ONLINE SUPPLEMENT

STIM1 is a Core Trigger of Airway Smooth Muscle Remodeling and Hyperresponsiveness in Asthma

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SUPPLEMENTARY METHODS

Cell Culture

Human Airways Smooth Muscle Cells (HASMCs) are isolated and cultured from human tracheas received from the National Disease Research Interchange (Philadelphia, PA, USA) and the International Institute for the Advancement of Medicine (Edison, NJ, USA). The tracheas are derived from de-identified donors, and hence research using these cells is not classified as human subject research by Penn State's and Rutgers's Institutional Review Board. The HASMCs derived from "normal" donors are donors without any pulmonary disease and died from other causes, whereas HASMCs derived from "asthmatic" donors are donors that died from asthma. As previously described (1), HASMCs were cultured in Ham's F-12 media supplemented with 1% L-Glutamine (Gibco), 1% Penicillin/Streptomycin (Gibco), 0.2% Primocin (InvivoGen), and 10% FBS (Hyclone) at 37°C, 5% CO2, and 100% humidity. Serum free media contains no FBS, but instead supplemented with 1 % BSA. All HASMCs experiments were completed between passages two to five.

Generation of Constructs and Stable Expression in HASMCs

Using Clontech's in-fusion HD cloning method and designed primers (see supplementary oligonucleotide table for all primers for cloning), the cDNA for NFAT4, mCherry, and pLenti PGK Blast V5-LUC were fused together to generate pLenti-NFAT4mCherry construct. The cDNA from NFAT4 was amplified out of pREP-NFAT4 (Addgene:11790). A set of mCherry primers were used to amplify the mCherry cDNA out of CAV1-mCherry (Addgene: 27705). A set of vector primers ("vector 1") were used to amplify the plasmid backbone from pLenti PGK Blast V5-LUC (Addgene: 19166), and the V5 tag and luciferase were removed from this vector. The three fragments were then fused together to generate the pLenti-NFAT4mCherry construct. The CA-NFAT4mcherry construct was made by cloning a synthesized oligonucleotide sequence

of CA-NFAT4 from amino acid 163 to 300 with various Serine to Alanine substitutions (see supplementary oligonucleotide table) into pLenti PGK Blast NFAT4mCherry between amino acid 163 to 300 using Clontech's in-fusion HD cloning method and two pairs of designed primers ("fragment for CA-NFAT3" and "Vector-2"). Similarly, the mCherry pLenti PGK Blast construct was generated by cloning out NFAT4 from the pLenti PGK Blast NFAT4mCherry using "Vector-3" primers. To generate lentiviruses, NFAT4mCherry, CA-NFAT4mCherry, and empty vector pLenti PGK Blast lentiviral constructs were packaged into HEK293FT using the ViraPower kit (Invitrogen) and Lipofectamine 2000 (Thermofisher Scientific). HASMCs were then infected with lentiviruses for 72 hrs and selected with 3 μg/ml of blasticidin for an additional 96hrs.

The shSTIM1 #1 and shSTIM1 #5 pLKO.1 lentiviral shRNA constructs were obtained from the Broad's Institute's RNAi Consortium Library at the Penn State's Genome Science core (see supplementary methods for sequences). To generate lentiviruses, shSTIM1 #1, shSTIM1 #5, and shScramble pLKO.1 lentiviral constructs were packaged into HEK293FT using the ViraPower kit (Invitrogen) and Lipofectamine 2000 (Thermofisher Scientific). HASMCs were then infected with lentiviruses for 72 hrs and selected with 2.5 μg/ml of puromycin for an additional 72 hrs. Western blotting and functional Ca^{2+} imaging was used to confirm knockdown. Stable knockdown and NFAT4-expressing HASMCs were used within two weeks for experiments and then discarded.

Western Blotting

After cells were washed with ice-cold phosphate-buffer saline (PBS), cells were lysed at 80-90 % confluency with chilled RIPA lysis buffer (Millipore Sigma) supplemented with fresh protease and phosphatase inhibitors (Thermofisher Scientific). For deglycosylation experiments, 5 μl of N-Glycosidase F (Millipore Sigma) were added to 50 μg of protein lysate and incubated at 37°C water bath overnight. 30 μg of protein lysate was then loaded into a 4-12% or 8% (for NFAT-4 experiments) Bis-Tris gel (Thermofisher Scientific). Migrated gels were transferred onto a PVDF membrane (Millipore Sigma) and blocked with Olympus Blocking Buffer (LI-COR) for 1 hour at RT. Blocked membranes were then incubated overnight with primary antibodies diluted in Olympus Blocking Buffer and 0.1% Tween (see supplementary methods for dilutions and sources of antibodies). The next day membranes were washed three times with 0.1% TBST for five min each and incubated in secondary antibody (1:10,000 680RD conjugated anti-mouse (LI-COR) and 1:5000 800CW conjugated anti-rabbit (LI-COR)) diluted in Olympus Blocking Buffer with 0.1% Tween for 1 hr at room temperature. After three additional washes with 0.1% TBST for five min each, blots were imaged using Odyssey Clx imaging system. Densitometry was quantified using ImageStudio Lite software. Each densitometry quantification was normalized to the expression of a housekeeping gene (i.e. α-tubulin, GAPDH).

Quantitative PCR

Using RNAeasy Minikit (Qiagen) total RNA was isolated and quantified using the nanodrop 2000 spectrophotometer (Thermofisher Scientific). 1 μg of total RNA was transcribed into cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). SYBR Green qPCR Master Mix (Applied Biosystems), corresponding primers (see supplementary

table), and cDNA was loaded into a 96 well plate. Target mRNA expression was then recorded with the QuantStudio 3 real-time PCR system (Applied Biosystems). The PCR protocol was a 50 °C 2-min activation step, a 95 °C 2-min melt step, and 40 cycles of 95°C for 15 s followed by 60 °C for 15 s and 72 °C for 30 s. Melt curves were generated and analyzed for each target. Target mRNA expression was quantified by comparative C_t method and normalized to housekeeping genes. qPCR experiments were performed in technical and biological triplicates.

Single Cell Ca^{2+} Imaging

Cells were seeded onto 25 mm glass coverslips for 24 hours in complete media. The coverslips were then mounted into an Attofluor cell chamber (Thermofisher Scientific) and incubated with 4 μM of Fura2-AM (Thermofisher Scientific) and 0.1% Pluronic F-127 (Thermofisher Scientific) in complete media for 45 min. HASMCs were then washed with HEPES-buffered salt solution (HBSS) containing 140 mM NaCl, 4.7 mM KCl, 1.13 mM $MgCl_2$, 10 mM HEPES, 2.0 mM $CaCl_2$, and 10 mM gluosce with a pH adjusted to 7.4 by NaOH. After last wash, HASMCs were incubated in HBSS for 10 min at RT. Chambers were then mounted to a Leica DMi8 Fluorescent Microscope equipped with a 20X Fluor objective. Fura 2 was alternatively excited at 340 and 380 nm using a faster shutter wheel (Sutter Instruments) and corresponding emissions was collected at 510 nm. For each pixel, the ratio of fluorescence at 340 and 380 ($F_{340}F/_{380}$) was recorded using a Hamamatsu Flash 4 camera. Using Lecia Applicate Suite X (LAS X) software, ROIs were drawn around the perimeter of each cell, and the $F_{340}F/_{380}$ was analyzed for 15-35 cells per coverslip.

NFAT Nuclear Translocation Imaging

HASMCs stably expressing NFAT4mCherry were seeded onto 25 mm glass coverslips for 24 hours in complete media. The next day coverslips were mounted into an Attofluor cell chamber (Thermofisher Scientific) and placed in HBSS for 10 min at RT. Chambers were then mounted to a Leica DMi8 Fluorescent Microscope equipped with a 20X Fluor objective. NFAT4mCherry was excited with a 561nm fast wheel filter and emissions was collected with a RFP filter cube. Fluorescence was recorded using a Hamamatsu Flash 4 camera. Using LAS X software, ROIs were drawn in the nucleus and cytosol of each cell. Background fluorescence was subtracted from each ROI. NFAT4 translocation of each cell was calculated by dividing the fluorescence from nuclear ROI to the cytoplasmic ROI.

HASMC Proliferation and Migration Assays

HASMC proliferation was measured using CyQUANT Cell Proliferation Assay (Thermofischer Scientific). 2,000 HASMCs in complete media were seeded in each well of 96 well tissue culture plate. For time zero recording, three wells from each condition were then washed with PBS and stained with CyQUANT dye for one hour at 37°C, 5% CO_2, and 100% humidity after one-hour post seeding. A plate reader (Flexstation 3) recorded fluorescence from these wells at 485 and 530 nm excitation and emission respectively. Background fluorescence was subtracted from all readings. The cells were then returned to 37°C, 5% CO_2, and 100% humidity and further readings were taken at 24, 48, and 72 hrs. Readings were normalized to time zero recording and represented as RFU/RFU_0. HASMC migration was measured by seeding 20,000 HASMCs into

silicon inserts with a 500 μm gap in a 6 well culture plate for 24 hrs. To allow cells to synchronize, HASMCs were then serum-starved for 24 hrs. The silicon inserts were removed and complete media supplemented with 10 μg/ml of mitomycin C (Sigma-Aldrich) was placed on HASMCs. Using Leica DMi8 Fluorescent Microscope equipped with a 4X objective, bright field images were taken at 0, 12, and 24 hours. Percent gap closure was calculated by normalizing the size of the gap area between 0 and 12 or 24 hrs by the area at 0 hrs.

For rescue experiments, proliferation and migration measurements were normalized to shScramble condition of that rescuing construct.

