SUPPLEMENTAL MATERIAL

Expanded Materials and Methods

Animals, tissue samples and cell culture. All protocols in this study have been conducted in accordance with approved local ethical guidelines. Mice of the following strains were bred in our animal facilities and used at adult age: FVBN 9x-NFAT-luciferase reporter (NFAT-luc, [1]), BalB/c NFATc3 knock-out ($NFATc3^{-/-}$, [2]) and wild-type littermates ($NFATc3^{+/+}$), C57Bl/6 OPN knock-out ($OPN^{-/-}$, [3]) and wild-type littermates ($OPN^{+/+}$). Also wild-type adult NMRI, C57Bl/6 and BalB/c were used (Taconic, Europe). In addition, NFAT-luc were backcrossed with $NFATc3^{-/-}$ on the BalB/c background for, at least, four generations and NFAT-luc/$NFATc3^{+/+}$, NFAT-luc/$NFATc3^{+/-}$ and NFAT-luc/$NFATc3^{-/-}$ were used.

Aortas, cerebral arteries (anterior, middle, posterior, cerebellar and basilar) and plasma were used. Unless perfusion fixed (see below), vessels were dissected free of surrounding tissue in ice-cold physiological saline solution (PSS, containing in mmol/L: NaCl, 135; KCl, 5.9; $MgCl_2$, 1.2; Hepes, 11.6; glucose, 11.5; pH 7.4). Arteries were used directly after dissection or after organ culture in DMEM and Ham's F12 (1:1, Biochrom AG, Berlin, Germany) supplemented with 50 U/mL penicillin and 50 μg/mL streptomycin.

For electrophoretic mobility shift assay (EMSA), VSMCs and Jurkat cells (Human T cell lymphoma) were used. VSMCs were obtained from explants of mouse aorta and used up to passage 7 as previously described [4]. Cells were cultured in DMEM and Ham's F12 (1:1) supplemented with 15% fetal bovine serum (FBS, Biochrom AG, Berlin, Germany). Cells were made quiescent by removal of serum for 24 h before experiments started. Jurkat cells were grown in RPMI medium supplemented with 10% FBS, 10 mmol/L HEPES, 2 mmol/L pyruvate, 50 μmol/L 2-mercaptoethanol and 50 μg gentamicin per mL (complete RPMI media) at 37°C and 5% CO_2.

Intraperitoneal glucose tolerance test (IP-GTT). Male C57Bl/6 mice were fasted for 16 hours followed by intraperitoneal (i.p.) injection of glucose (2 g per kg body weight) or equal volume of saline (vehicle). Blood glucose levels were measured in whole venous blood from the tail vein (OneTouch glucometer, LifeScan, Inc., CA). Mice were sacrificed and whole body perfusion fixed with 4% paraformaldehyde. Cerebral arteries were dissected out for measurement of NFATc3 nuclear accumulation by confocal immunofluorescence.

Streptozotocin (STZ) treatment. Mice were injected i.p. with STZ (60 mg per kg body weight in citrate buffer, pH 4.5) or equal volume of vehicle (citrate buffer), once a day during 4, 5 or 6 days depending on the mouse strain, for NFAT-luc, C57Bl/6 and BalB/c, respectively, since pilot experiments demonstrated different susceptibility to STZ treatment (not shown). Mice were weighed and glucose levels measured in whole venous blood (OneTouch glucometer, LifeScan, Inc., CA) at various time-points as indicated in the text. Diabetes was defined by blood glucose > 250 mg/dL (13.9 mmol/L). Animals with a loss of body weight >15% were excluded from the study (two C57Bl/6) and mice showing signs of dehydration or weight loss >10% received daily i.p. injections of saline (1 mL). All animals were fed normal chow diet and water *ad libitum*. Male and female NFAT-luc mice were used for measurement of NFAT-dependent transcriptional activity with a luciferase reporter assay. For these experiments, animals were euthanized at day 8, 12 or 16 after the first STZ injection and aortas dissected out

and frozen for further processing (see below). In experiments using NFAT-luc/NFATc3$^{+/+}$, NFAT-luc/NFATc3$^{+/-}$ and NFAT-luc/NFATc3$^{-/-}$, mice were euthanized at day 12 after the first STZ injection. Female C57Bl/6 mice were used for determination of OPN levels in plasma and protein expression in subvalvular aortic sections. In our hands, female mice are more prone to develop atherosclerotic lesions in aorta than male mice, so we predicted female mice to be more susceptible to macrovascular complications in diabetes and hence a better model to study the impact of NFAT signaling on OPN expression. Eight weeks after the first STZ injection, animals were anaesthetized by i.p. injection of 7.5 mg ketamine hydrochloride and 2.5 mg xylazine per 100 mg body weight, and euthanized by exsanguination through cardiac puncture for collection of blood. After whole body perfusion with phosphate-buffered saline (PBS) followed by Histochoice (Amresco Inc, OH), the aortic arch was dissected out and stored in Histochoice at 4^{o}C until further processing. Female BalBC mice were used for *in vivo* experiments using the NFAT inhibitor A-285222 (0.15–0.29 mg/kg body weight in saline solution administered i.p., once a day during the length of the experiment). Animals were euthanized 4 weeks after the first STZ injection, plasma was collected for insulin and glucagon measurements and aortas dissected out for western blotting experiments. A-285222 inhibits all NFAT isoforms and was kindly provided by Abbott Laboratories (Abbott Park, IL).

