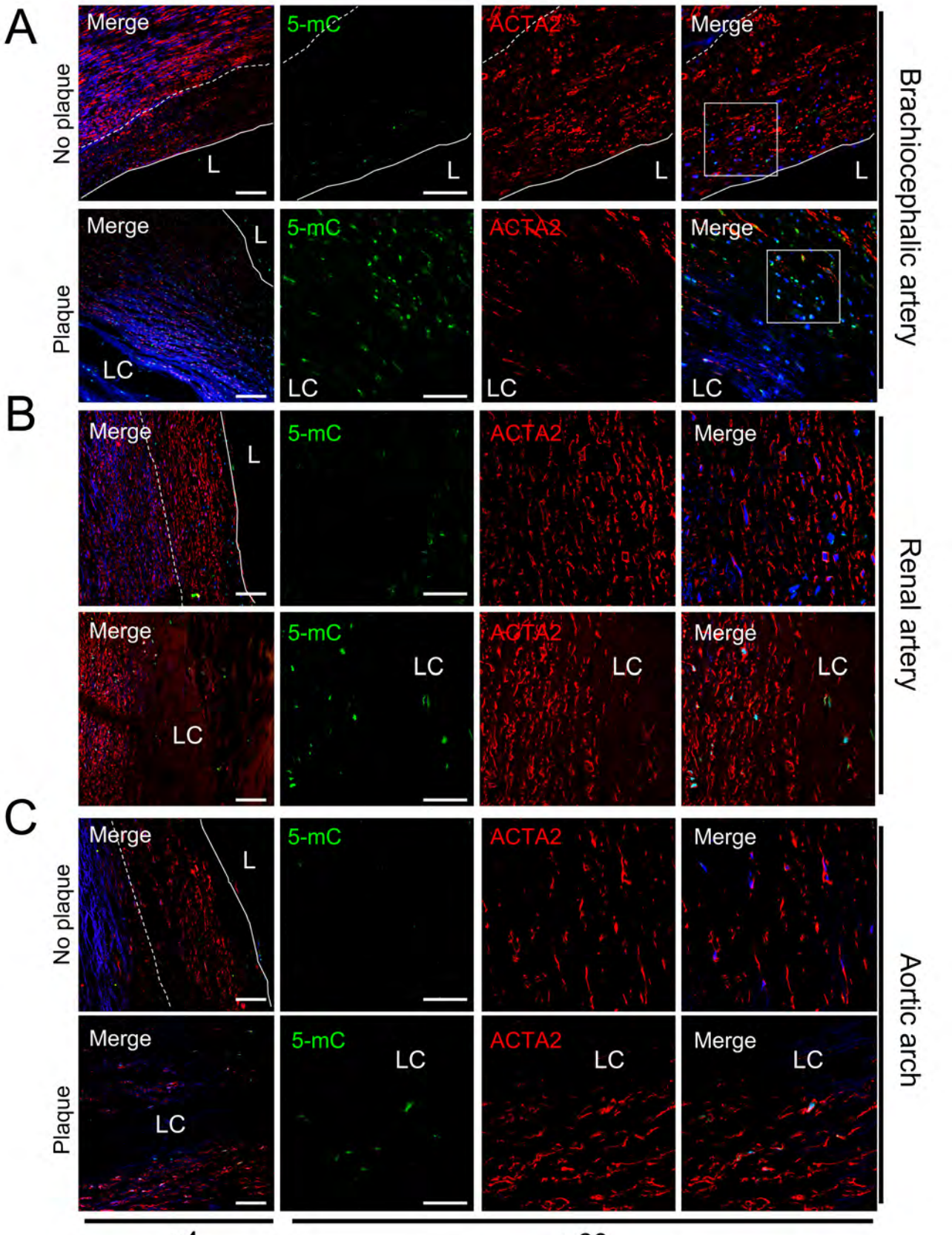

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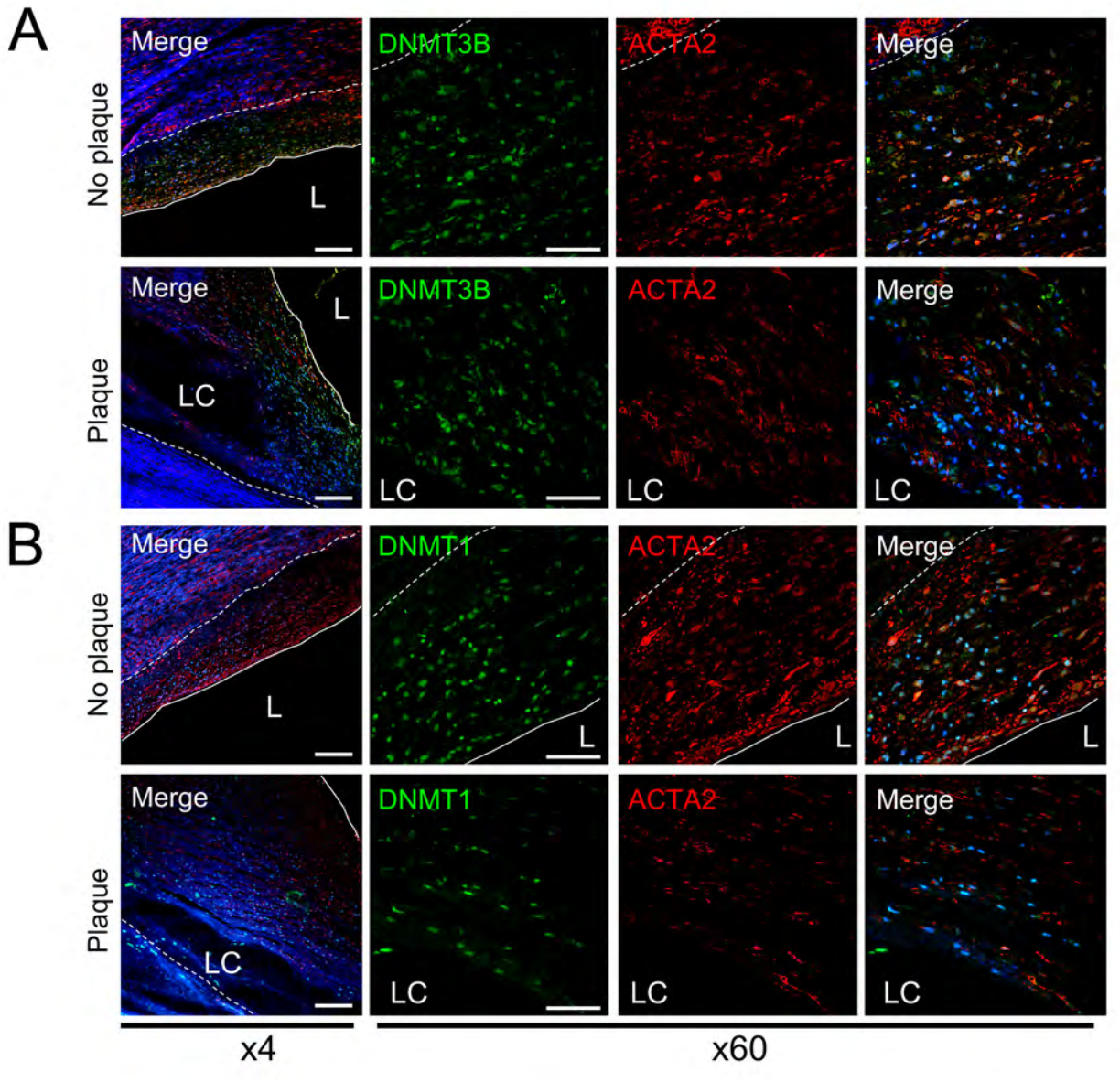

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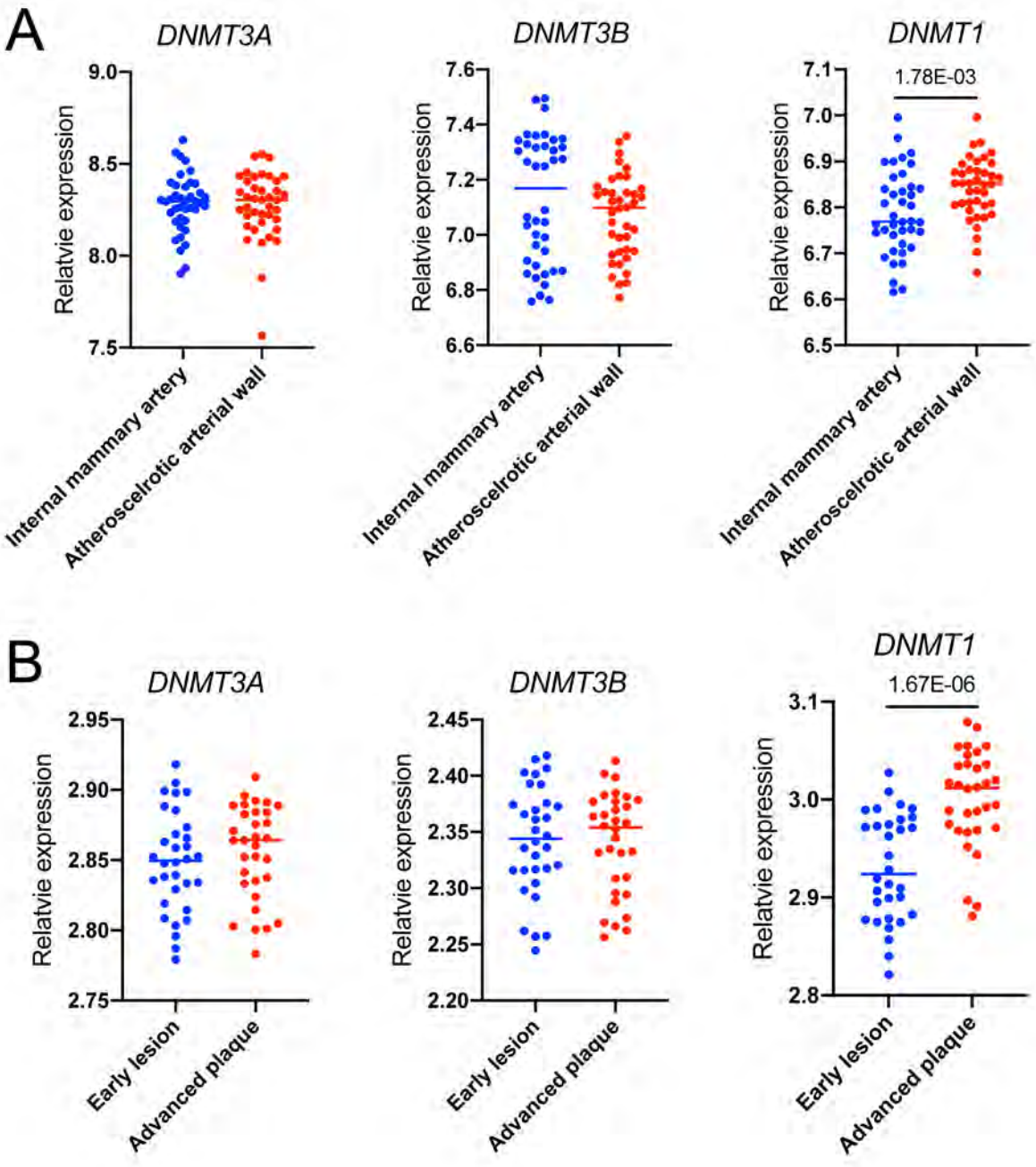