Flow Cytometry

HASMC apoptosis was measured using flow cytometry and costaining for FITC-Annexin V (Tonbo Biosciences) and 7-AAD (Tonbo Biosciences). 200,000 HASMCs were harvested per sample and wash once with cold PBS. Pellets were resuspended with 100 μl of Annexin V Buffer (10 mM HEPES, 150 mM NaCl, and 2.5 mM $CaCl_2$ in PBS with a pH adjusted to 7.4) and 5 μl of 7-AAD and 5 μl of FITC-Annexin V. Samples were then incubated for 30 min at RT. 400 μl of Annexin V buffer was then added and data was collected on LSRII flow cytometer (BD Biosciences) and further analyzed by FlowJo Software. Mitochondrial mass was measured by similarly harvesting 200,000 HASMCs per sample and staining cells with 1:10,000 of Mitotracker (Thermofischer Scientific) diluted in HBSS. Samples were stained for 20 minutes in 37°C non-CO_2 incubator. After washing one time with HBSS, data was collected on LSRII flow cytometer (BD Biosciences) and further analyzed by FlowJo Software.

Mitochondrial DNA Measurements

Following methods previously described (2, 3), $1*10^6$ HASMCs were collected, suspended in DirectPCR Lysis Reagent (Viagen Biotech), and digested using Proteinase K. Total DNA was then collected after the sequential additions of isopropanol and 70% ethanol. Using qPCR, SYBR Green qPCR Master Mix (Applied Biosystems), and three sets of primers that specifically amplify regions of mitochondrial DNA and two sets of primers that specifically amplify regions of genomic DNA (see supplementary methods for sequence of primers), target mitochondrial DNA was recorded for each sample. The PCR protocol was a 50 °C 2-min activation step, a 95 °C 2-min melt step, and 40 cycles of 95°C for 15 s followed by 56 °C for 15 s and 72 °C for 30 s. Melt curves were generated and analyzed for each target. Mitochondrial DNA copy number was quantified by comparative C_t method and normalized to genomic DNA.

Metabolic Fuel Flux Assays

To measure oxidative consumption rate (OCR) and extracellular acidification rate (ECAR) in HASMCs, the Seahorse XFp Extracellular Flux Analyzer (Seahorse Bioscience) was utilized. The Seahorse XFp Cell Mito Stress Test Kit and Seahorse Glycolytic Stress Test Kit (Agilent Technologies) were used to measure OCR and ECAR respectively. Following the manufacture's protocol, 22,500 cells were seeded per well in a 24-well XF Cell Culture Microplate with complete media at 37°C, 5% CO_2, and 100% humidity. After 24 hrs., the cells were washed with Seahorse XF DMEM media (pH 7.4), and left in Seahorse XF DMEM media for 1 hour at 37°C

in a non-CO_2 incubator. The plate was then placed in the Seahorse XFp Extracellular Flux Analyzer, and baseline measurements were recorded. For OCR experiments, 0.5 μM oligomycin, 1 μM FCCP (p-trifluoromethoxy carbonyl cyanide phenylhydrazone), and 0.5 μM Rotenone/Antimycin A were sequentially injected at indicated timepoints. Similarly, for ECAR experiments, 10 mM glucose, 1 μM oligomycin, and 50 mM 2-Deoxy-D-glucose (2-DG) were sequentially injected at indicated timepoints. Following the experiment, RIPA was added to each well, and protein concentrations were recorded for each sample using the Pierce Rapid Gold BCA Protein Assay. Measurements from OCR and ECAR experiments were normalized to these protein concentrations.

Glucose and Lactate Measurements

Using the YSI 7100 multichannel biochemistry analyzer (YSI Life Sciences), glucose consumption and lactate production was measured. 50,000 HASMCs were seeded per well in a 24-well tissue culture plate for 24 hrs. at 37°C, 5% CO_2, and 100% humidity. The media from each well was then collected and centrifuged at 5000 RPM for 5 min. The supernatant was then placed into a well of a 96 well plate. Fresh media was also placed into a separate well as a control. The YSI 7100 multichannel biochemistry analyzer then recorded the amount of glucose and lactate in each sample. Glucose consumption was calculated by subtracting the amount of glucose in the media of each sample by the amount of glucose in the fresh media. Lactate production was calculated by subtracting the amount of lactate in the fresh media by the amount of lactate in the media of each sample. Following the experiment, RIPA was added to each well of the 24-well tissue culture plate, and protein concentrations were recorded for each sample using the Pierce Rapid Gold BCA Protein Assay. Glucose and lactate measurements were normalized to these protein concentrations.

Transcriptome Sequencing (RNA-seq) and Differential Expression Analysis

Using RNAeasy Minikit (Qiagen) total RNA was isolated and quantified using the nanodrop 2000 spectrophotometer (Thermofisher Scientific). With 1 μg of RNA, construction of cDNA library was performed by Novogene using an NEBNext® Ultra 2 RNA Library Prep Kit for Illumina (cat NEB #E7775, New England Biolabs, Ipswich, MA, USA) according to the manufacturer's protocol. Briefly, mRNA was enriched using oligo(dT) beads followed by two rounds of purification. It was then fragmented randomly using fragmentation buffer. The first strand cDNA was synthesized using random hexamers primer, and the second strand was generated with a custom second-strand synthesis buffer (Illumina), dNTPs, RNase H and DNA polymerase I (ds cDNA). After a series of terminal repair, poly-adenylation, sequencing adaptor ligation, size selection, and PCR enrichment, the double-stranded cDNA library was ready. Using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and quantitative PCR. Size distribution was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), the 250-350 bp insert libraries were quantified. Qualified libraries were sequenced on an Illumina NovaSeq 6000 Platform (Illumina, San Diego, CA, USA) using a paired-end 150 run (2×150 bases). There were 20 M raw reads generated from each library. Alignments were parse using STAR (v2.5) program (4), which counts the number of paired-end clean reads per gene while mapping. Counts were produced using htseq-count with default

parameters, and FPKM of each gene was calculated using the length of the gene and reads count mapped to this gene (5).

Exploratory analyses were performed on the gene-level read count data, and lowly expressed genes were removed from the data set. The EDASeq R package (6) was used to create a SeqExpressionSet based on the read counts, then upper quantile normalization was applied. The RUVSeq R package (7) was then applied using k = 1 and a set of eleven housekeeping genes described by Eisenberg and Levanon (8) to identify factors of unwanted variation that were included as a covariate in the differential expression analysis performed with edgeR (9, 10). Differentially expressed genes were chosen based on a false discovery rate threshold of $q < 0.05$.

Gene Set Enrichment Analysis (GSEA)

Based on the edgeR output, GSEA software was used to perform pathway analysis (11). The genes in the edgeR output were ordered based on a signed version of the likelihood ratio statistic. In brief, the signed likelihood ratio statistic was computed by multiplying the observed likelihood ratio statistic by the sign of the log fold change. Using the Reactome Gene Sets from the Molecular Signature Database (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp), a GSEA pre-ranked analysis was performed.

Transmission Electron Microscopy Sample Preparation and Morphological Analysis

For HASMC experiments, 90% confluent cells were fixed with fixed in half strength Karnovsky fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde (pH 7.3). Samples were further fixed in 1% osmium tetroxide in 0.1 M Sorenson's buffer (pH 7.4) for 30 minutes. Samples were then dehydrated in a graduated ethanol series, acetone and embedded in LX-112 (Ladd Research, Williston, VT). Thin sections (60 nm) were stained with uranyl acetate and lead citrate and viewed in a JEOL JEM1400 Transmission Electron Microscope (JEOL USA Inc., Peabody, MA, USA). For experiments using mouse lung slices, the lungs from sacrificed mice were first inflated with 1 ml of half strength Karnovsky fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde (pH 7.3) using a tracheal catheter. Entire lungs were dissected and placed in half strength Karnovsky fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde (pH 7.3). Thin lung slices were then prepared and processed using similar techniques as described with the HASMCs.

The morphology and density of mitochondria was analyzed using NIH ImageJ software. At least 10 different micrographs were used for each sample, and analysis was blinded. Mitochondrial density was calculated by counting the number of mitochondria in the micrograph and normalizing to the area of cytoplasm. Morphological parameters (i.e. aspect ratio, circularity, roundness, and solidity) was calculated by manually tracing the outer mitochondrial membrane using NIH ImageJ software.

Metabolomics

90% confluent HASMCs were changed to fresh media 2 hours before harvest. Cells were trypsinized and washed with ice cold PBS, and pellet was then flash frozen in liquid nitrogen.

Metabolites were extracted by NYU Metabolomics Core Resource Laboratory following previously described methods (12). Briefly, the cell count was first used to scale the metabolite extraction to ratio of $1*10^6$ cells/ml of extraction solvent [80% LCMS grade methanol (aq) with 500 nM labelled amino acid internal standard (Cambridge Isotope Laboratories, Inc., Cat No. MSK-A2-1.2)]. Zirconium disrptuion beads (0.5 mm, RPI) were added to each sample and samples were homogenized 5 min at 4°C in a BeadBlaster with a 30 s on, 30 s off pattern. The lysate was centrifuged at 21,000g for 3 min, and 450 µl of supernatant was transferred for speed vacuum concentration. Dried extracts were dissolved in 50 µl of LCMS grade water and sonicated for 2 minutes. They were subsequently centrifuged and transferred to a glass insert for analysis.