Luciferase reporter assay. Phenotypically normal NFAT-luc transgenic mice [1] were used. These mice express nine copies of an NFAT binding site from the interleukin-4 promoter, positioned 5' to a minimal promoter from the α-myosin heavy chain gene (-164 to +16) and inserted upstream of a luciferase reporter gene. Also, NFAT-luc/NFATc3$^{+/+}$, NFAT-luc/NFATc3$^{+/-}$ and NFAT-luc/NFATc3$^{-/-}$ were used. Luciferase activity was measured in aorta after *in vivo* STZ-treatment or after *ex vivo* stimulation of intact arteries as specified in the text. Assays were performed as previously described [4, 5] and optical density measured using a Tecan Infinite M200 instrument (Tecan Nordic AB, Mölndal, Sweden).

Immunofluorescence. For measurement of NFATc3 nuclear accumulation, experiments were performed as previously described [4-6]. Briefly, primary antibody, rabbit anti-NFATc3 (1:250 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) and Cy5-labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) were used. Nuclei were stained with the nucleic acid dye SYTOX Green (Molecular Probes, Invitrogen, Paisley, UK). NFATc3 and nuclear regions were detected by monitoring Cy5 and green fluorescence on a Zeiss LSM 5 laser scanning confocal microscope. Images were obtained at 63X magnification and mean fluorescence intensity of nuclear NFATc3 was quantified using the Zeiss LSM 5 analysis software. For OPN staining, unless perfusion fixed after *in vivo* experiments, vessels were fixed in 4% formaldehyde in PBS. Aortic sections (10 μm) and intact cerebral arteries were permeabilized with 0.2% Triton X-100 in PBS for 10 minutes and blocked with 2% bovine serum albumin (BSA) in PBS for 2 hours. Rabbit anti-OPN (1:500 dilution, IBL, Hamburg, Germany) was applied overnight at 4°C and Cy5-anti-rabbit IgG (1:400 dilution) for 1 hour at room temperature. For quantification, multiple fields for each vessel were imaged (10X and 63X) and analyzed under blind conditions. Three to five boxes were randomly positioned within the vessel media layer and mean pixel intensity (range 0 to 255 grayscale values) after background subtraction was calculated using the Zeiss LSM 5 Pascal Analysis software. Specificity of immune staining was confirmed by the absence of staining when

primary or secondary antibodies were omitted from the protocol or in sections from $OPN^{-/-}$ mice.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared essentially as described before [4]. VSMCs were treated for 30 min with UTP (100 µmol/L) and then rinsed with ice-cold PBS, harvested by scraping, centrifuged (850 x g, 4°C, 2 min), and resuspended in 60 µL of hypotonic buffer containing (in mmol/L) Tris (pH 7.3) 10, KCl 10, and $MgCl_2$ 1.5. 2-Mercaptoethanol (5 mmol/L) and protease inhibitor cocktail (Sigma) were added to all buffers. After centrifugation (9000 x g, 2 min), cells were lysed by resuspension in 80 µL of lysis buffer containing (in mmol/L) 10 Tris (pH 7.3), 10 KCl, and 1.5 $MgCl_2$, and 0.4% Nonidet P-40. After incubation at 4°C for 10 min, nuclei were collected by centrifugation (9000 x g, 1 min). Pellets were washed once in 500 µl of 20 mmol/L KCl buffer that contained (in mmol/L) 20 Tris (pH 7.3), 20 KCl, 1.5 $MgCl_2$, 0.2 EDTA, and 21.75% glycerol. Isolated nuclei were resuspended in 15 µl of 20 mmol/L KCl buffer and 60 µl of 600 mol/L KCl buffer containing (in mmol/L) 20 Tris (pH 7.3), 600 KCl, 1.5 $MgCl_2$, 0.2 EDTA, and 21.75% glycerol. Nuclear proteins were extracted by incubation on ice for 30 min. After centrifugation (9000 x g, 4°C, 15 min), the supernatant containing nuclear proteins was transferred to precooled microcentrifuge tubes and used for EMSA. Purity of nuclear fractions was demonstrated by lack of immunoreactivity for the cytosolic marker GAPDH.