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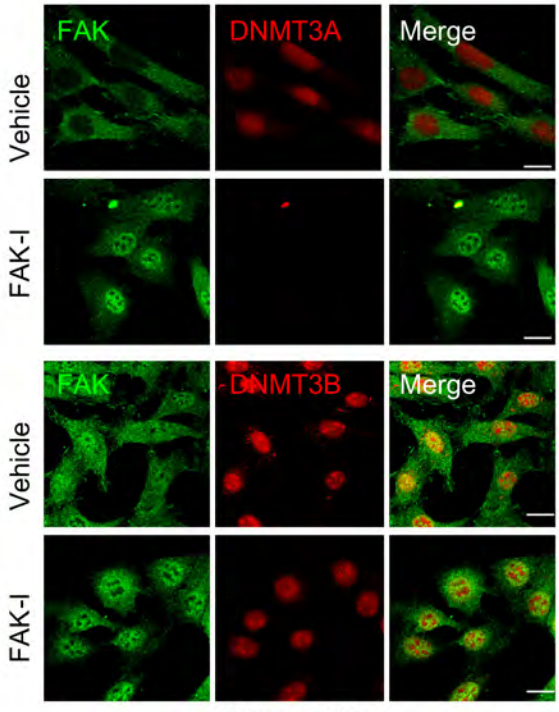

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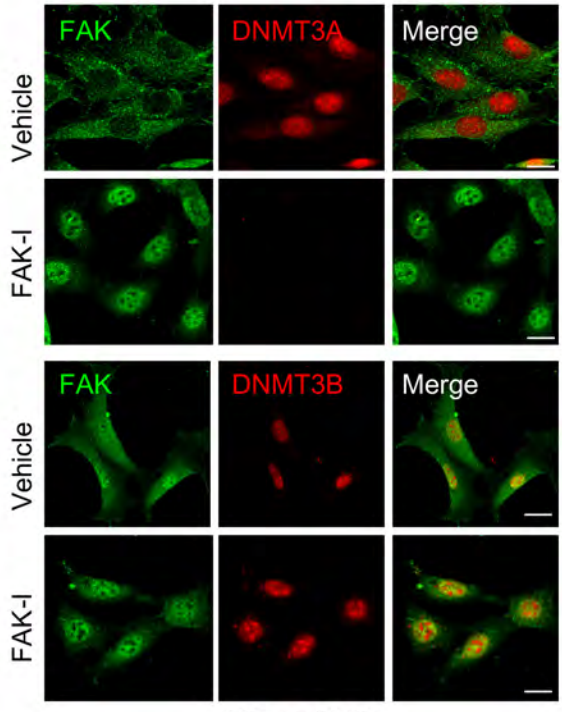

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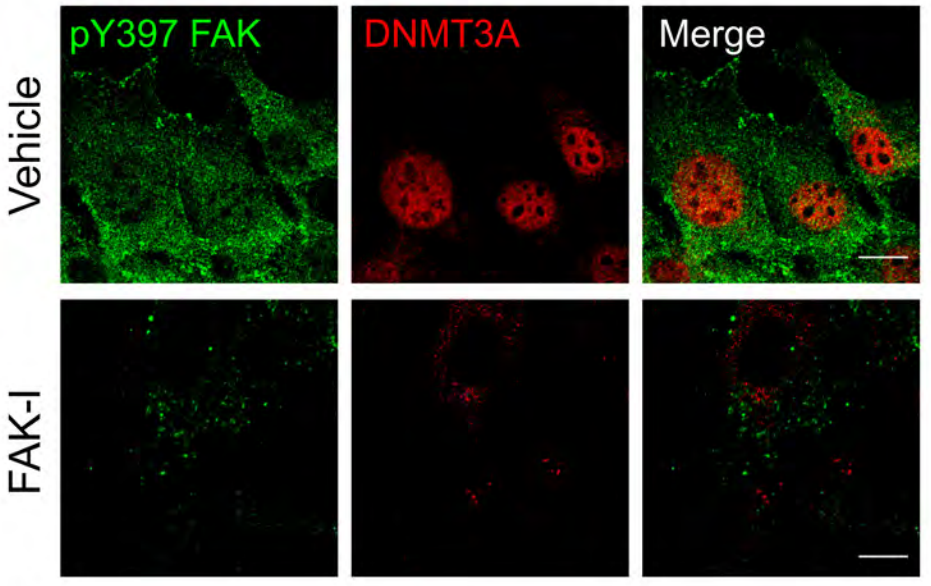

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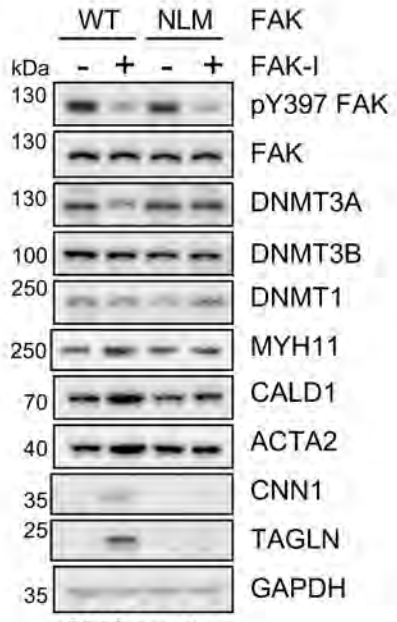

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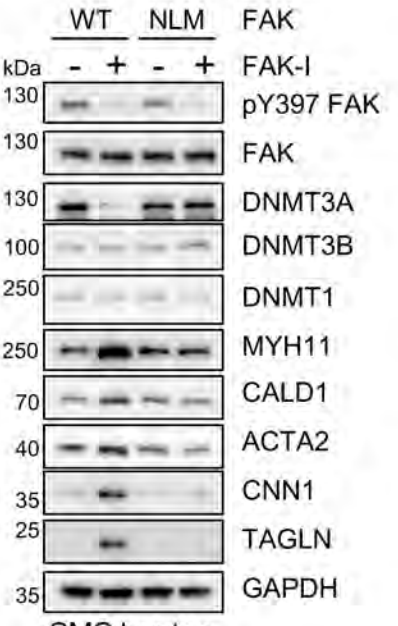

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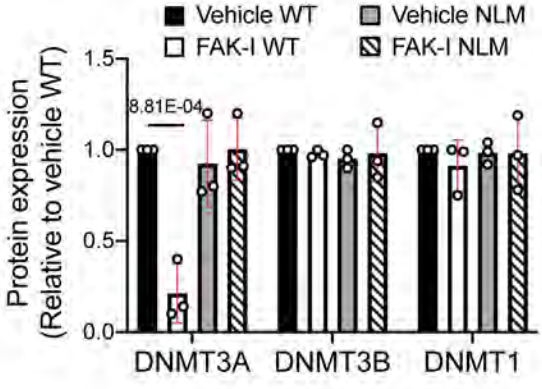

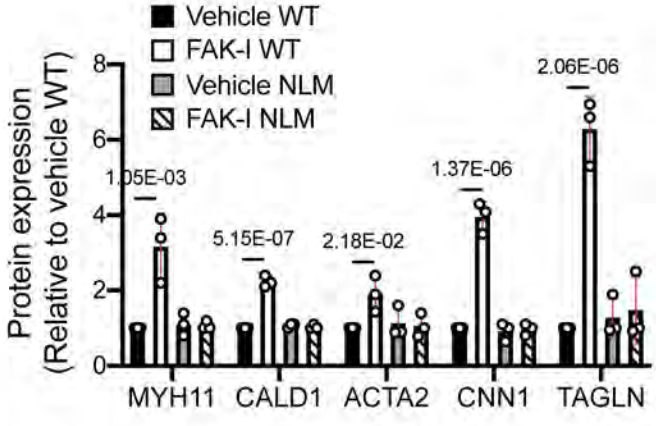

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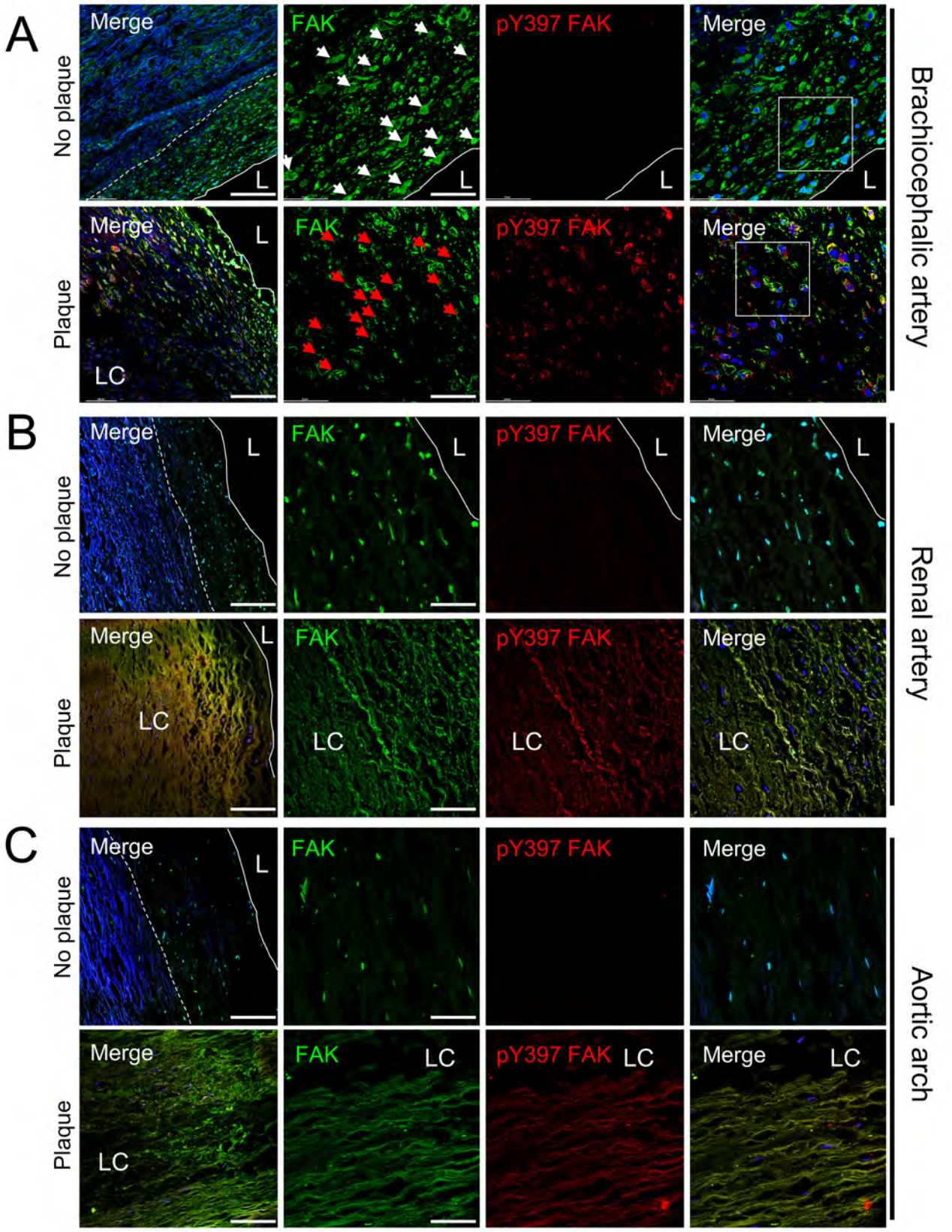