The LC column for the LC-MS/MS analysis was Millipore ZIC-pHILIC (2.1 x150 mm, 5 µm) coupled to a Dionex Ultimate 3000TM system. The column oven temperature was set to 25oC for the gradient elution. A flow rate of 100 µL/min was used with the following buffers; A) 10 mM ammonium carbonate in water, pH 9.0, and B) neat acetonitrile. The gradient profile was as follows; 80-20%B (0-30 min), 20-80%B (30-31 min), 80-80%B (31-42 min). Injection volume was set to 2 µL for all analyses (42 min total run time per injection). LC system coupled to a Thermo Q Exactive HF mass spectrometer operating in heated electrospray ionization mode (HESI) was used to measure MS. The method duration was 30 min with a polarity switching data-dependent Top 5 method for both positive and negative modes. The spray voltage for both positive and negative modes was 3.5kV and capillary temperature was set to 32°C with a sheath gas rate of 35, aux gas of 10, and max spray current of 100 µA. Full MS scan for both polarities was utilized 120,000 resolution with an AGC target of 3e6 and a maximum IT of 100 ms, and the scan range was from 67-1000 *m/z*. Tandem MS spectra for both positive and negative mode used a resolution of 15,000, AGC target of 1e5, maximum IT of 50 ms, isolation window of 0.4 m/z, isolation offset of 0.1 m/z, fixed first mass of 50 m/z, and 3-way multiplexed normalized collision energies (nCE) of 10, 35, 80. The minimum ACG target was $1*10^4$ with an intensity of $2*10^5$. Data was acquired in profile mode.

Metabolomic data was processed through Python and R libraries. Peak hight intensities were extracted by the established accurate mass and retention time for each metabolite (13), and verified with authentic standards and/or high-resolution MS/MS manually curated against the NIST14MS/MS (14) and METLIN (15) spectral libraries. The theoretical m/z of the metabolite molecular ion was used with a ± 10 ppm mass tolerance window, and a ± 0.2 min peak apex retention time tolerance within the expected elution window (1–2 min). The median mass accuracy vs the theoretical m/z for the library was −0.6 ppm (n = 121 detected metabolites). Median retention time range (time between earliest and latest eluting sample for a given metabolite) was 0.25 min (30 min LCMS method). A signal to noise ratio (S/N) of 3X was used compared to blank controls throughout the sequence to report detection, with a floor of 10,000 (arbitrary units). Labeled amino acid internal standards in each sample were used to assess instrument performance (median CV% = 12%).

Mouse Models

Institutional Animal Care and Use Committee approved all animal studies. $STIM1^{fl/fl}$ (16), Myh11 Cre (17), and $GCaMP6f^{fl/fl}$ (18) mice have been previously described. All mice are on a

pure C57BL/6. All experimental mice were genotyped by cutting tail tips and running PCRs with primers specific for each transgenic mouse (see supplementary methods for primer sequences). To induce activation of Myh11 Cre, 6-7-week-old mice were intraperitonially injected with 100 μl of tamoxifen (Millipore Sigma) dissolved in sunflower oil (10 mg/ml) daily for five consecutive days. Mice were ready for further experiments one week later.

Mouse Model of HDM-Induced Asthma

Following anesthesia with isoflurane, male STIM1 smKO and littermate controls ($STIM1^{fl/fl}$ and Myh11 Cre) were intranasally challenged with 25 μg of HDM (*Dermatphagoides pteronyssinus*) (Greer Laboratories) suspended in 40 μl of PBS at 8-9 weeks of age. At same time controls were challenged with PBS alone. Mice were challenged for five consecutive days followed with two days of rest for five weeks. 72 hrs after last challenge mice were measured for AHR and tissue/BAL were collected.

Measurement of AHR to Methacholine

13-14-week-old STIM1 smKO mice or littermates that were either challenged to HDM or saline were anesthetized with 7 mg/ml of pentobarbital intraperitoneally. Tracheal catheter was surgically placed on mice. Mice were intubated and ventilated with the small animal ventilator, Flexivent FX1 (Scireq). Dynamic airway resistance was measured when mice were nebulized to 0, 6.25, 12.5, 25, 50, and 100 mg/ml of methacholine using the parameters tidal volume (10 mL/kg), frequency (150/min), and positive end-expiratory pressure (3 cm H_2O).

***Ex Vivo* Ca^{2+} Imaging in Lung Slices**

As described previously (19, 20), male GCaMP6f, STIM1 smKO and littermate controls (GCaMP6f, Myh11 Cre) that were either challenged to HDM or saline mice were anesthetized with 2.5% isoflurane and sacrificed by cervical dislocation. The trachea was cannulated and lungs were inflated with 0.8 ml of 2.5% low-melting agarose in HBSS. An ice pack was then placed on top of the mouse, and agarose was allowed to freeze for 5 minutes. After dissecting the lungs from thorax cavity, the trachea was clamped shut and the lungs were placed in HBSS on ice for 30 minutes. The left lung was then placed into a mold and embedded in 2.5% agarose. The mold was allowed to freeze on ice for 30 minutes. The embedded lung was then sectioned at 200 μm thickness using the Leica VT 1000S vibratome. Lung Slices were temporarily placed into serum free smooth muscle media. A single lung slice was then placed onto a 25 mm glass coverslip and mounted into an Attofluor cell chamber (Thermofisher Scientific). 20 μl of 0.8% low-melting agarose in HBSS with 2 mM Ca^{2+} was pipetted over the lung slice. After allowing the agarose to solidify over the lung slice for 3 minutes at RT, HBSS with 2 mM Ca^{2+} was placed over the agarose plug and lung slice. Using a Leica DiM8 confocal microscope with a ×10 objective, GCaMP6f was excited with a 488 nm laser and the corresponding emission was captured with a GFP filter. At one minute, the HBSS with 2 mM Ca^{2+} was carefully aspirated, and lung slices were stimulated with 6.25 mg/ml of MeCl in HBSS with 2 mM Ca^{2+}. Images were acquired every 7.5 seconds for 14 minutes. ROIs were then drawn around the perimeter of each smooth muscle that circumvents an airway, and the fluorescence intensity (F) was recorded using the LAS X software. Fluorescence from each ROI was subtracted from its background and

normalized to its baseline fluorescence ($\Delta F/F_0$). As previously described (21), traces of each ROI were transferred onto layouts and the number of oscillations per each ROI was manually counted. A Ca^{2+} oscillation was defined as a spike that returned to baseline and that had an amplitude of at least 1.25 fold of the basal fluorescence value. Cells that had sustained Ca^{2+} signals for at least 5 minutes after stimulation and that receded to ≥ 25% of the initial peak were designated as cells with "plateaus." The proportion of cells with plateaus were calculated for each coverslip. For the cells without plateaus, the number of oscillations for that entire 14 minutes were reported.

In vivo measurements of Airway Remodeling

Lung processing: After sacrificing mice, lungs were inflated with 1 ml of 10% neutral buffer formalin (NBF) using a tracheal catheter. Entire lungs were dissected and placed in 10% NBF overnight. The next day lungs were placed in 70% ethanol for storage. The midsection of the left lung was then paraffin embedded.

Immunostaining for α-Smooth Muscle Actin: After deparaffinization and rehydration, antigens from paraffin-embedded lung sections of mice were unmasked using near boiling temperatures of 10 mM sodium citrate. Unmaksed sections were then quenched for endogenous peroxidase activity using 3% hydrogen peroxide, and quenched sections were blocked with 5% goat serum in TBST. Using anti-α-smooth muscle actin primary antibody (CST) at 1:400 dilution in 1% BSA TBST, blocked sections were immunostained overnight at 4°C. Primary antibody was then stained using Signal Stain DAB reagent (CST). An anti-rabbit isotype control was used to measure nonspecific staining. The isotype control was labeled and subsequently stained with DAB using identical concentrations, incubation times, and washes as the other slides labeled with anti-α-smooth muscle actin. Following immunostaining, slides were counterstained with hematoxylin, dehydrated and mounted. Slides were imaged using a 20x dry objective on Leica DMi8 microscope. As previously described (22), α-Smooth muscle actin was quantified using Image J software by first outlining stained peribronchial area and then this area was normalized to the length of the basement membrane of the bronchiole. Slides were blinded during image acquisition, and each individual data point represents the average normalized α-Smooth muscle actin area from three different bronchioles from a single mouse.

Peribronchial Masson's trichrome staining: Deparaffinized lung slices were stained with Masson's trichrome stain kit. Slides were imaged using a 20x dry objective on Leica DMi8 microscope. Blue positive stained area around bronchials was outlined and quantified using Image J software. This area was then normalized to the length of the basement membrane of that bronchiole. Slides were blinded during image acquisition, and each individual data point represents the average normalized blue Masson's trichrome stained area from three different bronchioles from a single mouse.

Immunostaining for STIM1: Lung sections were deparaffinized, rehydrated, and unmasked using 10 mM sodium citrate at near boiling temperatures. After blocking with 5% goat serum in TBST, sections were immunostained for STIM1 (CST) at 1:650 dilution in 1% BSA TBST over night at 4°C. Sections were then incubated with 2 μg/ml of goat anti-rabbit Alexa Flour 647 (CST) for 45 minutes in 0.2 % BSA in TBST at room temperature. To label smooth muscle tissue, sections were then immunostained with α-smooth muscle actin antibody conjugated to Cy3 at 1:200 dilution in 1% BSA over night at 4°C. The next day sections were then counterstained for Hoescht at 2 μg/ml in PBS at room temperature for 15 minutes and finally

mounted. An anti-rabbit isotype control was used to measure nonspecific staining. The isotype control was labeled and subsequently stained with DAB using identical concentrations, incubation times, and washes as the other slides labeled with antiSTIM1. Slides were imaged using a 40x oil objective on a confocal Leica DMi8 microscope. ROIs were drawn around cells stained for α-smooth muscle actin, and STIM1 fluorescence was then quantified. Slides were blinded during image acquisition, and each individual data point represents the fluorescence of a single smooth muscle cell pooled from at least 4 mice from each cohort.