DNA probes were labeled with $\gamma[^{32}P]$ ATP (Amersham Biosciences, Piscataway, NJ) by incubation with T4 polynucleotide kinase (Roche Diagnostics, Mannheim, Germany), annealed and purified on a mini Quick Spin Oligo Column (Roche Diagnostics, Sweden). Nuclear extracts were incubated with labeled probe (20,000 cpm, 3 fmol) for 30 min at room temperature in binding buffer (in mmol/L: 10 HEPES [pH 7.9], 70 KCl, 1 dithiothreitol, 1 EDTA, 2.5 $MgCl_2$, 4% glycerol) with 0.75 µg Poly(dI/dC) (Amersham Pharmacia Biotech, UK). Samples were separated on a 6% polyacrylamide TBE gel, which was dried and subjected to autoradiography. Oligonucleotides were as follows:
NFAT site 1-2 sense: 5′-TAAACAACT**GGAAAAT**CCCACTTAG**GGAAAAT**GTCAGCAAC
NFAT site 1-2 antisense: 5′-GTTGCTGAC**ATTTTCC**CTAAGTGGG**ATTTTCC**AGTTGTTTA
NFAT site 3 sense: 5’-GTGATCTACTCTTC**CTTTCCT**TATGGATCC
NFAT site 3 antisense: 5’-GGATCCATA**AGGAAAG**GAAGAGTAGATCAC
NFAT site 4 sense: 5’-TTGTGTGT**GTTTCCT**TTTCTTC
NFAT site 4 antisense: 5’-GAAGAAA**AGGAAAC**ACACACAA
NFAT consensus sense: 5′-CGCCCAAAGA**GGAAAAT**TTGTTTCATA
NFAT consensus antisense: 5′-TATGAAACAA**ATTTTCC**TCTTTGGGCG
NFAT binding sites [7] are indicated in bold and underlined sequences were mutated to AA (sense strand) in NFATmut.

Quantitative RT-PCR. Total RNA was extracted from cultured VSMCs or from intact aortas using TRI Reagent. cDNAs were synthesized with the RevertAid™ H Minus M-MuLV Reverse Transcriptase (Fermentas GMBH, St. Leon-Rot, Germany) according to the manufacturer’s instructions. For quantification of mRNA, real-time PCR was performed on a 7900HT TaqMan (Applied Biosystems, Carlsbad, CA) using TaqMan Gene Expression Assays Mm00436767_m1 directed against mouse OPN (SPP1) and Mm00478295_m1 directed against cyclophilin B (PPIB) which served as endogenous control (Applied Biosystems, Carlsbad,

CA). The relative quantity of OPN mRNA was calculated using the comparative threshold method ($\Delta\Delta C_t$). All experiments were performed in triplicate.

Chromatin Immunoprecipitation (ChIP). ChIP was performed on VSMCs from aorta explants and on intact aortas. Samples were treated as described in the text, after which they were transferred to 1% formaldehyde to cross-link protein-DNA and protein-protein interactions within intact chromatin. Chromatin was purified with the ChIP-IT Express Enzymatic kit (Active Motif, Rixensart, Belgium) according to the manufacturer's instructions. Briefly, aortas were homogenized using a glass homogenizer in lysis buffer and incubated on ice for 30 min before transfer to a dounce homogenizer. Sheared chromatin was immunoprecipitated with 3 μg antibody against NFATc3 (sc-8321, Santa Cruz Biotechnology) or no antibody. Real-time PCR was performed on immunoprecipitated DNA and on input DNA using primers designed to flank the NFAT site 3 (forward primer: CAAGGTCTCTGTGAGGGTGATCT, reverse primer: GAATCCCGGAAGAGCATCAG, probe: CTCTTCCTTTCCTTATGGAT) in the OPN promoter (see Fig. 3, all primers were from Applied Biosystems, Carlsbad, CA). The NFATc3 antibody successfully increased the number of immunoprecipitated chromatin fragments compared to no antibody control. The relative increase in NFATc3 binding to the OPN promoter was calculated using the comparative threshold method ($\Delta\Delta C_t$) with input DNA as the reference. All experiments were performed in triplicate.

Western blotting. For western blotting, frozen arteries were pulverized in liquid nitrogen, and protein extracted with SDS sample buffer (62.5 mmol/L Tris-HCl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromphenol blue). Protein concentration was determined with the EZQ protein quantitation kit (Molecular Probes, Invitrogen, Paisley, UK). An equal amount of protein was loaded onto 12.5% Tris-HCL gels (Bio-Rad Laboratories, Sundbyberg, Sweden) and separated by gel electrophoresis, after which proteins were transfered to Hybond-P polyvinylidene difluoride membranes (Amersham Biosciences, Piscataway, NJ). After blocking in 3% BSA/5% non-fat dry milk, membranes were incubated with primary antibody against OPN (1:500 dilution in 3% BSA, IBL, Hamburg, Germany) and HRP-conjugated secondary antibody (Cell Signaling, Danvers, MA), and bands detected with chemiluminescence (Supersignal West Femto, Pierce Biotechnology, Rockford, IL). Recombinant histidine-tagged OPN was produced by 293-EBNA cells, which had been transfected with a plasmid coding for full-length murine OPN [8]. Vessels from $OPN^{-/-}$ mice were use as negative control. Coomassie blue staining of the upper portions of the gels or western blotting of β-actin (1:1000 dilution, GenScript Corporation, Piscataway, NJ) were used as loading controls.

OPN ELISA assay. The levels of OPN in plasma were assayed using Quantikine mouse OPN ELISA kit (R&D Systems, Abingdon UK) according to the manufacturer's instructions. Absorbance was measured at 450 nm and the lower limit of detection was 5.7 pg/mL.