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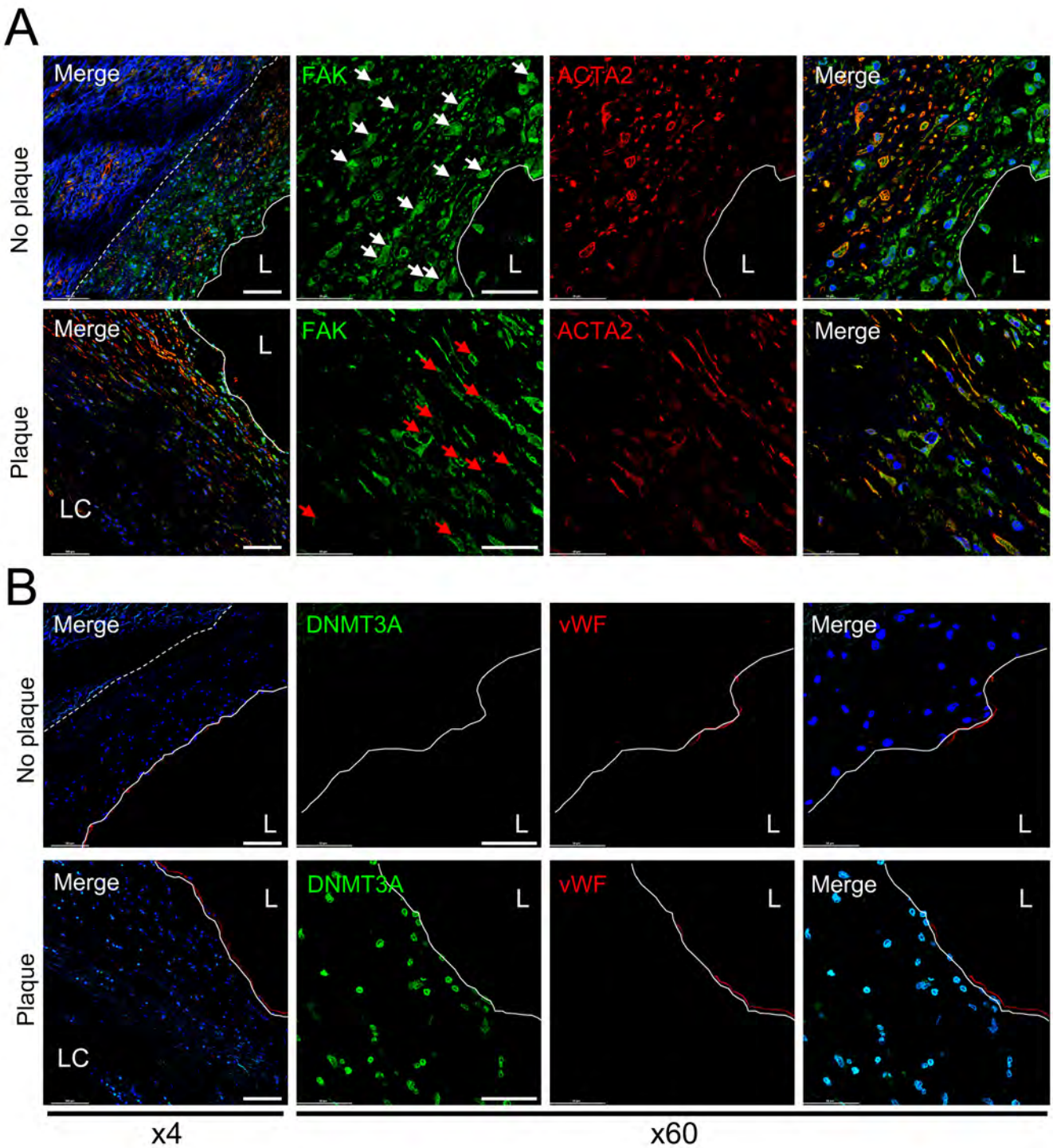

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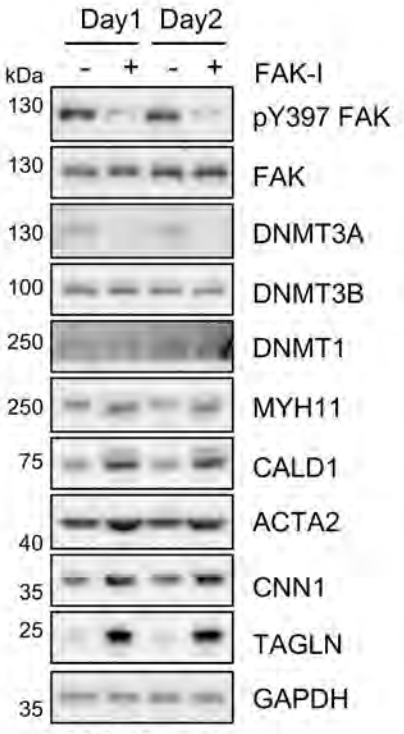

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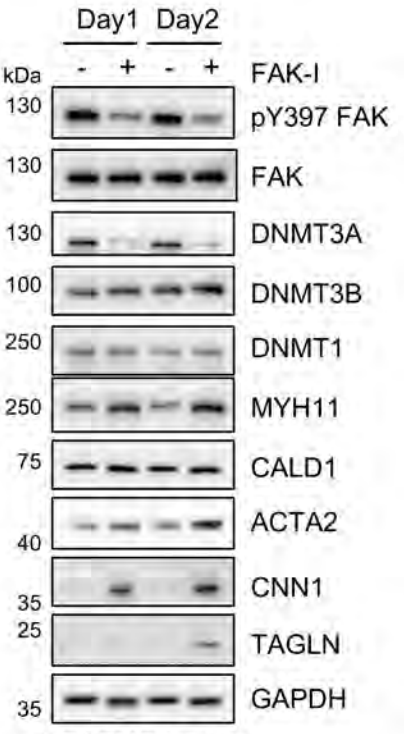

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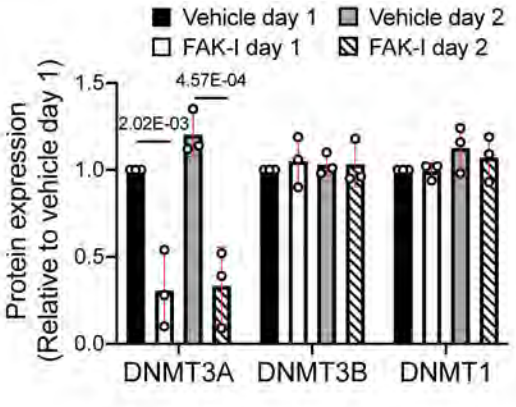
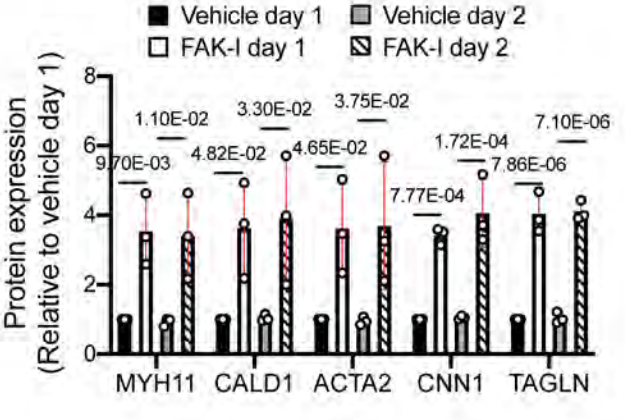
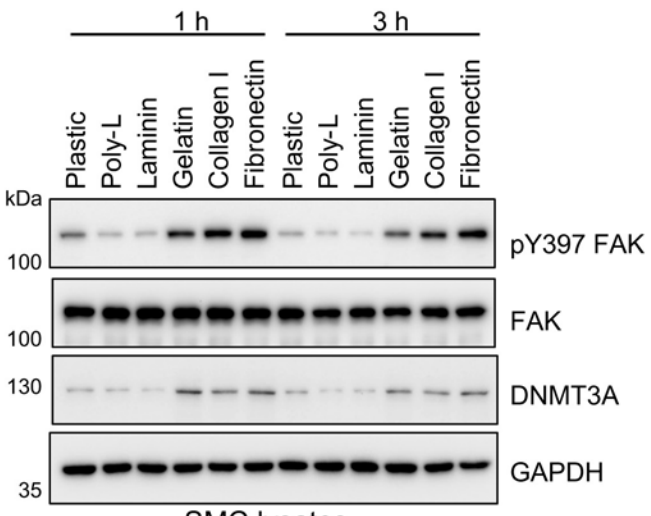




Online Figure XX



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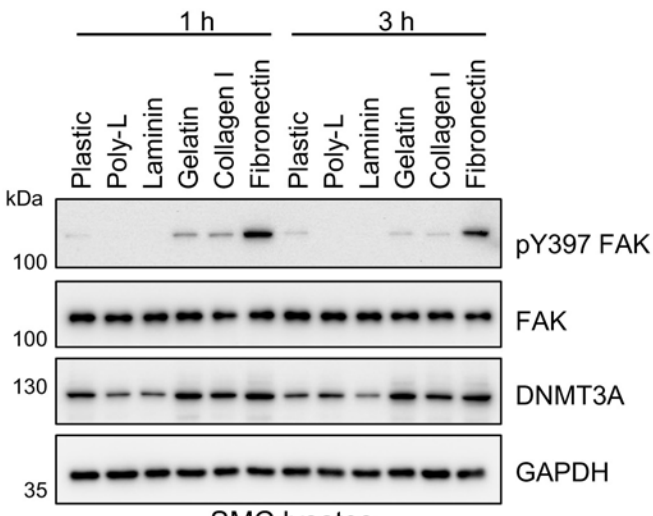