Bronchial Airway Lavage Fluid Collection

Following methods previously described (23), mice were first anesthetized with 2.5% isoflurane and sacrificed by cervical dislocation. Lungs were inflated with 1 ml of PBS with 0.1 mM EDTA using a tracheal catheter, and 1 ml of BAL was then collected. BAL was then centrifuged at 1,500 rpm and the supernatant was stored at -80°C for ELISA experiments. The cell pellet was resuspended with Ammonium-Chloride-Potassium (ACK) buffer and incubated on ice for 5 mins. The samples were then again centrifuged at 1,500 rpm and supernatant was aspirated. The cell pellet was resuspended with 500 µl of RPMI. Total BAL cells were then counted using a hemocytometer. For differential cell counts, 50,000 cells were resuspended in 200 µl of RPMI and loaded into a Cytofunnel (Thermofisher Scientific) and centrifuged for 10 min at 600 rpm. Slides were then removed from Cytofunnel and stained using Diff-Quick stain kit. Cells were differentially counted based on morphology and staining patterns.

Quantification of Cytokines using ELISA

For *in vitro* experiments, 25,000 HASMC were seeded per well in 24-well tissue culture plates. After 24 hrs., cells were placed in serum free media for 48 hrs. Cells were then stimulated with either 20 ng/ml of human Platelet Derived Growth Factor-BB (R&D Systems), 500 nM Bradykinin (Millipore Sigma), 100 µM Acetyl-β-methylcholine (Millipore Sigma), and 10 µg/ml of House Dust Mite (*Dermatphagoides pteronyssinus*) (Greer Laboratories) diluted in serum free media for 18 hours. Certain samples were also pretreated for 1 hr with 1 µM of cyclosporin A (Caymen Chemicals) prior to stimulation with agonists. Supernatants from each sample were then collected and stored at -80°C until ready for use. Following the manufacture's protocol, IL-6 was measured using an ELISA kit (Thermofischer Scientific). These measurements were normalized to total protein in supernatant, which was measured using the Pierce Rapid Gold BCA Protein Assay (Thermofischer Scientific). For *in vivo* experiments, mouse IL-6 and IgE were measured from supernatants of BAL using ELISA kits (Thermofischer Scientific) and following the manufacture's protocol. These measurements were normalized to total protein in these supernatants, which was measured using the Pierce Rapid Gold BCA Protein Assay.

Statistical Methods

All statistical analyses used Graphpad Prism 9 software. Data are represented as mean ± SEM. Experiments were repeated at least three independent times. For statistical analyses with only two experimental groups, two-sample t-test were performed and for statistical analyses with more than two experimental groups one-way ANOVA was performed with Dunnett's method for

multiple comparisons. *, **, and *** indicates p-values of < 0.05, < 0.01, and < 0.001, respectively. Differences were considered statistically significant when $p < 0.05$.

Table S1. REAGENTS

Reagent	Source	Identifier	Dilution
Rabbit monoclonal anti-α-Smooth Muscle Actin	Cell Signaling Technologies	Cat#19245	1:400
Rabbit polyclonal anti-NFAT4	Cell Signaling Technologies	Cat#4998	1:1000
Rabbit monoclonal anti-GLUT1	Cell Signaling Technologies	Cat#12939	1:1000
Rabbit monoclonal anti-OPA1	Cell Signaling Technologies	Cat#80471	1:1000
Rabbit monoclonal anti-MFN2	Cell Signaling Technologies	Cat#9482	1:1000
Rabbit polyclonal anti-Caspase3	Cell Signaling Technologies	Cat#9662	1:1000
Rabbit monoclonal anti-PGC-1α	Cell Signaling Technologies	Cat#2178	1:1000
Rabbit monoclonal anti-PPARγ	Cell Signaling Technologies	Cat#2435	1:1000
Rabbit monoclonal anti-STIM1	Cell Signaling Technologies	Cat#5668	1:1000 for WB, 1:650 for IF
Mouse monoclonal anti-α-tubulin	Cell Signaling Technologies	Cat#3873	1:2000
Rabbit polyclonal anti-ORAI1	MilliporeSigma	Cat#O8264	1:1000
Mouse monoclonal anti-GAPDH	MilliporeSigma	Cat#MAB374	1:4000
Mouse monoclonal anti-OXPHOS	Abcam	Cat# ab110413	1:1000
Rabbit polyclonal anti-STIM2	Cell Signaling Technologies	Cat#4917	1:1000
Mouse monoclonal anti-Actin, α-smooth muscle-Cy3	MilliporeSigma	Cat#C6198	1:200
IL-6 Human Uncoated ELISA Kit with Plates	Thermofisher Scientific	Cat#88-7066-22	
IL-6 Mouse Uncoated ELISA Kit with Plates	Thermofisher Scientific	Cat#88-7064-22	
IgE Mouse Uncoated ELISA Kit with Plates	Thermofisher Scientific	Cat#88-50460-22	
Goat anti-Rabbit IgG Cross-Absorbed Secondary Antibody Alexa Fluor 647	Thermofisher Scientific	Cat#A21245	2 μg/ml
680 CW goat anti-Mouse	LI-COR	Cat#926-68070	1:5000
800 CW donkey anti-Rabbit	LI-COR	Cat#926-32213	1:10000

Hoechst 33342, trihydrochloride trihydrate	Thermofisher Scientific	Cat#H3570	1 mg/ml
Fura-2, AM	Thermofisher Scientific	Cat#F1221	
MitoTracker Green FM	Thermofisher Scientific	Cat # M7514	
Pluronic™ F-127 (20% Solution in DMSO)	Thermofisher Scientific	Cat# P3000MP	
Thapsigargin	Calbiochem	Cat#586005	
Acetyl-β-methylcholine chloride	MilliporeSigma	Cat#A2251	
Bradykinin acetate salt	MilliporeSigma	Cat#B3259	
Recombinant Human PDGF-BB Protein, CF	R&D Systems	Cat#220-BB-010	
Oligomycin A	MilliporeSigma	Cat#75351-5MG	
Antimycin A from Streptomyces sp.	MilliporeSigma	Cat#A8674-25MG	
Rotenone	MilliporeSigma	Cat#45656	
2-deoxy-D-Glucose	Cayman Chemical Company	Cat#14325	
Mitomycin C from *Streptomyces caespitosus*	MilliporeSigma	Cat#M4287	
CyQUANT™ NF Cell Proliferation Assay	Thermofisher Scientific	Cat#C35006	
Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP)	Cayman Chemical Company	Cat#15218	
House Dust Mite (*Dermatphagoides pteronyssinus*)	Greer Laboratories	Cat#XPB82D3A2.5	
Pancuronium bromide	MilliporeSigma	Cat#P1918	
7AAD	Tonbo Biosciences	Cat#13-6993-T200	
FITC Annexin V	Tonbo Biosciences	Cat#35-6409-T100	

Table S2. RECOMBIANT DNA

Recombinant DNA	**Source**
pLenti-PGK-Empty Vector	Trebak Lab; This paper
pLnti-PGK-CA-NFAT4mCherry	Trebak Lab; This paper
pLenti-PGK-NFAT4mCherry	Trebak Lab; This paper

shSTIM1 #1	TRC 1.0 shRNA Library
shSTIM1 #5	TRC 1.0 shRNA Library
shScramble	TRC 1.0 shRNA Library

Table S3. OLIGONUCLEOTIDES

Primers	Forward	Reverse
GAPDH (real-time PCR)	5'-CCCTTCATTGACCTCAACTACA-3'	5'-ATGACAAGCTTCCCGTTCTC-3'
NONO (real-time PCR)	5'-TCCGAGGAGATACCAGTCGG-3'	5'-CCTGGGCCTCTCAACTTCGAT-3'
ORAI2 (real-time PCR)	5'-TGGCGGAAGCTCTACCTGAG-3'	5'-CGGGTACTGGTACTGCGTC-3'
ORAI3 (real-time PCR)	5'-CTGGAGAGTGACCACGAGTA-3'	5'-TGGAGACCATGAGTGCAAAG-3'
α-TUBULIN (real-time PCR)	5'-AGTCCAAGCTGGAGTTCTCTAT-3'	5'-CAATCAGAGTGCTCCAGGGT-3'
COL5A2 (real-time PCR)	5'-TTCCAGGCTCTGATGGTTTAC-3'	5'-GAGGTCCTTGTTCTCCTCTTTC-3'
LOX (real-time PCR)	5'- GCCACTATGACCTGCTTGAT-3'	5'- GATGTCCTGTGTAGCGAATGT-3'
TGFβR1 (real-time PCR)	5'- GTTCCGTGAGGCAGAGATTTA-3'	5'- CTGATGGGTCAGAAGGTACAAG-3'
mtDNAR1 (real-time PCR)	5'-CGGGCTACTACAACCCTTCG-3'	5'-GATGGTAGATGTGGCGGGTT-3'
mtDNAR2 (real-time PCR)	5'-CTATCCTGCCCGCCATCATC-3'	5'-AAGGGAGGGATCGTTGACCT-3'
mtDNAR3 (real-time PCR)	5'-TCATCCAAACCCCCTGAAGC-3'	5'-CGACTGTGAGTGCGTTCGTA-3'
gDNAR1 (real-time PCR)	5'-GCAACTTAGAGGTGGGGAGC-3'	5'-AACAAGCTTTGAGTGCAAGAGA-3'
gDNAR2 (real-time PCR)	5'GGGTGGGGGAGAGATTACCT-3'	5'CAATCTGCCCAGAGAAGCCA-3'