Glucagon and insulin measurements. Plasma levels of glucagon and insulin were determined using in-house radioimmunoassays [9, 10]. Insulinogenic index was calculated as an indicator of pancreatic B-cell function, by dividing plasma insulin concentration (pmol/L) by plasma glucose concentration (mg/dL).

Statistics. Results are expressed as means ± SEM, where applicable (n represents number of mice). Statistical analysis was performed using GraphPad (Prism 4.0) and significance was determined using two-tailed Student's t-test, or one-way analysis of variance followed by Bonferroni's multiple comparison tests. Correlation analysis was performed using two-tailed Pearson's correlation test.

Supplemental Data

Supplemental Figure I. Insulin does not affect NFATc3 nuclear accumulation. Summarized data from confocal immunofluorescence experiments showing fluorescence intensity of nuclear NFATc3 in cerebral arteries treated *ex vivo* with 2 and 20 mmol/L glucose (36 and 360 mg/dL, respectively) for 30 min with or without insulin (0.6 and 6 ng/mL) or insulin alone (6 ng/mL). N=8, 37 to 70 images analyzed in each group, *P < 0.05 for 20 mmol/L glucose vs. 20 mmol/L glucose + insulin (6 ng/mL), ***P < 0.001 vs. control.

Supplemental Figure II. NFAT binds to elements in the OPN promoter. A. Mouse OPN 5′ flanking region of the mouse OPN gene. Transcription start site is indicated (+1). Location and sequence of suggested NFAT-binding sites 1-2, site 3 and site 4 are underlined. The mutated site 3 is shown in italics (*AA* instead of *CC*). **B.** EMSA experiments using VSMCs and T-cells and a labeled NFAT consensus probe (left gels) or NFAT-binding site 3 from the OPN promoter (right gel). Arrows indicate the slower (C2) and faster migrating complexes (C1), the free probe and wells. Experiments were repeated three times.

Supplemental Figure III. Representative image from EMSA experiments using nuclear extracts from VSMCs and NFAT-binding site 3 from the OPN promoter. The slower migrating complex (C2) was disturbed by the addition of an antibody against NFATc3. The inclusion of a control antibody (anti-actin) or antibodies against NFATc1, NFATc2 or NFATc4 did not disturb the binding of any of the complexes. Experiment was repeated three times.

Supplemental Figure IV. UTP-induced OPN expression is mediated via NFAT in mouse aorta. A. Confocal immunofluorescence images showing OPN (red) expression in aorta. The green represents autofluorescence from the elastic lamina. Aortas were cultured for 4d with or without UTP (100 μmol/L, supplemented daily) and A-285222 (1 μmol/L, added once at the beginning of the culture period). Bars=50 μm. **B**. Western blot showing OPN protein expression in mouse aorta cultured for 3d in the presence or absence of UTP and A-285222 as in A. Recombinant OPN was used for identification of bands and Coomassie blue staining of the upper part of the gel was used for equal loading and normalization to total protein in each lane. Summarized data from 4 separate experiments including vessels from 16 mice is shown above the gel, values are normalized to UTP (*P < 0.05, **P < 0.01). **C**. Stimulation for 30 min with UTP (100 μmol/L) increases NFAT-dependent transcriptional activity (RLU/μg) and this is prevented by A-285222 (1 μmol/L). Values are measured after 6 h in culture (4 experiments including vessels from 20 mice). **P < 0.01 vs. UTP.

Supplemental Figure V. STZ-induced hyperglycemia increases OPN expression in mouse aorta. A. Confocal images showing OPN expression (in white) in subvalvular sections of mouse aorta under normoglycemic and hyperglycemic conditions. Inserts show the same sections as in the larger images, stained with SYTOX Green for visualization of the vessel morphology and OPN (in red). Arteries from C57Bl/6 were harvested 8 weeks after the first STZ injection. Bar=200 μm. **B.** Summarized data from experiments in A showing mean fluorescence intensity of OPN staining in the vessel media and the valve leaflets. Six sections for each animal were analyzed, N=11 or 16 in each group, ***P < 0.001 vs. vehicle). **C.**

Correlation between OPN expression in arterial media layer and blood glucose levels (N=27). **D.** Plasma levels of OPN were measured using ELISA in mice treated with STZ or vehicle. N=9 or 12 in each group, *P < 0.05 vs. vehicle. **E.** Correlation between plasma levels of OPN and blood glucose levels (N=21).

Supplemental Figure VI. Insulin and glucagon levels in plasma were measured by radioimmunoassay from plasma collected at the end of the experiments described in Figure 5C and D. Insulinogenic index (left panel) was calculated as an indicator of pancreatic β-cell function, by dividing plasma insulin concentration (pmol/L) by plasma glucose concentration (mg/dL). Glucagon concentration (right panel) is expressed in pmol/L. N=7-8 for each group. *P < 0.001 for control vs. STZ and for A-285222 vs. STZ + A-285222.