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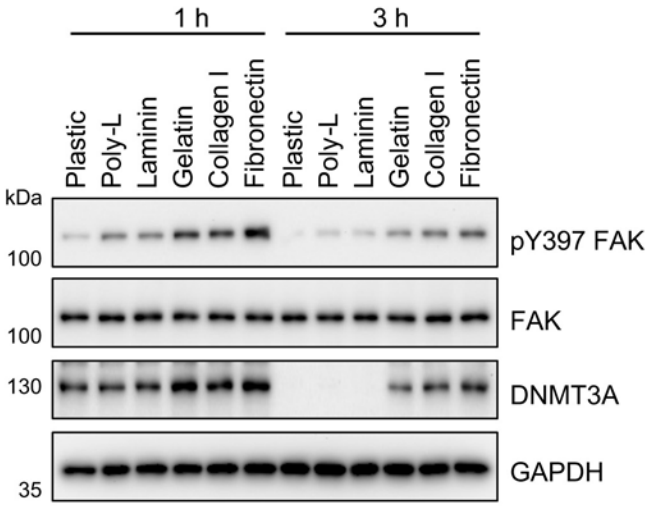
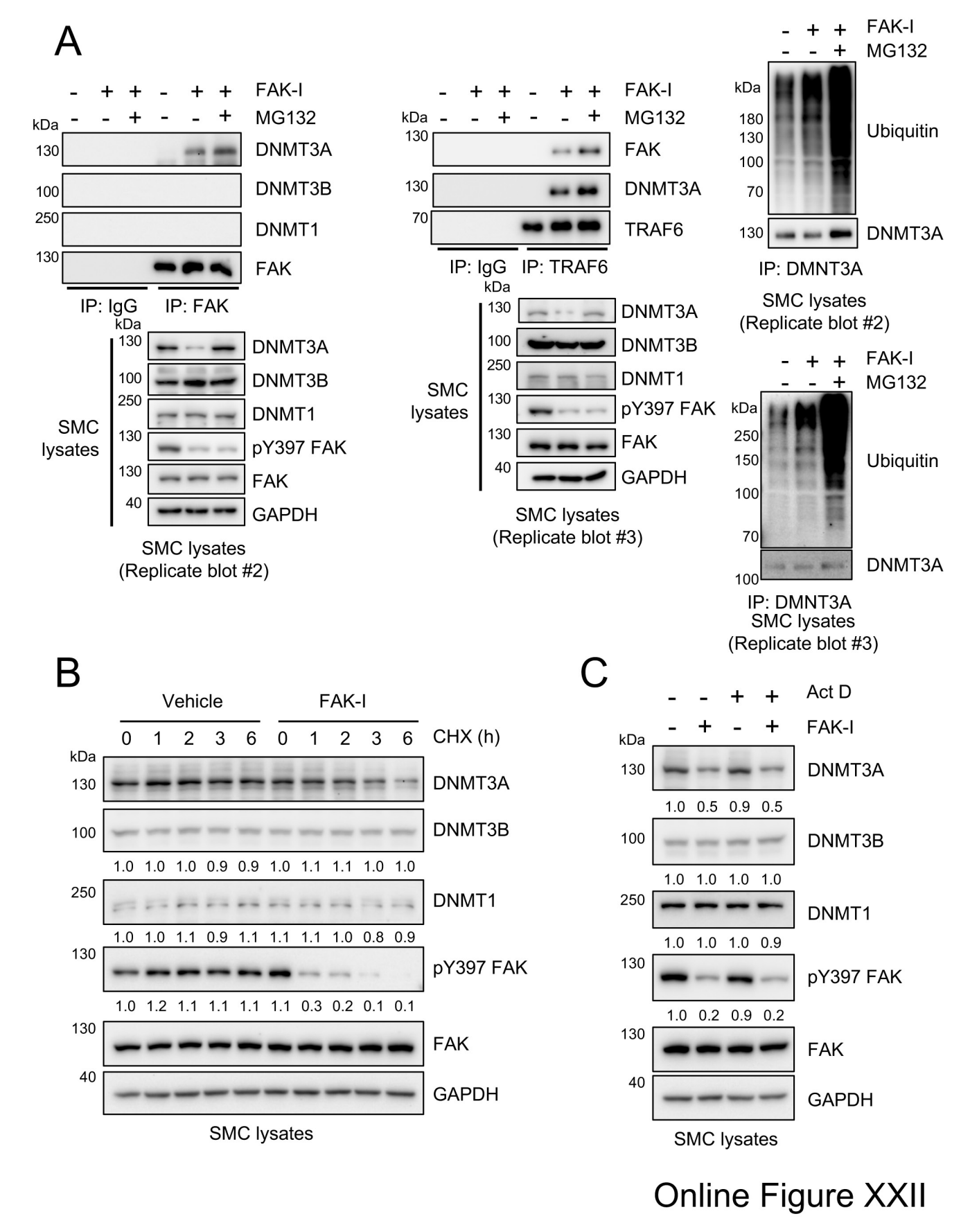
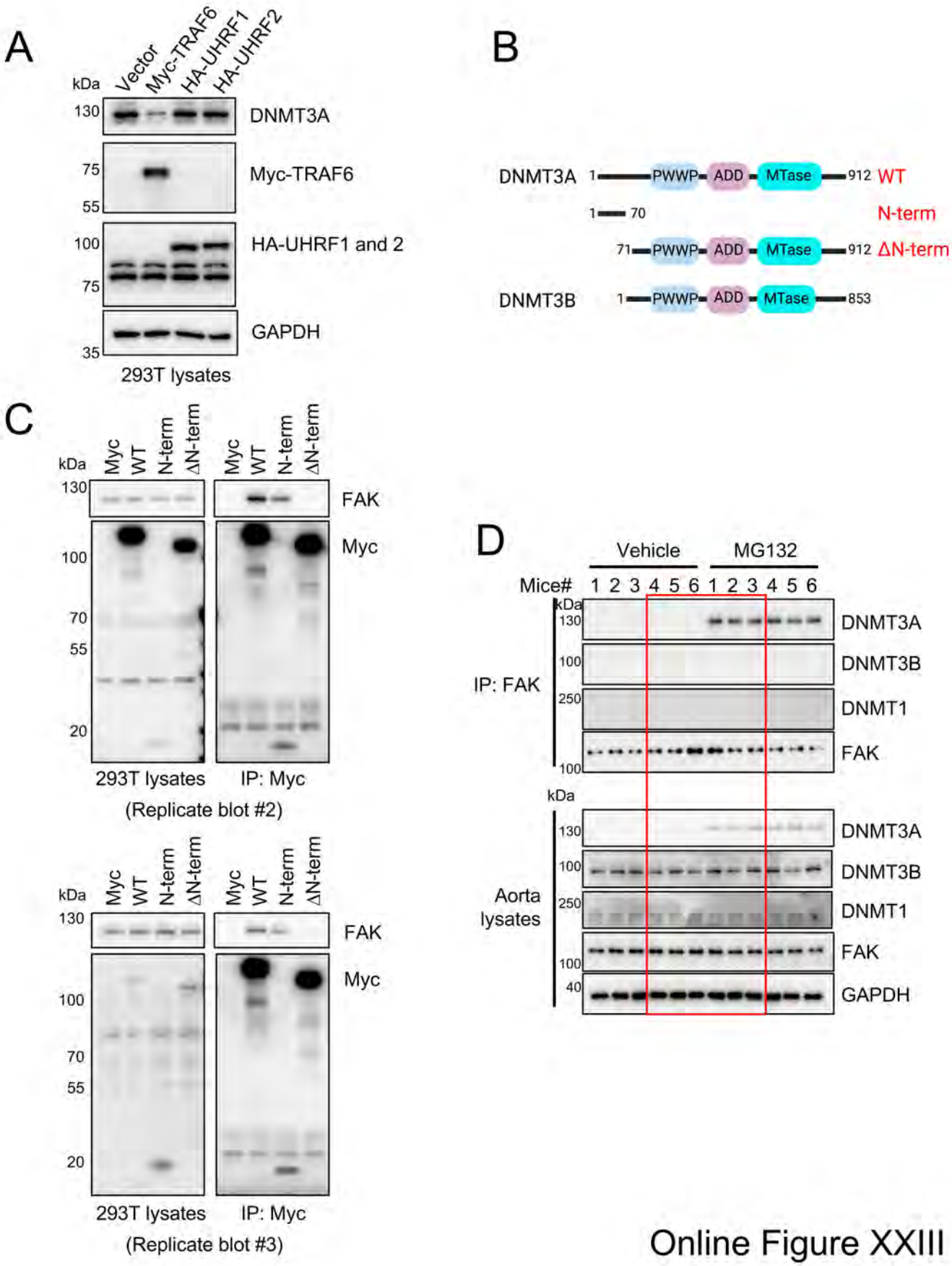


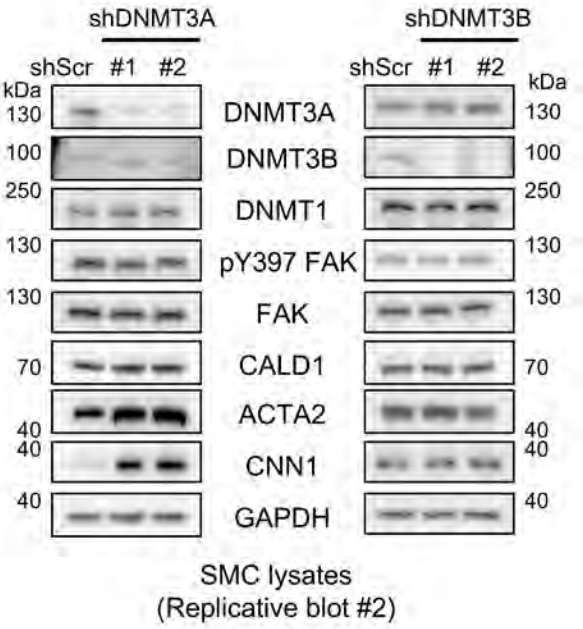
SMC lysates
(Replicate blot #3)

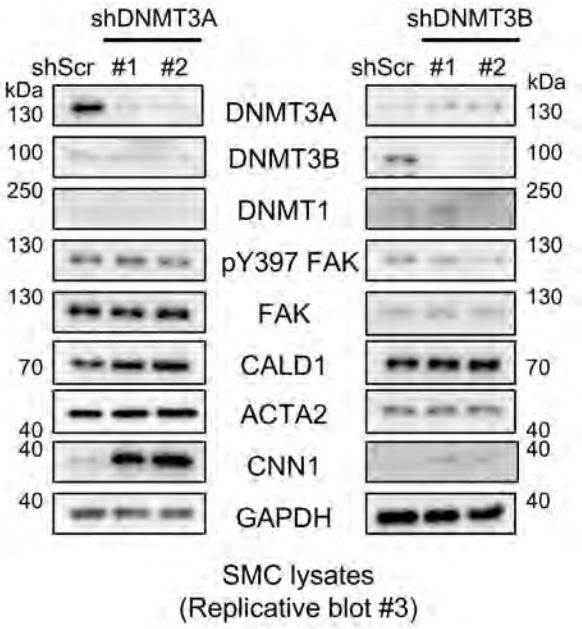
Online Figure XXI

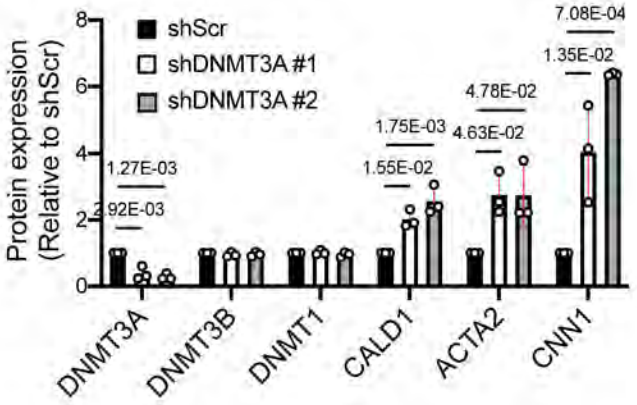


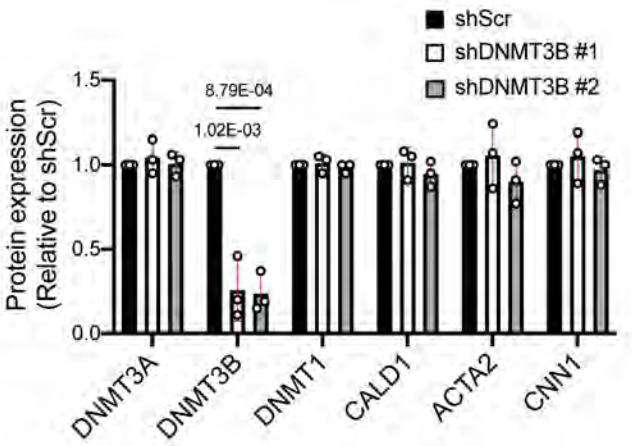
Online Figure XXII


A
Vector
Myc-TRAF6
HA-UHRF1
HA-UHRF2
kDa
130
DNMT3A
75
Myc-TRAF6
55
100
HA-UHRF1 and 2
75
GAPDH
35
293T lysates
B
DNMT3A
1
PWWP
ADD
MTase
912
WT
1
70
N-term
71
PWWP
ADD
MTase
912
ΔN-term
DNMT3B
1
PWWP
ADD
MTase
853
C
kDa
Myc
WT
N-term
ΔN-term
130
FAK
Myc
100
70
55
20
293T lysates
IP: Myc
(Replicate blot #2)
(Replicate blot #3)
D
Vehicle
MG132
Mice#
1 2 3 4 5 6 1 2 3 4 5 6
IP: FAK
DNMT3A
DNMT3B
DNMT1
FAK
Aorta lysates
GAPDH
Online Figure XXIII