NFAT4 (from pREP-NFAT4)	5'GCTCCACCATGACTACTGCAAACTGTGG	5'TCACCATTAGTAAACCATCATGACTCCTCG-3'
mCherry fagment (from CAV1mcherry)	5'GTTTACTAATGGTGAGCAAGGGCGAG-3'	5'AATTCTTACTTGTACAGCTCGTCCATGC-3'
Vector-1(from pLenti PGK Blast V5-LUC)	5'GTACAAGTAAGAATTCTTCTAGACCCAGCTTTCTTG-3'	5'TAGTCATGGTGGAGCCTGCTTTTTTGTAC-3'
fragment for (163) CA-NFAT4 (300)	5'CCTCTTGAGCCATCCTACCG-3'	5'TCTTCTGTCACACTTCCCCTG-3'
Vector-2 (from pLenti PGK-NFAT4mCherry)	5'AAGTGTGACAGAAGATACGTGGC-3'	5'GGATGGCTCAAGAGGAAGATAG-3'
Vector-3 (from pLenti PGK-NFAT-mCherry)	5'CTCCACCATGGTGAGCAAGGGCGA-3'	5'CTCACCATGGTGGAGCCTGCTTTTTTG-3'
STIM1 Reaction A (Genotyping)	5'-GCAACTTAGAGGTGGGGAGC-3'	5'-GGCTCTGCTGACCTGGAACTATAGTG-3'
STIM1 Reaction B (Genotyping)	5'-AACGTCTTGCAGTTGCTGTAGGC-3'	5'-GGCTCTGCTGACCTGGAACTATAGTG-3'
Myh11 Cre Transgene (Genotyping)	5'-AACGTCTTGCAGTTGCTGTAGGC-3'	5'-AGTCCCTCACATCCTCAGGTT-3'
Myh11 Cre Internal Control (Genotyping)	5'-CAGCCAACTTTACGCCTAGC-3'	5'-CCTGGGCCTCTCAACTTCGAT-3'
GCaMP6 WT Allele (Genotyping)	5'-AAGGGAGCTGCAGTGGAGTA-3'	5'CCGAAAATCTGTGGGAAGTC-3'
GCaMP6 Flox Allele (Genotyping)	5-ACGAGTCGGATCTCCCTTTG-3'	5'CCGAAAATCTGTGGGAAGTC-3'

Oligonucleotides	**Sequences**

shSTIM1 #1	CCGGACATGAGGTGGAGGTGCAATACTCGAGTATTGCACCTCC ACCTCATGTTTTTTG
shSTIM1 #5	CCGGTGGTGGTGTCTATCGTTATTGCTCGAGCAATAACGATAG ACACCACCATTTTTG
(163) CA-NFAT4 (300)	CCTCTTGAGCCATCCTACCGGGAGTCTTCTCTTGCACCTGCACC TGCCGCAGCGATCGCAGCGAGGGCATGGTTCGCAGATGCAGCC GCATGTGAAGCACTTGCACATATTTATGATGATGTGGACGCAG AGTTGAATGAAGCTGCAGCCCGATTTACCCTTGGAGCGCCTCT GACTGCGCCTGGTGGCGCGCCAGGGGGCTGCCCTGGAGAAGA AACTTGGCATCAACAGTATGGACTTGGACACTCATTAGCACCC AGGCAAGCGCCTTGCCACGCGCCTAGAGCTGCCGTCACTGATG AGAATTGGCTGAGCCCCAGGCCAGCCTCAGGACCCTCATCAAG GCCCACATCCCCCTGTGGGAAACGGAGGCACTCCAGTGCTGAA GTTTGTTATGCTGGGTCCCTTGCTCCCCATCACGCGCCTGTTCC TGCGCCTGGTCACGCACCCAGGGGAAGTGTGACAGAAGA

SUPPLEMENTARY FIGURE LEGENDS

Fig. S1: Genotyping for STIM1^{smKO} mice, STIM2 expression in ASM, and leukocytes from BAL of Mice. (A) Schematic of binding sites of genotyping primers used to amplify DNA products for STIM1 from Myh11^{Cre}, STIM1$^{fl/fl}$, and STIM1^{smKO} mice. Table lists predicted band sizes of each PCR reaction in each mouse cohort. See supplementary methods for sequence of primers. **(B)** Gel showing PCR products from genotyping reactions in B from the tracheas of Myh11^{Cre}, STIM1$^{fl/fl}$, and STIM1^{smKO} mice. **(C)** Representative Western blot of denuded tracheal and spleen samples from Myh11^{Cre} mice. STIM1 and STIM2 were probed on the left and right of the dotted line, respectively. α-tubulin was used as a loading control. **(D)** Representative confocal images of a lung slice labeled with an isotype control, Alexa Fluor 647 secondary, and Cy3-congugated to α-SMA (left) and a lung slice labeled only with isotype control and Alexa Flour 647 secondary (right). Slices are also counterstained with Hoescht. (Scale bar: 25 μm). **(E)** Lung slice labeled with an isotype control and stained with DAB for immunohistochemistry. Lung slice is counterstained with Hematoxylin. (Scale bar: 100 μm). **(F)** Quantification of the total number of bronchial airway lavage (BAL) total leukocytes and **(G)** leukocyte subsets from saline-challenged Myh11^{Cre} *(n=5)*, HDM-challenged Myh11^{Cre} *(n=5)*, saline-challenged STIM1^{smKO} *(n=5)*, and HDM-challenged STIM1^{smKO} mice *(n=5). *P<0.05, **P<0.01, ***P<0.001,* when compared to Saline-challenged Myh11 Cre (One-Way ANOVA with Dunnett's test for multiple comparisons).

Fig. S2: STIM1/2 protein expression and SOCE in HASMCs cultured from normal and asthmatic donors. (A) Table of demographics from normal (white) and asthmatic (grey) HASMC donors including race, age, and gender. **(B)** Western blots showing STIM1 and STIM2 protein expression in HASMC from all normal and asthmatic donors. Quantification of STIM1 **(C)** and STIM2 **(D)** protein expression between normal (cool colors; *n=4)* and asthmatic (warm colors; *n=4)* HASMCs from B using densitometry normalized to α-tubulin. **(E)** Cytosolic Ca^{2+} measurements using the standard Ca^{2+} off/ Ca^{2+} on protocol with 2 μM thapsigargin showing SOCE in HASMCs from normal donors (Donor 1: dark blue, *[n=57]*; Donor 2: light blue, *[n=57];*, Donor 3: teal, *[n=56]*) and asthmatic donors (Donor 1: red, *[n=47]*; Donor 2: pink, *[n=52]*; Donor 3: fuchsia *[n=57]*). **(F)** Quantification of maximal Ca^{2+} entry from D. **(G)** Quantification of maximal Ca^{2+} entry from normal and asthmatic donors. *ns=not significant* (unpaired Student's *t* test).

Fig. S3: ORAI expression in STIM1 KD HASMCs and STIM1 in proliferation and migration of HASMCs from another donor. (A) Representative Western blot showing ORAI1α (long) and ORAI1β (short) protein expression (using deglycosylated protein samples) in shScramble, shSTIM1 #1, and shSTIM1 #5 HASMCs. **(B)** Quantification of ORAI1α and **(C)** ORAI1β protein expression from A in shScramble *(n=3)*, shSTIM1 #1 *(n=3)*, and shSTIM1 #5 *(n=3)* HASMCs using densitometry normalized to α-tubulin. Quantification of ORAI2 **(D)** and ORAI3 **(E)** mRNA expression in shScramble *(n=4)*, shSTIM1 #1 *(n=4)*, and shSTIM1 #5 *(n=4)* HASMCs using RT-qPCR. **(F)** Representative Western blot showing STIM1 protein expression in shScramble, shSTIM1 #1, and shSTIM1 #5 HASMCs from Donor 2. **(G)** Quantification of STIM1 protein expression in shScramble *(n=3)*, shSTIM1 #1 *(n=3)*, and shSTIM1 #5 *(n=3)* HASMCs from F using densitometry normalized to GAPDH. **(H)** Proliferation assays with quantification of normalized relative fluorescent units (RFU) of shScramble *(n=4)*, shSTIM1 #1

(n=4), and shSTIM1 #5 *(n=5)* HASMCs from Donor 2 over 72 hrs. **(I)** Quantification of HASMC migration in Donor 2 at 12 and 24 hrs in shScramble *(n=8),* shSTIM1 #1 *(n=8),* and shSTIM1 #5 *(n=4)* HASMCs. ***P<0.01, ***P<0.001, ****P<0.0001, ns=not significant* (unpaired Student's *t* test for two comparisons and ANOVA with Dunnett's test for multiple comparisons).

Fig. S4: STIM1 does not affect apoptosis of HASMCs. (A) Representative flow cytometry dot plots of gating strategy and co-staining for 7AAD and Annexin V in shScramble, shSTIM1 #1, shSTIM1 #5, and unstained HASMCs. **(B)** Quantification of % of cells in early apoptosis (quadrant 3), late apoptosis (quadrant 2), and total apoptosis (quadrant 2+3) in shScramble *(n=4),* shSTIM1 #1 *(n=4),* and shSTIM1 #5 *(n=4)* HASMCs from flow cytometry dot plots in A. **(C)** Representative Western blot showing Caspase 3 and Cleaved Caspase 3 protein expression in shScramble, shSTIM1 #1, and shSTIM1 #5 HASMCs. **(D)** Quantification of Caspase 3 and **(E)** Cleaved Caspase 3 protein expression from C in shScramble *(n=3),* shSTIM1 #1 *(n=3),* and shSTIM1 #5 *(n=3)* HASMCs using densitometry normalized to GAPDH. **P<0.05, ns=not significant* (ANOVA with Dunnett's test for multiple comparisons).