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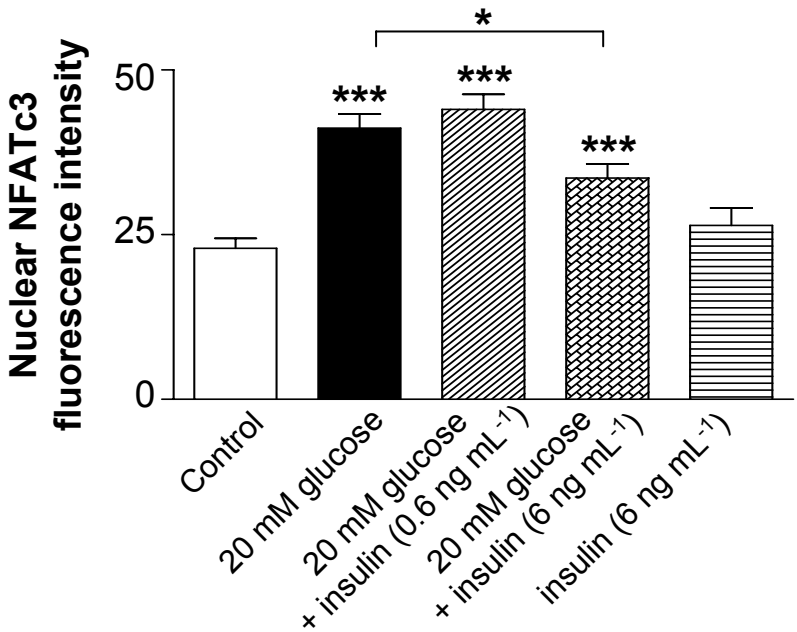
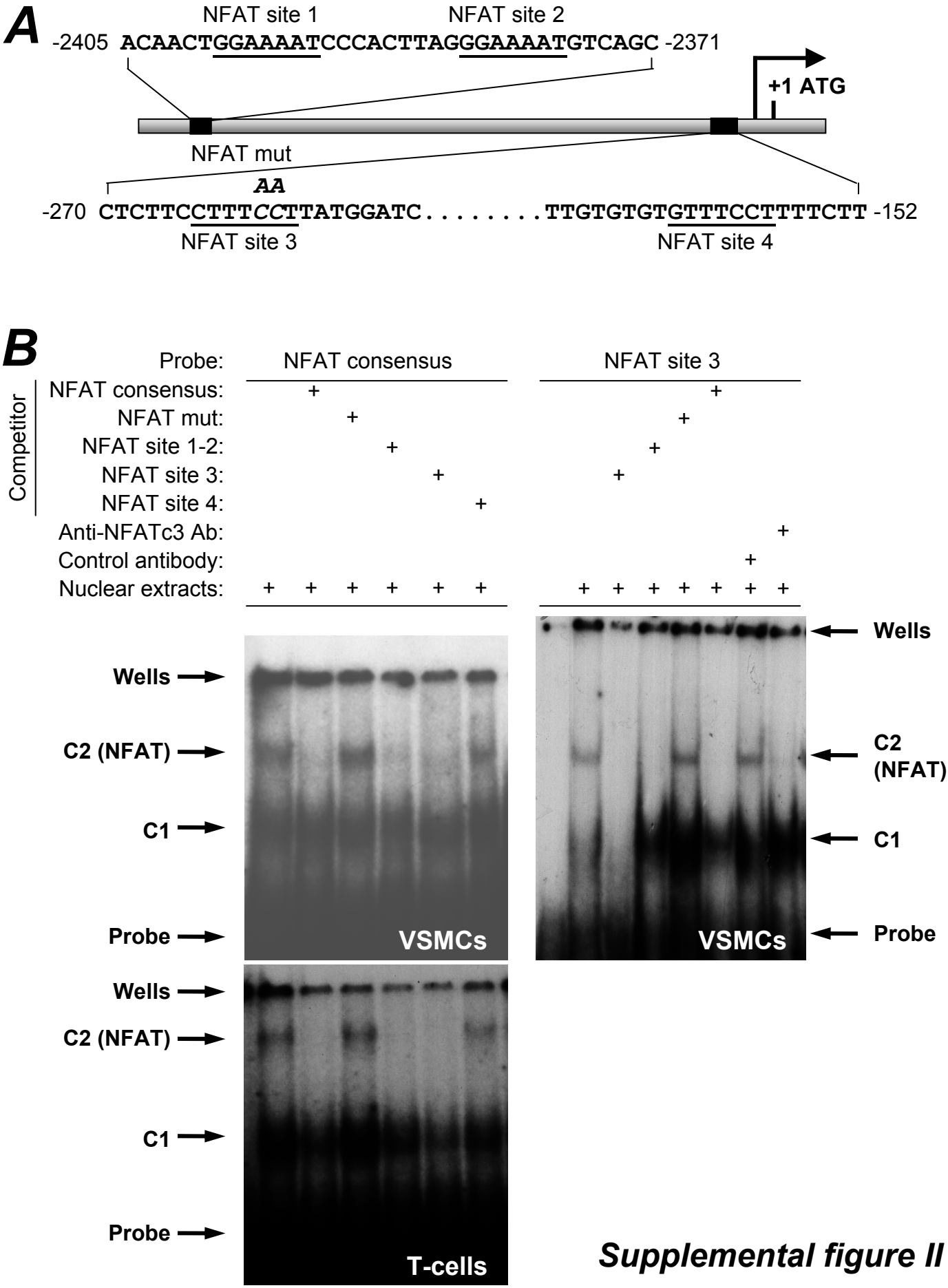