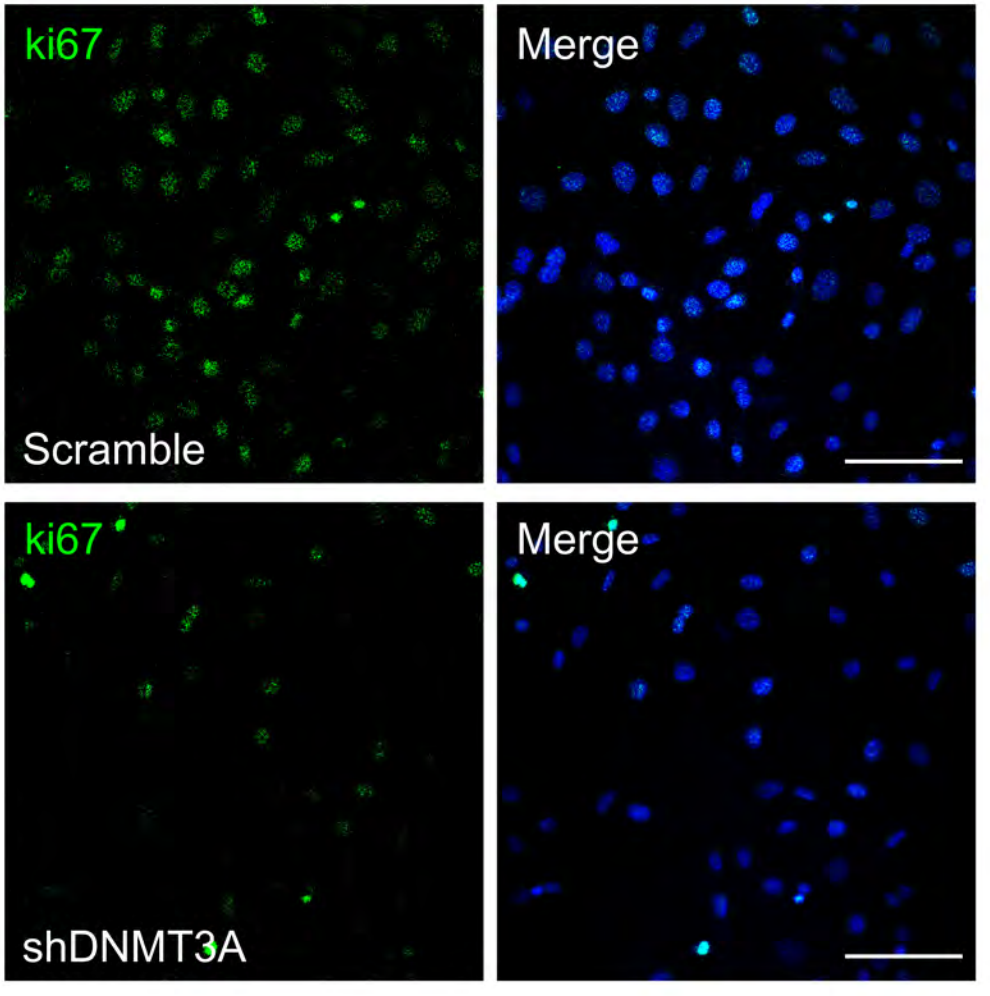




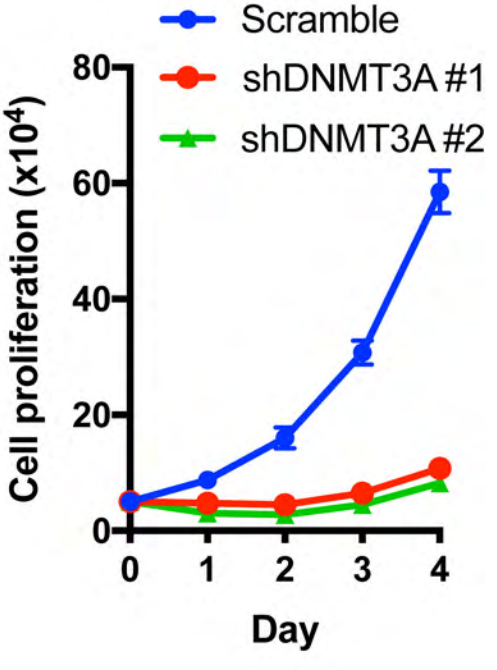


Online Figure XXIV

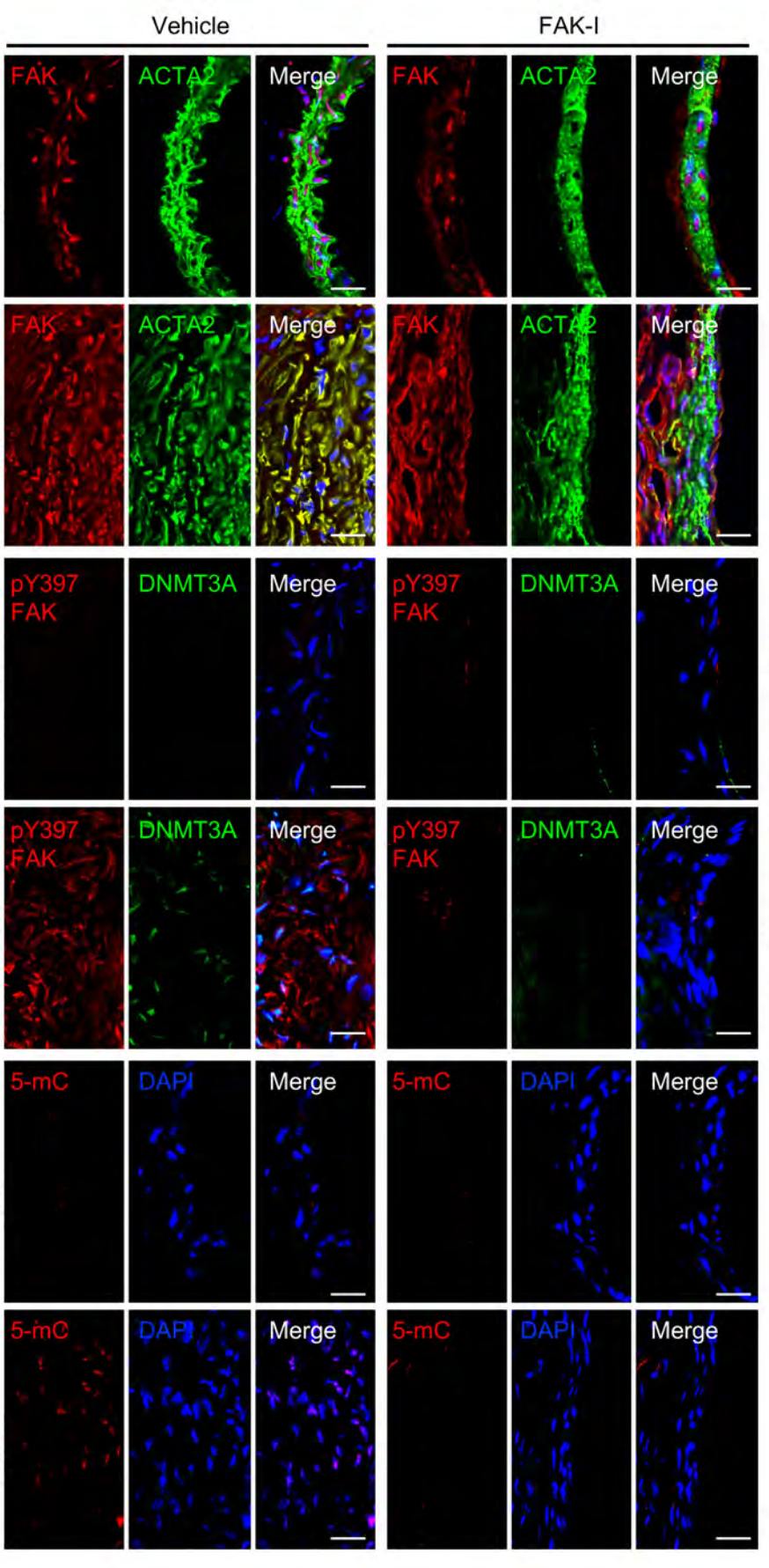
A



B

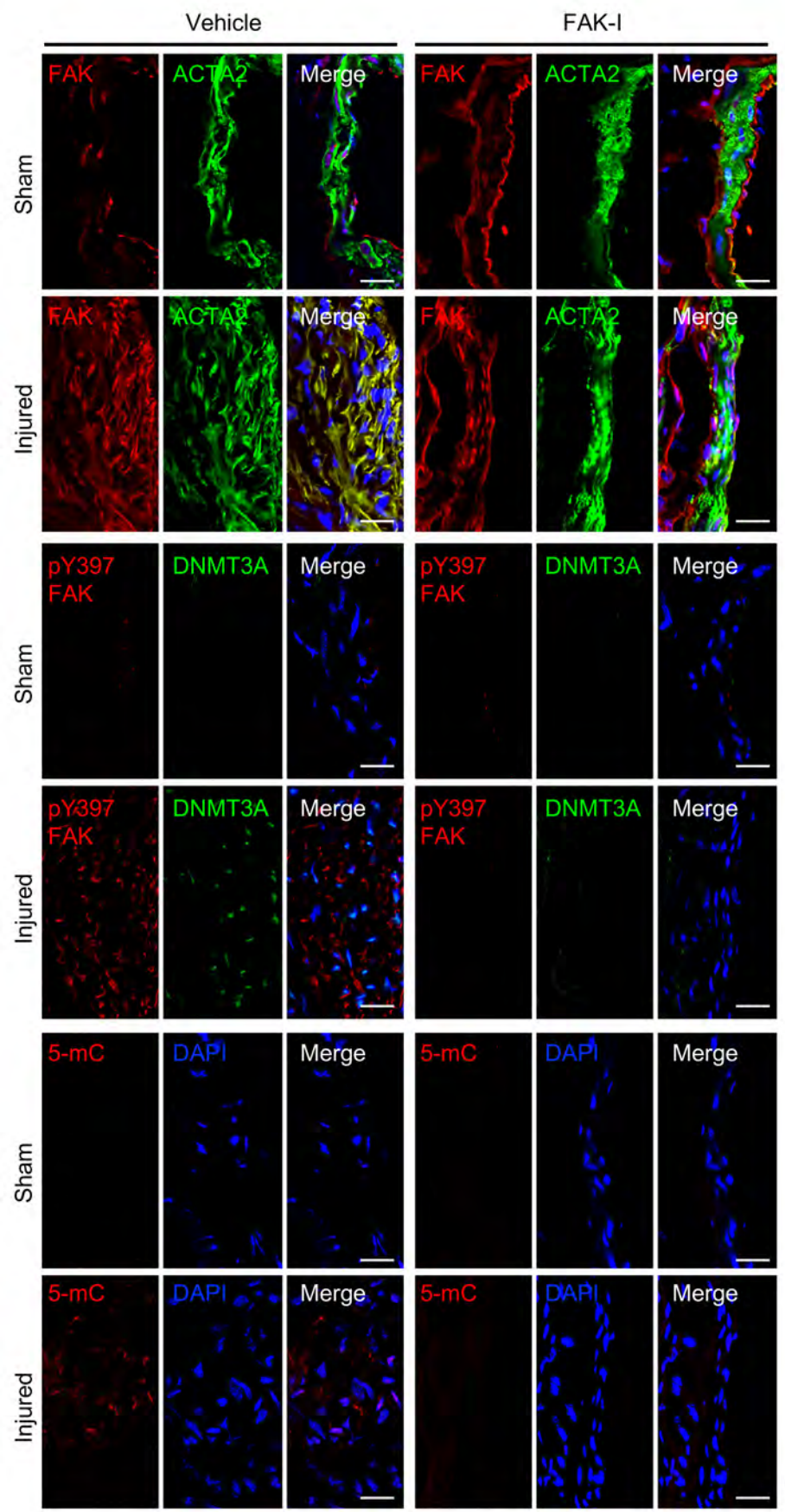


Online Figure XXV



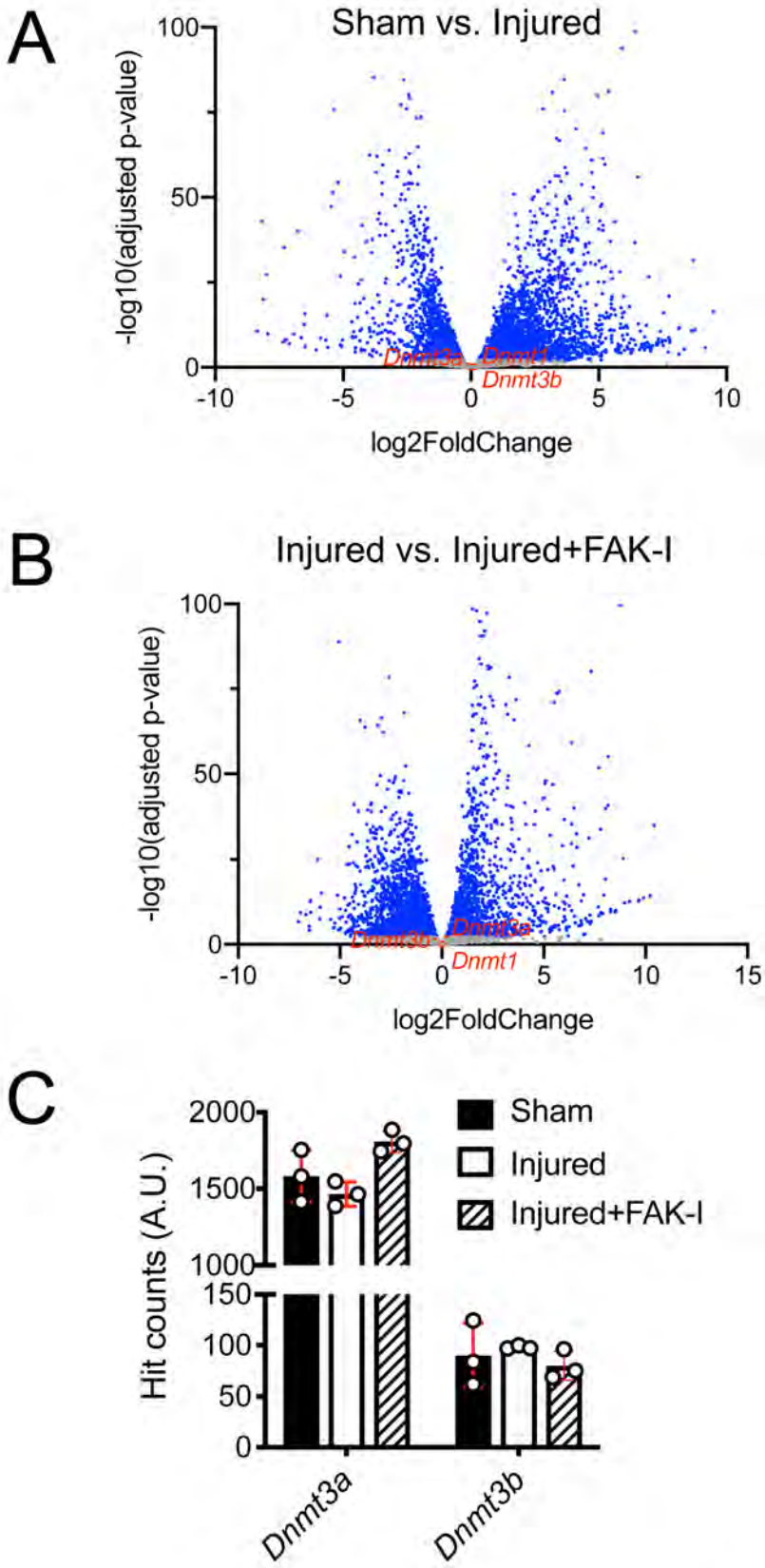
Replicate staining #2