Fig. S5: STIM1 regulates fibrosis and activation of NFAT4 and IL-6 in ASM cells. (A) Volcano plot comparing differentially expressed genes between shScramble and shSTIM1 #5 HASMCs. Genes are plotted by Log_2 Fold Change and $-Log_{10}$ False Discover Rate (FDR), with a threshold of an FDR <0.05 (dotted black line). In the shSTIM1 #5 samples, significantly upregulated and downregulated genes are light red and light blue respectively. Heatmap display of (B) Collagen Synthesis and (C) Muscle Contraction genes from the REACTOME pathway analysis. Genes appear in rows, samples appear in columns. Gene expression values were mean centered by gene. Quantification of **(D)** COL5A2, **(E)** LOX, and **(F)** TGFβR1 mRNA expression in shScramble *(n=4),* shSTIM1 #1 *(n=4),* and shSTIM1 #5 *(n=4)* HASMCs using RT-qPCR. **(G)** Representative Western blot and **(H)** quantification of phosphorylated and dephosphorylated NFAT4 (NFAT4-P and NFAT4-deP respectively) in HASMCs stimulated with of 2 μM thapsigargin in the presence of 2 mM Ca^{2+} at time 0, 5, 15, and 30 minutes. Indicated samples were preincubated for 10 minutes with either 5 μM Gd^{3+} or 1 μM Cyclosporine. **(I)** Using ELISA, IL-6 secretion is measured in shScramble (gray circles; *n=4),* shSTIM1 #1 (green circles; *n=4),* and shSTIM1 #5 (blue circles; *n=4)* HASMCs stimulated with either vehicle, 20 ng/ml PDGF, 100 μM Methacholine (MeCh), or 10 μg/ml of house dust mite (HDM). **(J)** IL-6 secretion is measured in shScramble (gray circles; *n=4),* shSTIM1 #1 (green circles; *n=4),* and shSTIM1 #5 (blue circles; *n=4)* HASMCs from donor 2 stimulated with either 500 nM Bradykinin, 20 ng/ml PDGF, 100 μM Methacholine (MeCh), or 10 μg/ml of house dust mite (HDM). **(K)** Quantification of IgE in the BAL of saline-challenged $Myh11^{Cre}$ *(n=5),* HDM-challenged $Myh11^{Cre}$ *(n=8),* saline-challenged $STIM1^{smKO}$ *(n=6),* and HDM-challenged $STIM1^{smKO}$ *(n=8)* mice. For each sample in I-K, IL-6 or IgE secretion is normalized to total protein content in sample. **P<0.05, **P<0.01, ****P<0.0001* when compared to shScramble or saline-challenged $Myh11^{Cre}$, *#P<0.05, ####P<0.0001* when compared to HDM-challenged $Myh11^{Cre}$. *ns=not significant* (One-Way ANOVA with Dunnett's test for multiple comparisons).

Fig. S6: STIM1 controls proliferation and migration through NFAT4 in ASM cells. (A) Schematic of the amino acid sequence of the generated constitutively-active NFAT4 mutant (CA-NFAT4). Serine to Alanine substitutions are highlighted in yellow. Representative confocal

images of HASMCs expressing NFAT4mCherry (left), expressing NFAT4mCherry **(B)** and either unstimulated (left) or stimulated with 2 μM of thapsigargin for 25 minutes (middle), HASMCs expressing CA-NFAT4mCherry are also shown (right). (Scale bar: 10 μm). **(C)** Normalized quantification of proliferation of shScramble expressing mCherry (top gray; *n=6)*, shSTIM1 #1 expressing mCherry (top green; *n=6)*, shSTIM1#5 expressing mCherry (top blue; *n=6)*, shScramble expressing NFAT4mCherry (middle gray; *n=6)*, shSTIM1 #1 expressing NFATmCherry (middle green; *n=6)*, shSTIM1 #5 expressing NFATmCherry (middle blue; *n=6)*, shScramble expressing CA-NFAT4mCherry (bottom gray; *n=6)*, shSTIM1 #1 expressing NFATmCherry (bottom green; *n=6)*, shSTIM1 #5 expressing NFATmCherry (bottom blue; *n=6)* HASMCs over 72 hrs. **(D)** Normalized quantification of migration of shScramble expressing mCherry (bottom gray; *n=11)*, shSTIM1 #1 expressing mCherry (bottom green; *n=10)*, shSTIM1 #5 expressing mCherry (bottom blue; *n=11)*, shScramble expressing NFAT4mCherry (middle gray; *n=12)*, shSTIM1 #1 expressing NFATmCherry (middle green; *n=12)*, shSTIM1 #5 expressing NFATmCherry (middle blue; *n=12)*, shScramble expressing CA-NFAT4mCherry (top gray; *n=12)*, shSTIM1 #1 expressing NFATmCherry (bottom green; *n=12)*, shSTIM1 #5 expressing NFATmCherry (bottom blue; *n=12)* HASMCs over 24 hrs. ***P<0.01, ***P<0.001, ****P<0.0001, ns=not significant* (ANOVA with Dunnett's test for multiple comparisons).

Fig. S7: STIM1 regulates metabolic respiration, glycolysis and production of metabolic intermediates in ASM. (A) Heat map of Diseases of Metabolism from the Reactome pathway analysis. Scale is to the right of heat maps. Red is enhanced expression, blue is decreased expression. Quantification of **(B)** Basal Respiration, **(C)** Maximal Respiration, and **(D)** Non-mitochondrial Respiration from OCR trace in Figure 3B. Quantification of **(E)** Basal Acidification, **(F)** Glycolysis, **(G)** Glycolytic Capacity, and **(H)** Non-glycolytic Acidification from ECAR trace in Figure 3K. **(I)** OCR of shScramble (black trace; *n=4)*, shSTIM1 #1 (green trace; *n=5)*, and shSTIM1 #5 (blue trace; *n=6)* HASMCs from Donor 2. Measurements are normalized to total protein expression in each sample. **(J)** ECAR of shScramble (black trace; *n=6)*, shSTIM1 #1 (green trace; *n=6)*, and shSTIM1 #5 (blue trace; *n=6)* HASMCs in Donor 2. Measurements are normalized to total protein expression in each sample. **(K)** Heat map of relative concentrations of metabolites that were significantly different between shScramble *(n=4)*, shSTIM1 #1 *(n=4)*, and shSTIM1 #5 *(n=4)* HASMCs. **P<0.05, **P<0.01, ***P<0.001, ns=not significant* (ANOVA with Dunnett's test for multiple comparisons).

Fig. S8: Mitochondrial density is reduced in STIM1 knockdown HASMCs independent of PGC1α and PPARγ. (A) Two additional representative TEM images of shScramble, shSTIM1 #1, and shSTIM1 #5 HASMCs. (Scale bar: 1 μm). **(B)** Quantification of Mitotracker Green FM median fluorescence intensity (MFI) using flow cytometry in shScramble *(n=4)*, shSTIM1 #1 *(n=4)*, and shSTIM1 #5 *(n=4)* HASMCs. Using TEM, quantification of the **(C)** Circularity, **(D)** Roundness, **(E)** Solidity of mitochondria from shScramble *(n=99)*, shSTIM1 #1 *(n=72)*, and shSTIM1 #5 *(n=70)* HASMCs. **(F)** Representative Western blot showing protein expression of the electron transport chain (ETC) proteins: ATP5A (Complex V), UQCRC2 (Complex III), MTCO1 (Complex IV), SDHB (Complex II), and NDUFB8 (Complex I) in shScramble, shSTIM1 #1, and shSTIM1 #5 HASMCs. **(G)** Quantification of ETC proteins in shScramble *(n=3)*, shSTIM1 #1 *(n=3)*, and shSTIM1 #5 *(n=3)* HASMCs from F using densitometry normalized to GAPDH. **(H)** Representative Western blot showing PGC1α, PPARγ1, and PPARγ2 protein expression in shScramble, shSTIM1 #1, and shSTIM1 #5 HASMCs. Quantification of **(I)**

PGC1α, **(J)** PPARγ1, and **(K)** PPARγ2 protein expression from H in shScramble *(n=3),* shSTIM1 #1 *(n=3),* and shSTIM1 #5 *(n=3)* HASMCs using densitometry normalized to GAPDH. **P<0.05, **P<0.01, ***P<0.001, ns=not significant* (ANOVA with Dunnett's test for multiple comparisons).

Fig. S9: STIM1 knockdown in HASMCs is associated with altered amino acids content. Metabolomics profiling was performed in HASMCs stably expressing either shScramble, shSTIM1#1, or shSTIM1#5 as described in the Methods section. Analysis in (**A**) considered clustering of all shSTIM1#1 and shSTIM1#5 samples, while (B, C) considered shSTIM1#1 (**B**) and shSTIM1#5 (**C**) comparisons to their respective shScramble controls.