Supplemental figure I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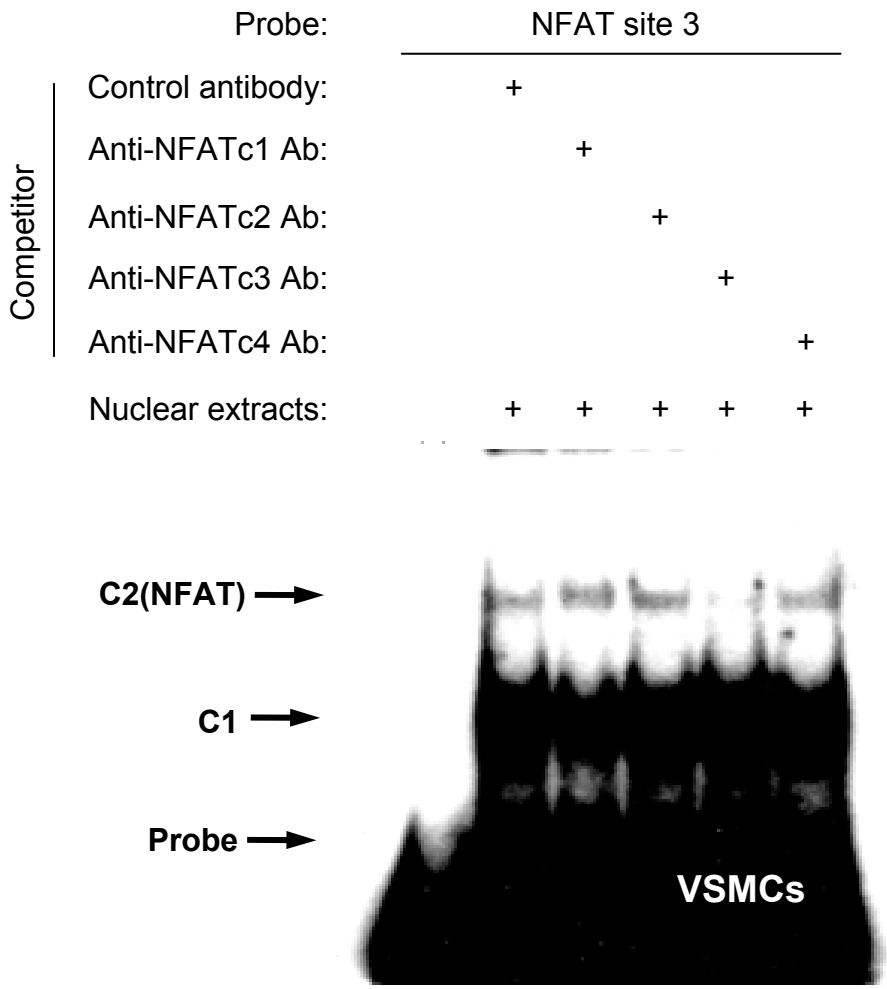

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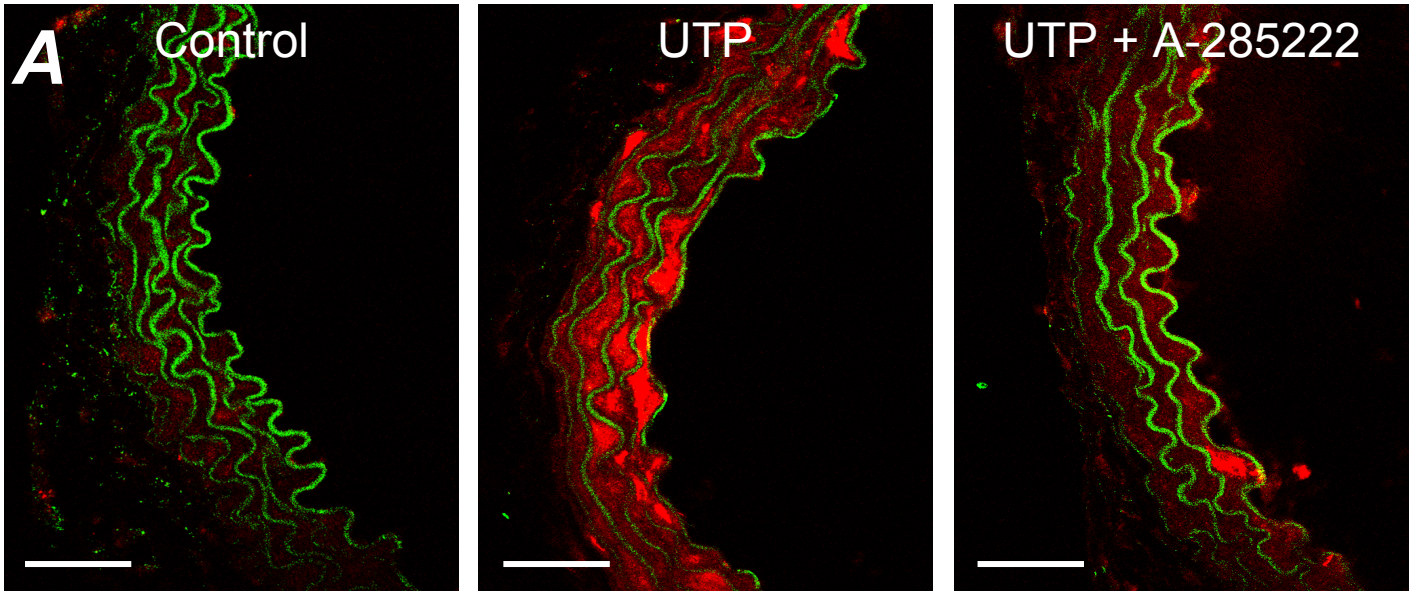