Online Figure XXVI

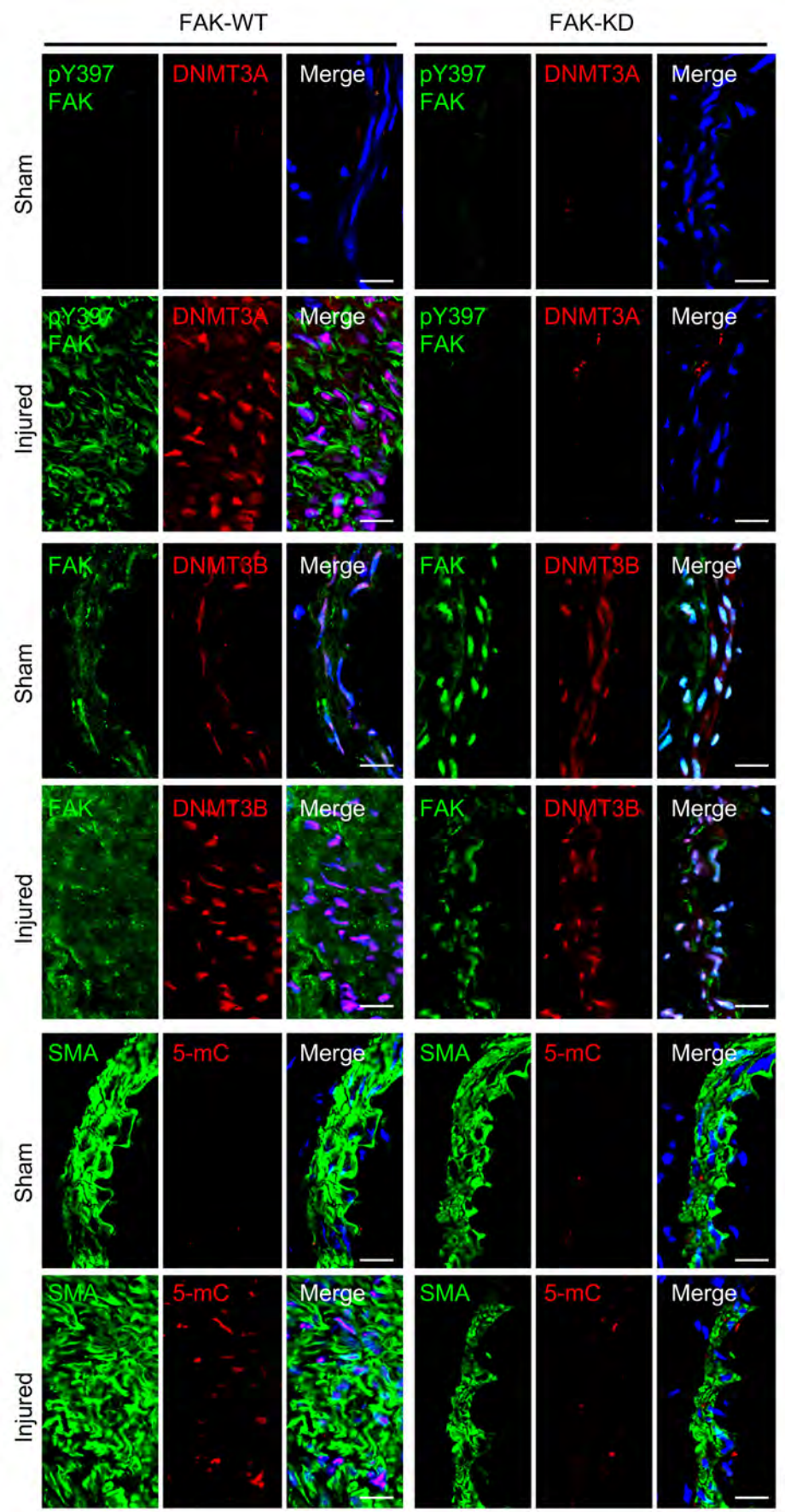


Replicate staining #3

Online Figure XXVII

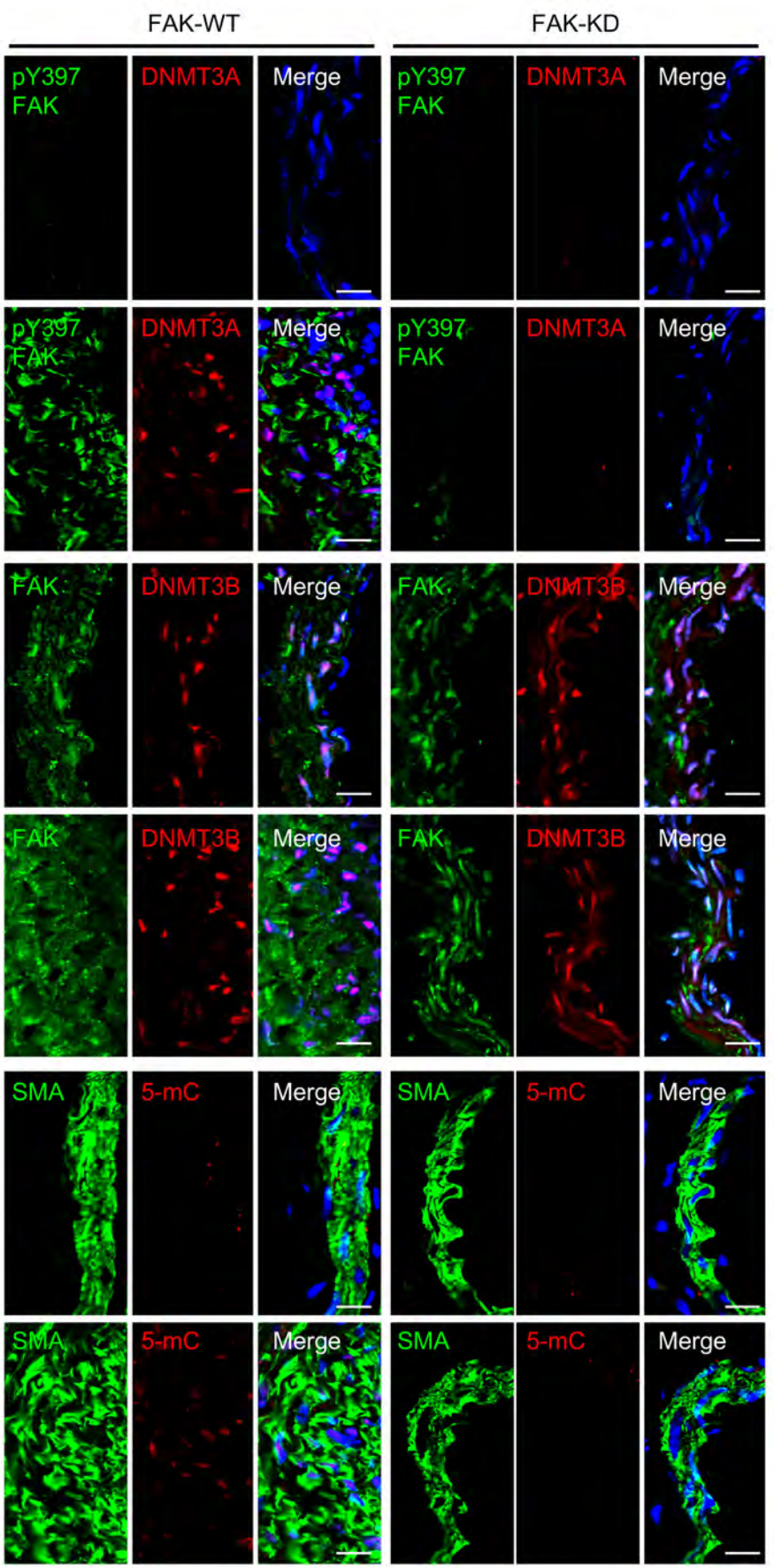


Online Figure XXVIII



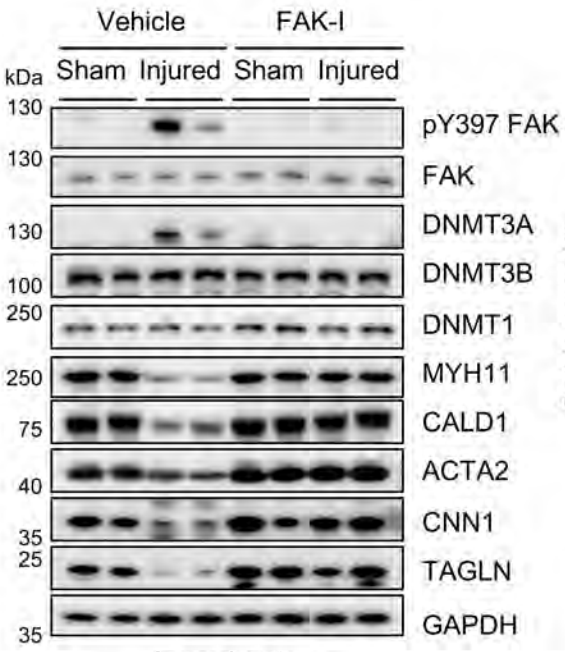
Replicate staining #2

Online Figure XXIX

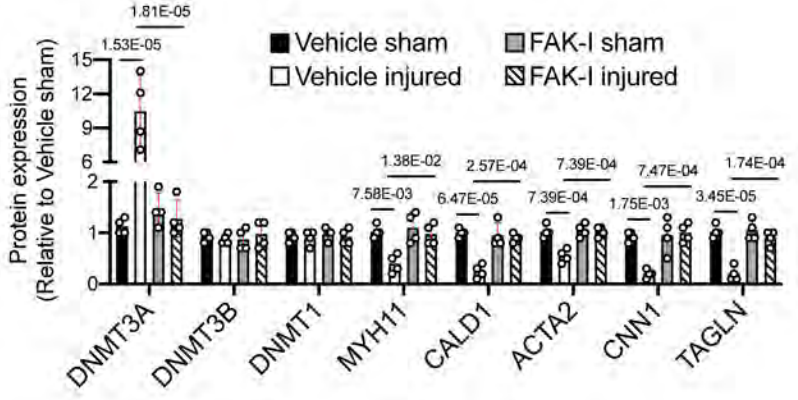