Fig. S10: ASM from HDM-challenged STIM1^{smKO} mice have reduced mitochondrial mass. **(A)** Representative TEM images of airways from Myh11^{Cre} and STIM1^{smKO} mice challenged with either saline or HDM. Red arrow heads point to ASM layer of airways. Top images are 800x, middle are 4000x, and bottom are 10,000x. (Scale bars: 5, 1, and 0.5 μm respectively). **(B)** Quantification of the mitochondrial density or number of mitochondria per μm^2 of cytoplasmic area of transmission electron micrographs from saline-challenged Myh11^{Cre} *(n=11),* HDM-challenged Myh11^{Cre} *(n=13),* saline-challenged STIM1^{smKO} *(n=10),* and HDM-challenged STIM1^{smKO} *(n=13)* mice. **(C)** Using TEM, quantification of the aspect ratio of mitochondria from saline-challenged Myh11^{Cre} *(n=65),* HDM-challenged Myh11^{Cre} *(n=228),* saline-challenged STIM1^{smKO} *(n=52),* and HDM-challenged STIM1^{smKO} mice *(n=81). *P<0.05, ****P<0.0001* when compared to Saline-challenged Myh11 Cre, *####P<0.0001* when compared to HDM-challenged Myh11^{Cre}. (One-Way ANOVA with Dunnett's test for multiple comparisons).

Fig. S11: Genotyping of GCaMP6-expressing mice. (A) Table of predicted band sizes for each PCR reaction. **(B)** Gel showing PCR products from genotyping reactions in A from Myh11^{Cre}, Myh11^{Cre} expressing GCaMP6, STIM1^{smKO}, and STIM1^{smKO} expressing GCaMP6 mice.

Fig. S12: Representative Ca^{2+} traces of ASM in response to 6.25 mg/ml of methacholine. ASM cells respond with **(A)** single spike, **(B)** regenerative oscillations returning to baseline, **(C)** all varieties of Ca^{2+} plateaus, **(D)** a subset of Ca^{2+} plateaus superimposed with Ca^{2+} oscillations.

SUPPLEMENTARY VEDIO LEGENDS

Supplementary Video 1. Representative video of MeCh-evoked Ca^{2+} signaling events in lung slices from saline-challenged control Myh11^{Cre} mice. Ca^{2+} imaging on live slices of airways from saline-challenged control Myh11^{Cre} mice expressing GCaMP6f specifically in smooth muscle cells. Slices were challenged with Methacholine (MeCh, 6.25 mg/ml). Also shown are time lapse traces of Ca^{2+} signals of CaMP6f-expressing lung slices taken from color-coded regions of interest (ROI).

Supplementary Video 2. Representative video of MeCh-evoked Ca^{2+} signaling events in lung slices from HDM-challenged control Myh11^{Cre} mice. Ca^{2+} imaging on live slices of

airways from house dust mite-challenged control Myh11^{Cre} mice expressing GCaMP6f specifically in smooth muscle cells. Slices were challenged with Methacholine (MeCh, 6.25 mg/ml). Also shown are time lapse traces of Ca^{2+} signals of CaMP6f-expressing lung slices taken from color-coded regions of interest (ROI).

Supplementary Video 3. Representative video of MeCh-evoked Ca^{2+} signaling events in lung slices from saline-challenged control STIM1^{smKO} mice. Ca^{2+} imaging on live slices of airways from saline-challenged control STIM1^{smKO} mice expressing GCaMP6f specifically in smooth muscle cells. Slices were challenged with Methacholine (MeCh, 6.25 mg/ml). Also shown are time lapse traces of Ca^{2+} signals of CaMP6f-expressing lung slices taken from color-coded regions of interest (ROI).

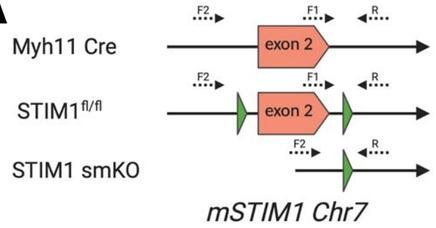
Supplementary Video 4. Representative video of MeCh-evoked Ca^{2+} signaling events in lung slices from HDM-challenged control STIM1^{smKO} mice. Ca^{2+} imaging on live slices of airways from house dust mite-challenged control STIM1^{smKO} mice expressing GCaMP6f specifically in smooth muscle cells. Slices were challenged with Methacholine (MeCh, 6.25 mg/ml). Also shown are time lapse traces of Ca^{2+} signals of CaMP6f-expressing lung slices taken from color-coded regions of interest (ROI).

REFERENCES

1. Panettieri RA, Murray RK, DePalo LR, Yadvish PA, & Kotlikoff MI (1989) A human airway smooth muscle cell line that retains physiological responsiveness. *Am J Physiol* 256(2 Pt 1):C329-335.
2. Vaeth M, *et al.* (2017) Store-Operated Ca(2+) Entry Controls Clonal Expansion of T Cells through Metabolic Reprogramming. *Immunity* 47(4):664-679 e666.
3. Miller FJ, Rosenfeldt FL, Zhang C, Linnane AW, & Nagley P (2003) Precise determination of mitochondrial DNA copy number in human skeletal and cardiac muscle by a PCR-based assay: lack of change of copy number with age. *Nucleic Acids Res* 31(11):e61.
4. Dobin A, *et al.* (2013) STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29(1):15-21.
5. Mortazavi A, Williams BA, McCue K, Schaeffer L, & Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods* 5(7):621-628.
6. Risso D, Schwartz K, Sherlock G, & Dudoit S (2011) GC-content normalization for RNA-Seq data. *BMC Bioinformatics* 12:480.
7. Risso D, Ngai J, Speed TP, & Dudoit S (2014) Normalization of RNA-seq data using factor analysis of control genes or samples. *Nat Biotechnol* 32(9):896-902.
8. Eisenberg E & Levanon EY (2013) Human housekeeping genes, revisited. *Trends Genet* 29(10):569-574.
9. Robinson MD, McCarthy DJ, & Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26(1):139-140.

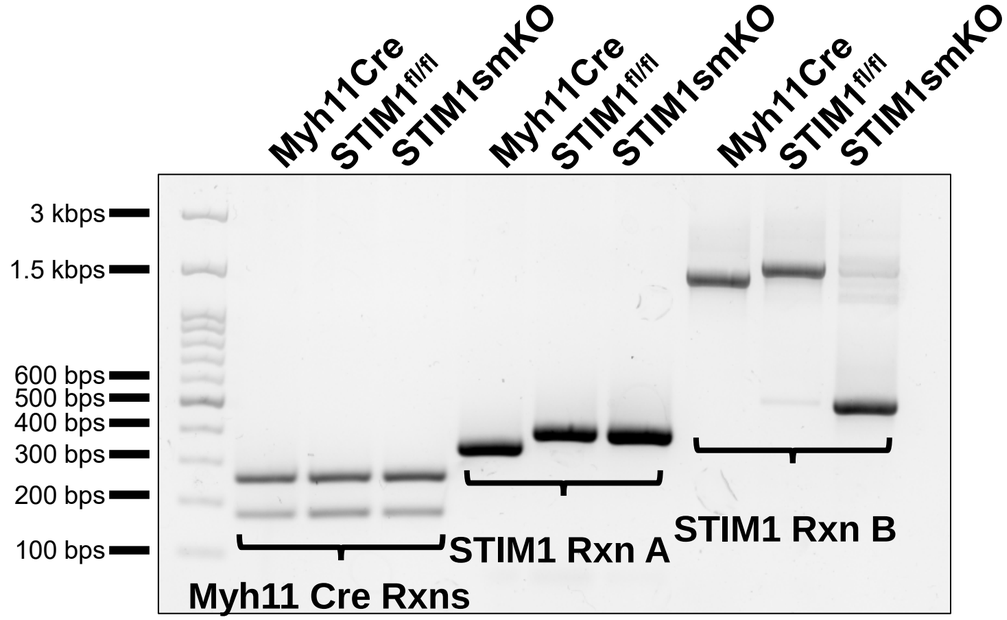
10. McCarthy DJ, Chen Y, & Smyth GK (2012) Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Res* 40(10):4288-4297.
11. Subramanian A, *et al.* (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 102(43):15545-15550.
12. Pacold ME, *et al.* (2016) A PHGDH inhibitor reveals coordination of serine synthesis and one-carbon unit fate. *Nat Chem Biol* 12(6):452-458.
13. Chen WW, Freinkman E, Wang T, Birsoy K, & Sabatini DM (2016) Absolute Quantification of Matrix Metabolites Reveals the Dynamics of Mitochondrial Metabolism. *Cell* 166(5):1324-1337 e1311.
14. Simon-Manso Y, *et al.* (2013) Metabolite profiling of a NIST Standard Reference Material for human plasma (SRM 1950): GC-MS, LC-MS, NMR, and clinical laboratory analyses, libraries, and web-based resources. *Anal Chem* 85(24):11725-11731.
15. Smith CA, *et al.* (2005) METLIN: a metabolite mass spectral database. *Ther Drug Monit* 27(6):747-751.
16. Oh-Hora M, *et al.* (2008) Dual functions for the endoplasmic reticulum calcium sensors STIM1 and STIM2 in T cell activation and tolerance. *Nat Immunol* 9(4):432-443.
17. Wirth A, *et al.* (2008) G12-G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension. *Nat Med* 14(1):64-68.
18. Madisen L, *et al.* (2015) Transgenic mice for intersectional targeting of neural sensors and effectors with high specificity and performance. *Neuron* 85(5):942-958.
19. Akram KM, *et al.* (2019) Live imaging of alveologenesis in precision-cut lung slices reveals dynamic epithelial cell behaviour. *Nat Commun* 10(1):1178.
20. Cooper PR & Panettieri RA, Jr. (2008) Steroids completely reverse albuterol-induced beta(2)-adrenergic receptor tolerance in human small airways. *J Allergy Clin Immunol* 122(4):734-740.
21. Yoast RE, *et al.* (2020) The native ORAI channel trio underlies the diversity of Ca2+ signaling events. *Nature Communications* 11(1):2444.
22. Das S, *et al.* (2016) GSDMB induces an asthma phenotype characterized by increased airway responsiveness and remodeling without lung inflammation. *Proc Natl Acad Sci U S A* 113(46):13132-13137.
23. Han H & Ziegler SF (2013) Bronchoalveolar Lavage and Lung Tissue Digestion. *Bio Protoc* 3(16).