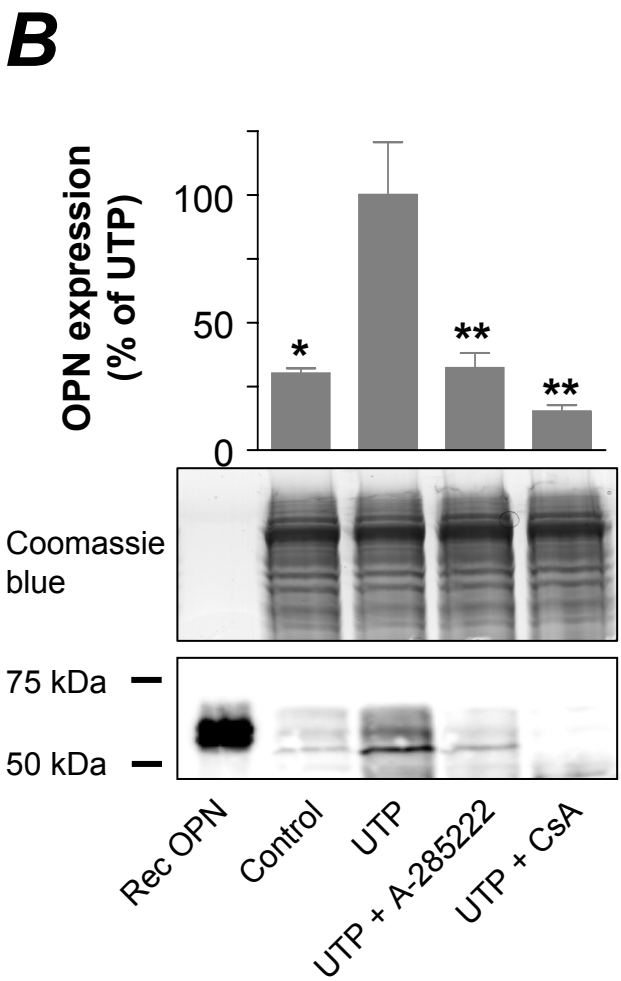

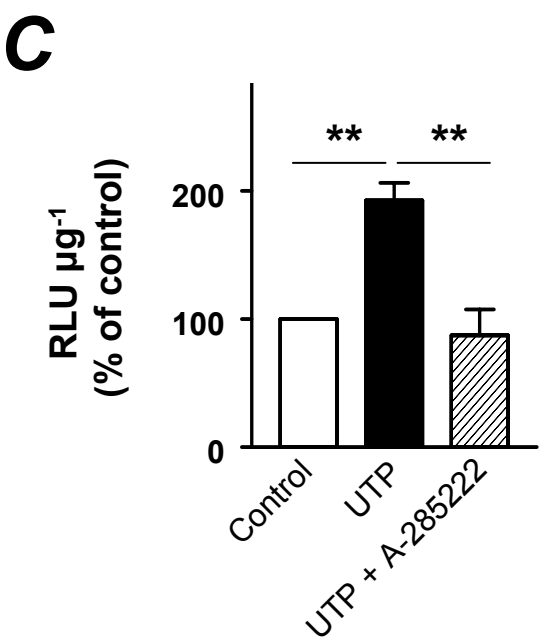

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