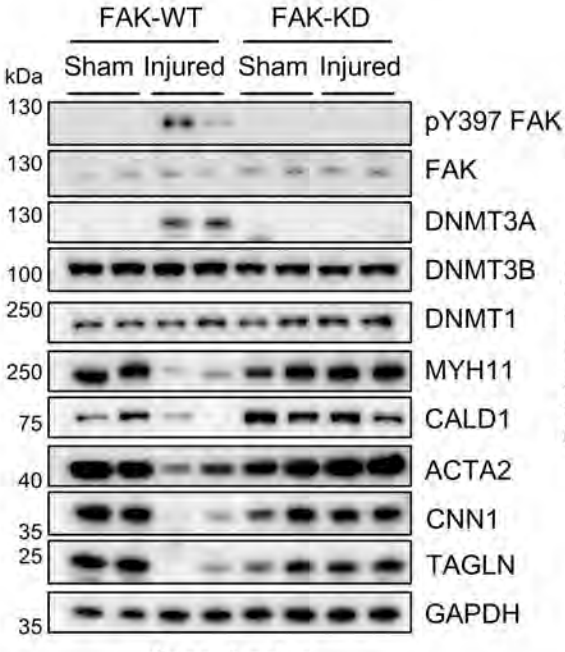
Replicate staining #3

Online Figure XXX

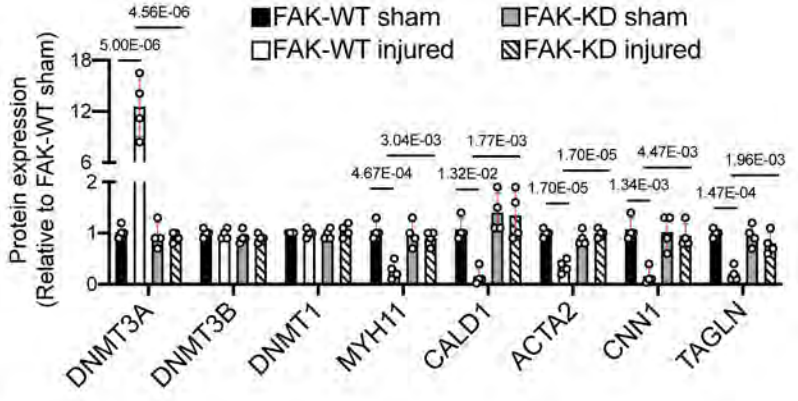


Artery lysates
(Replicate blot #2)

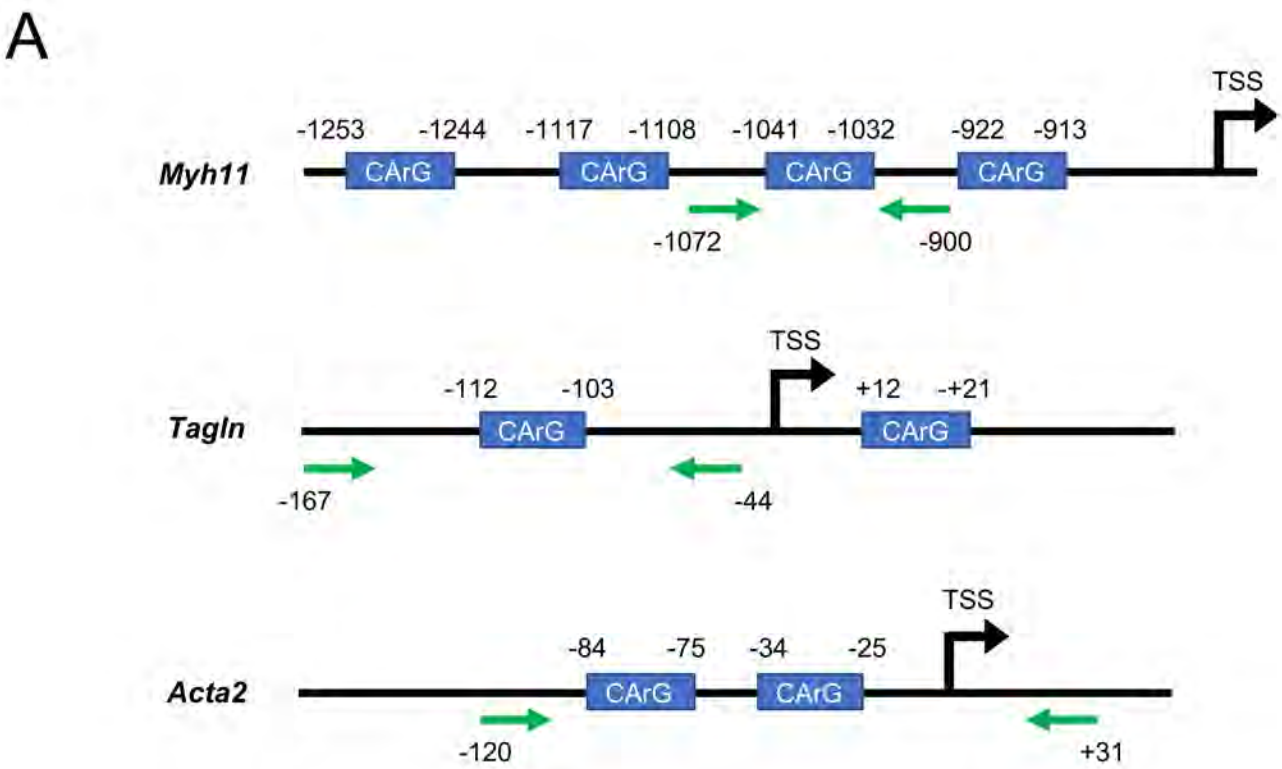


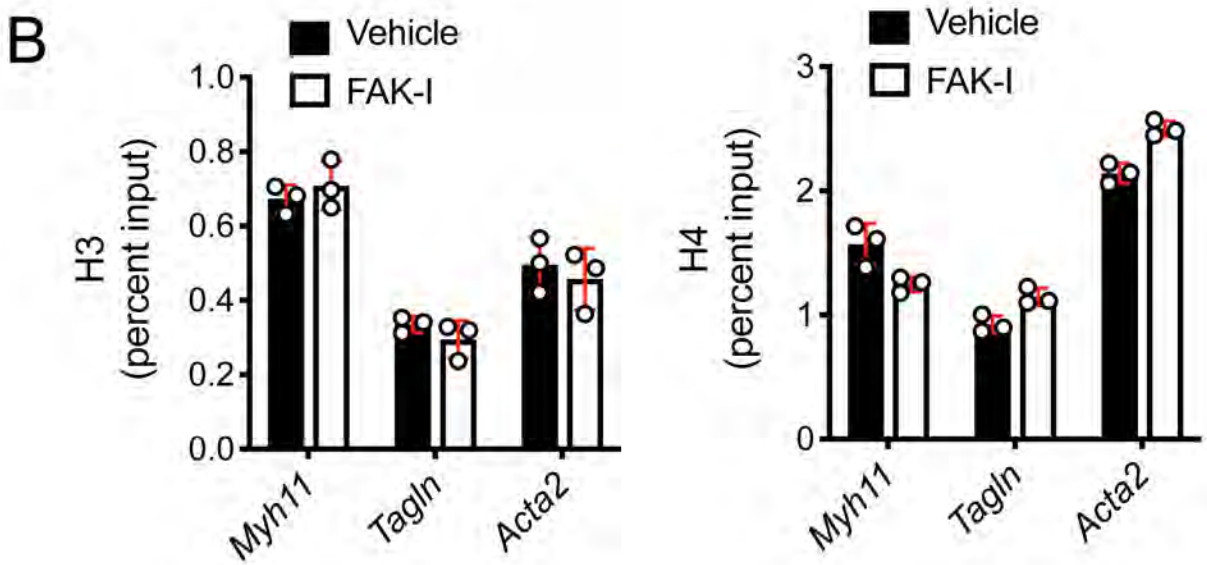


Artery lysates
(Replicate blot #2)

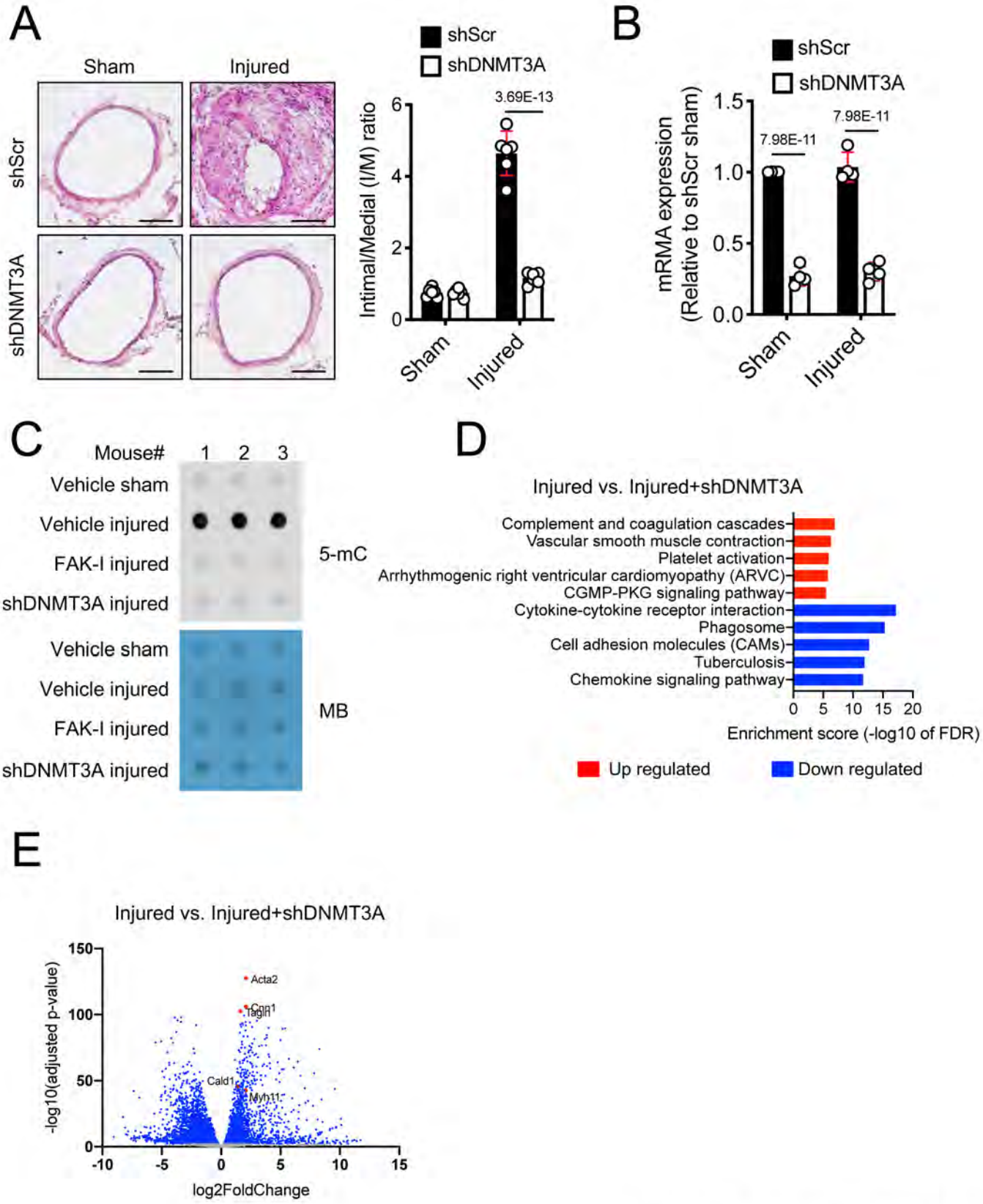


Online Figure XXXI

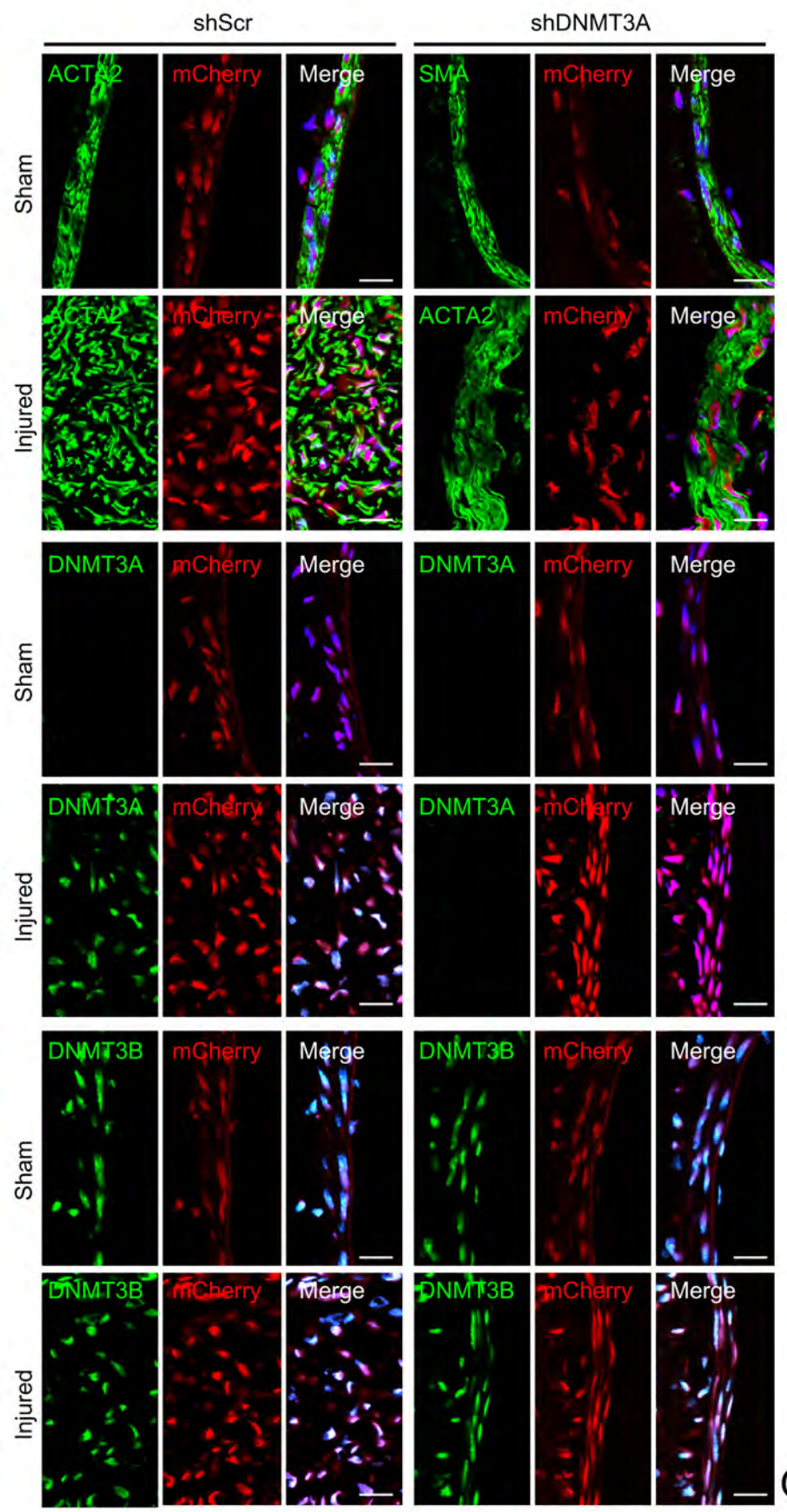




Online Figure XXXII

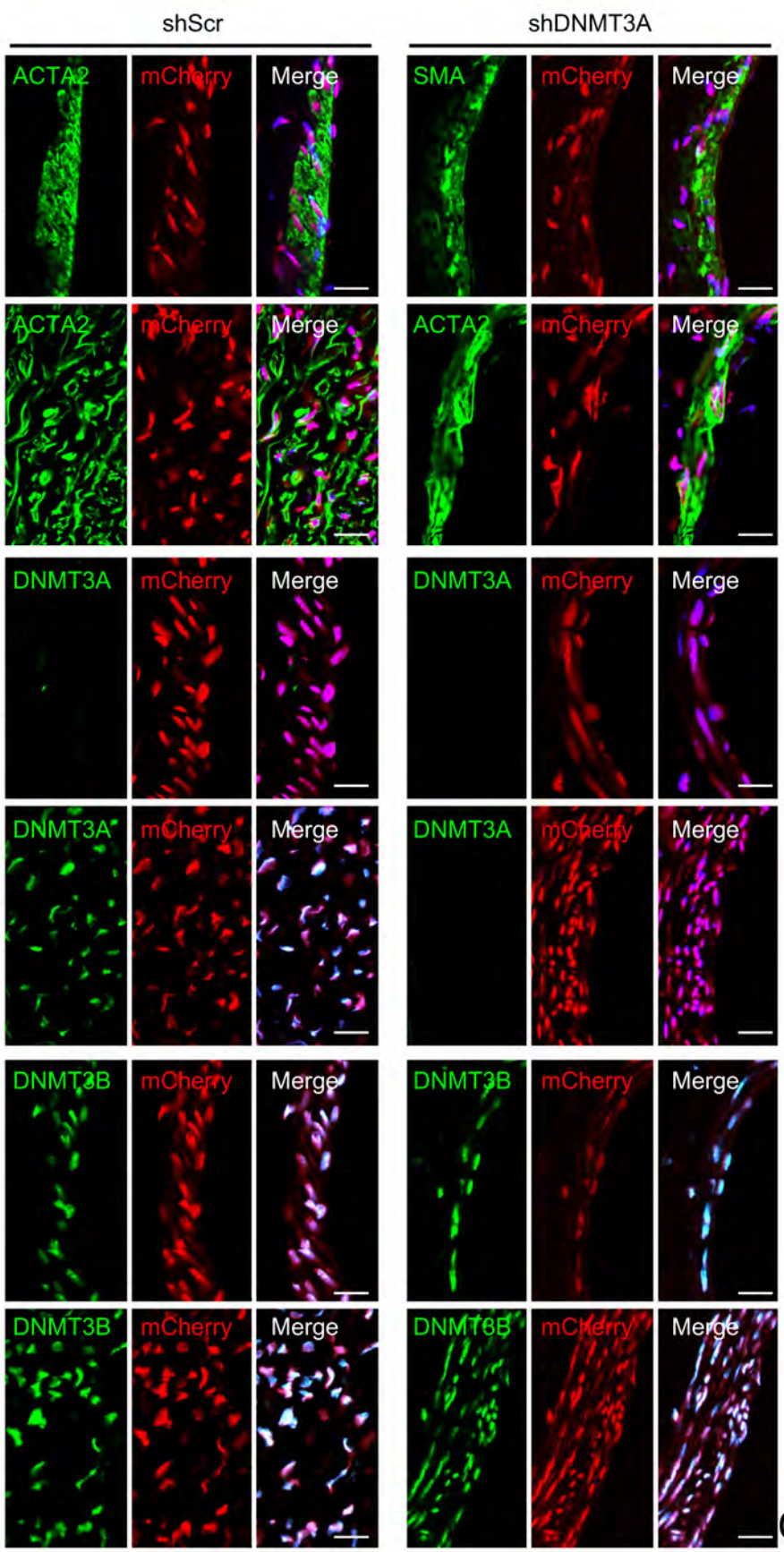


Online Figure XXXIII



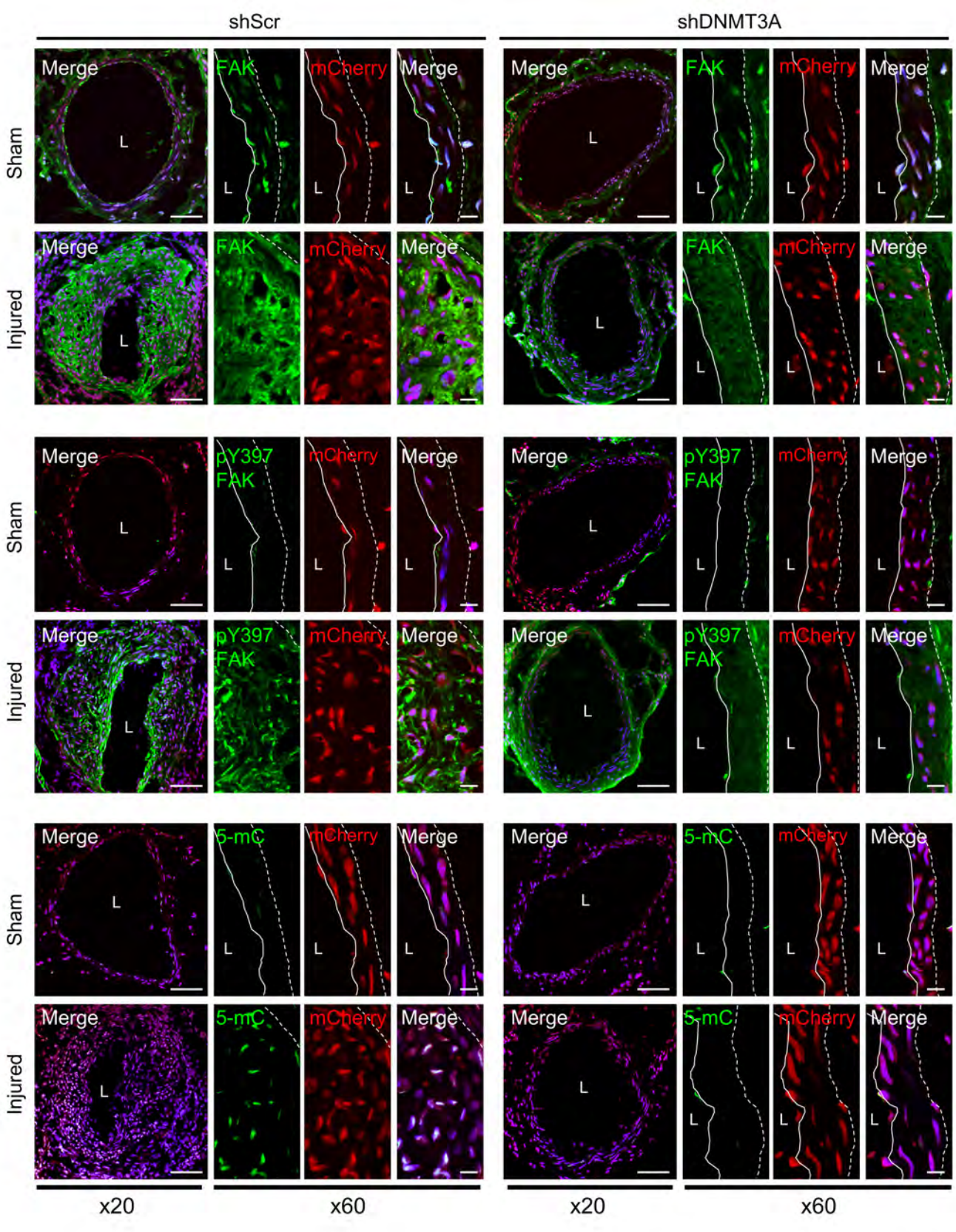
Replicate staining #2

Online Figure XXXIV



Replicate staining #3

Online Figure XXXV



Online Figure XXXVI