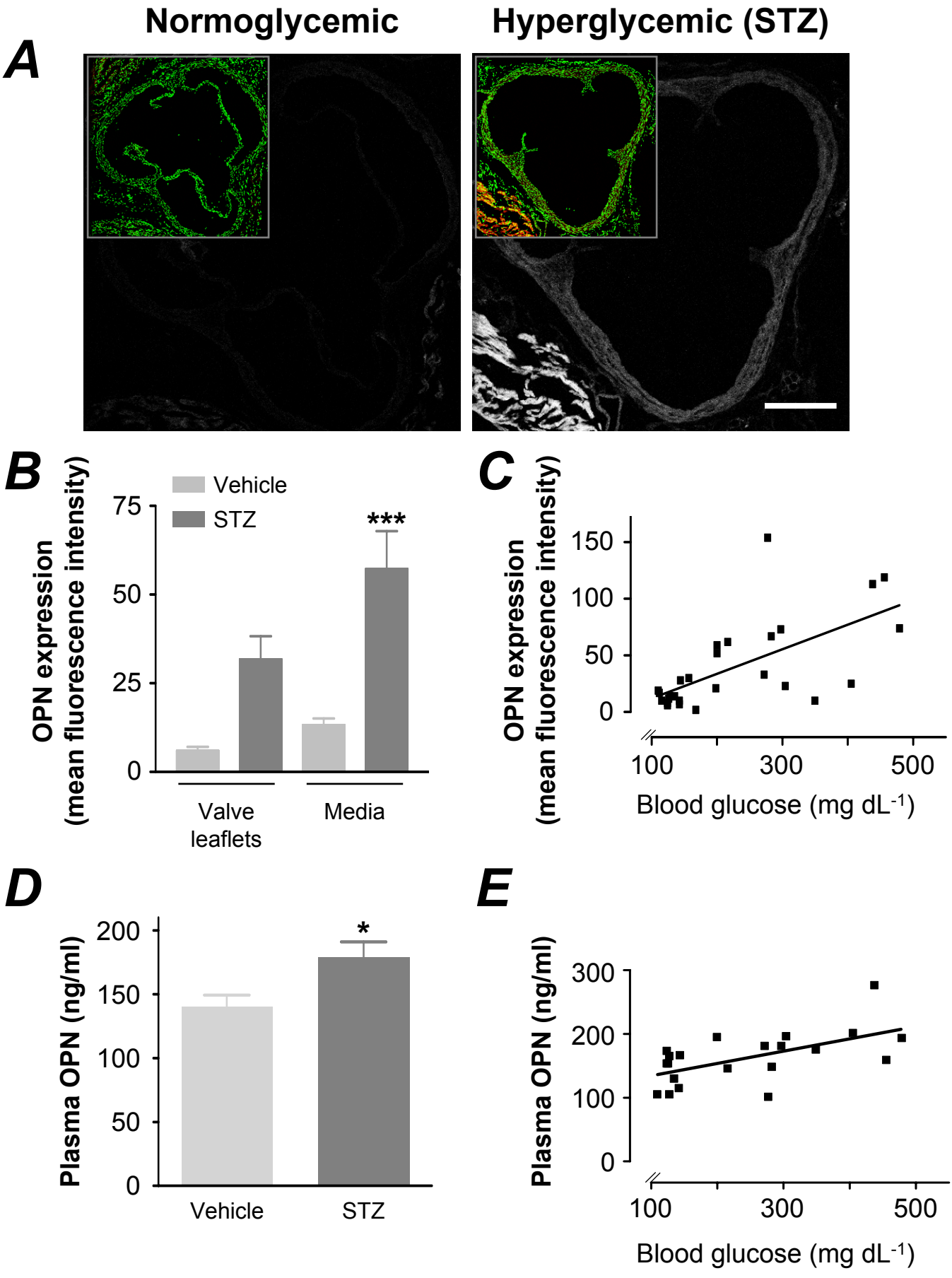

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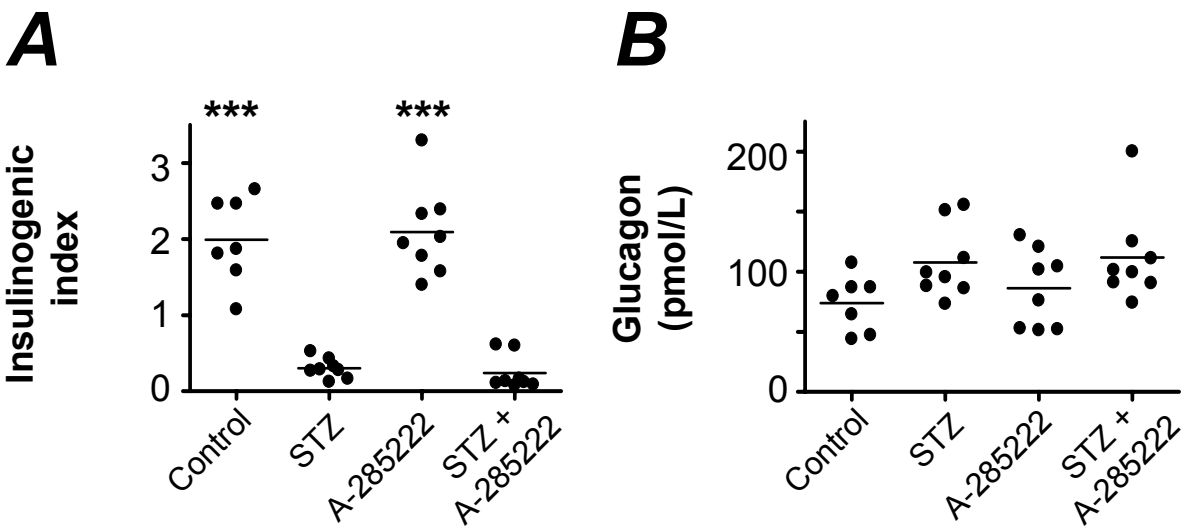

Supplemental figure VI