A



	Primers	Myh11 Cre	STIM1$^{fl/fl}$	STIM1 smKO
Myh11 Cre Transgene	TF→ ←TR	287 bps	287 bps	287 bps
Myh11 Cre Internal Control	CF→ ←CR	180bps	180bps	180bps
STIM1 Reaction A	F1→ ←R	348 bps	399 bps	No Product
STIM1 Reaction B	F2→ ←R	1211 bps	1262 bps	580 bps

B



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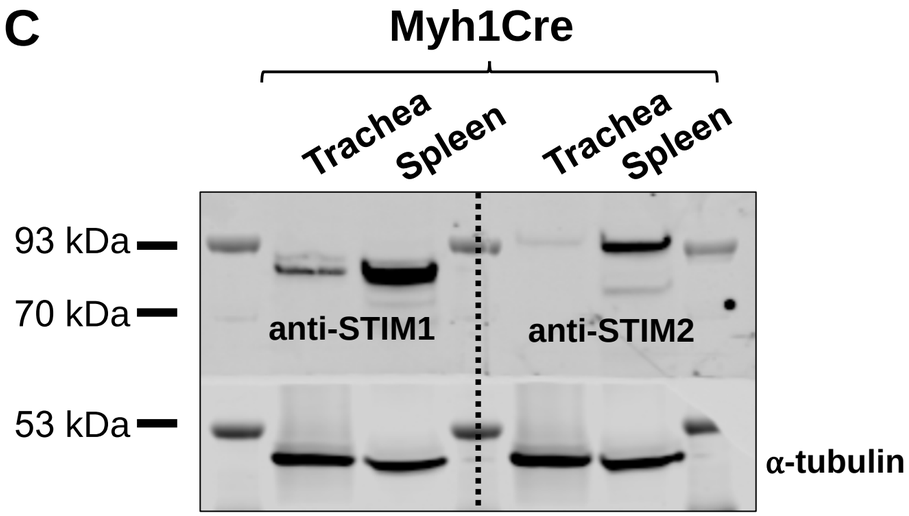

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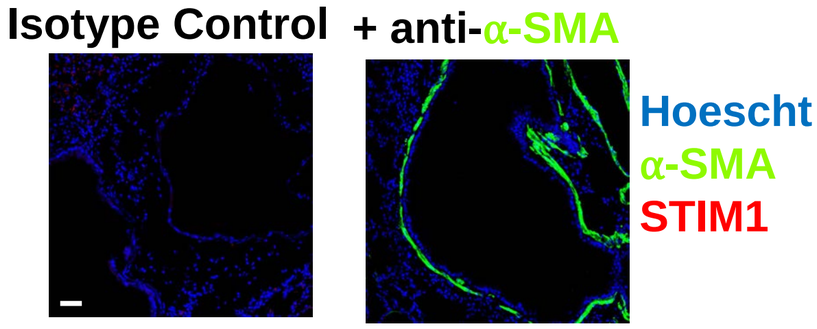

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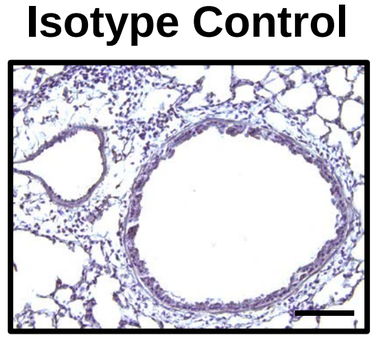

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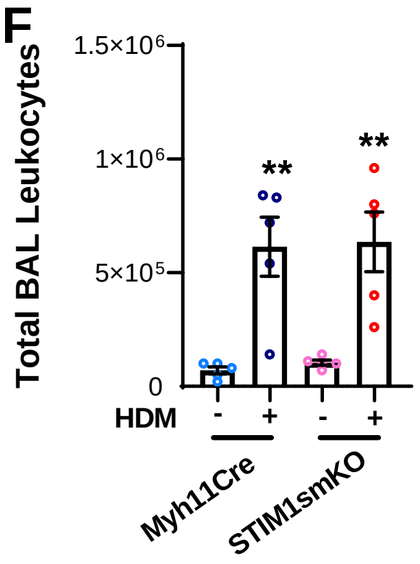

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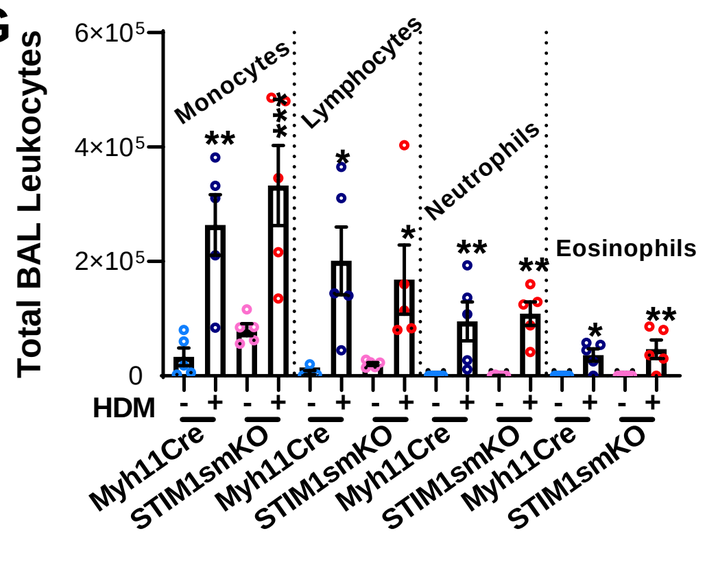
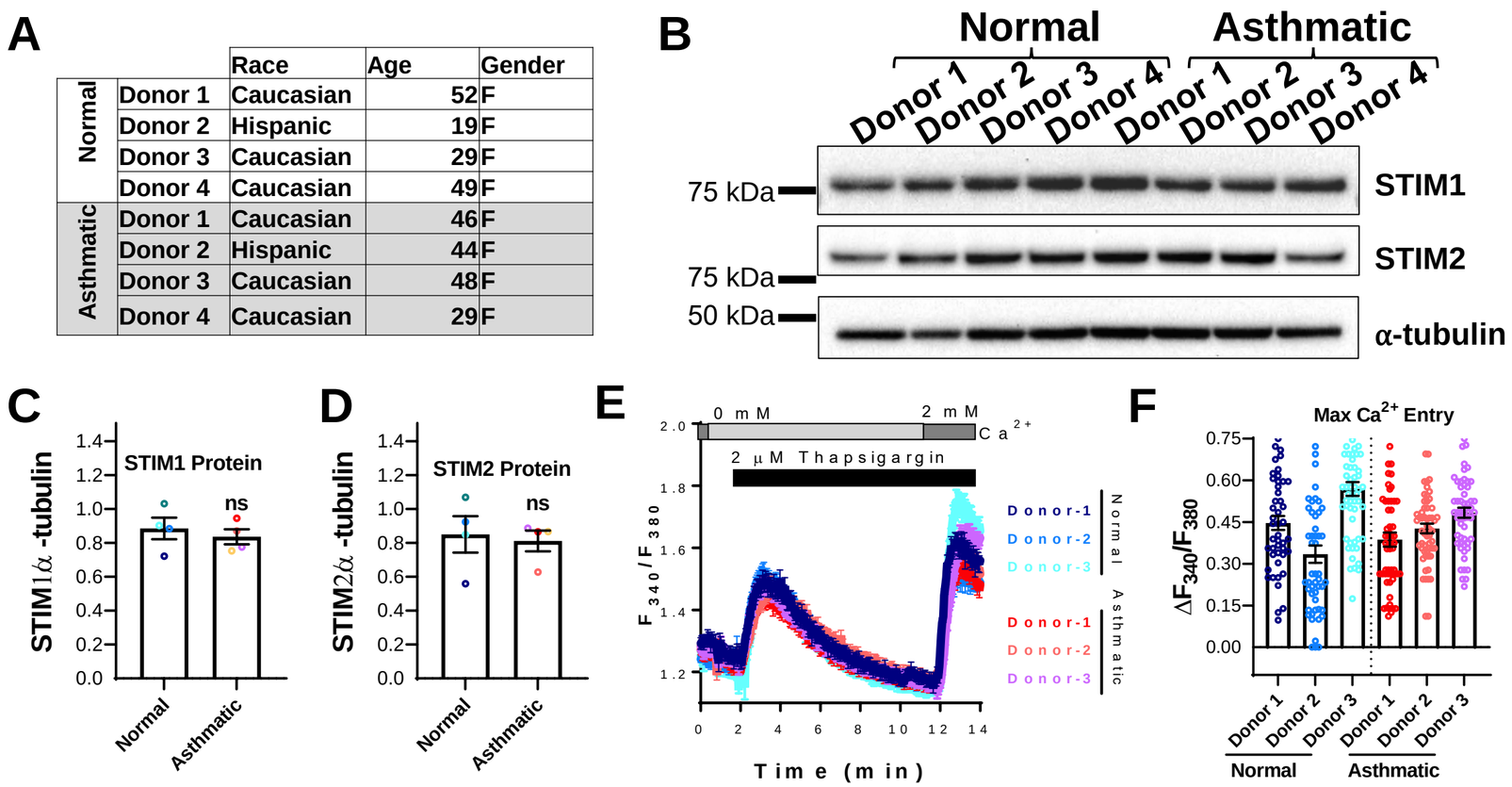


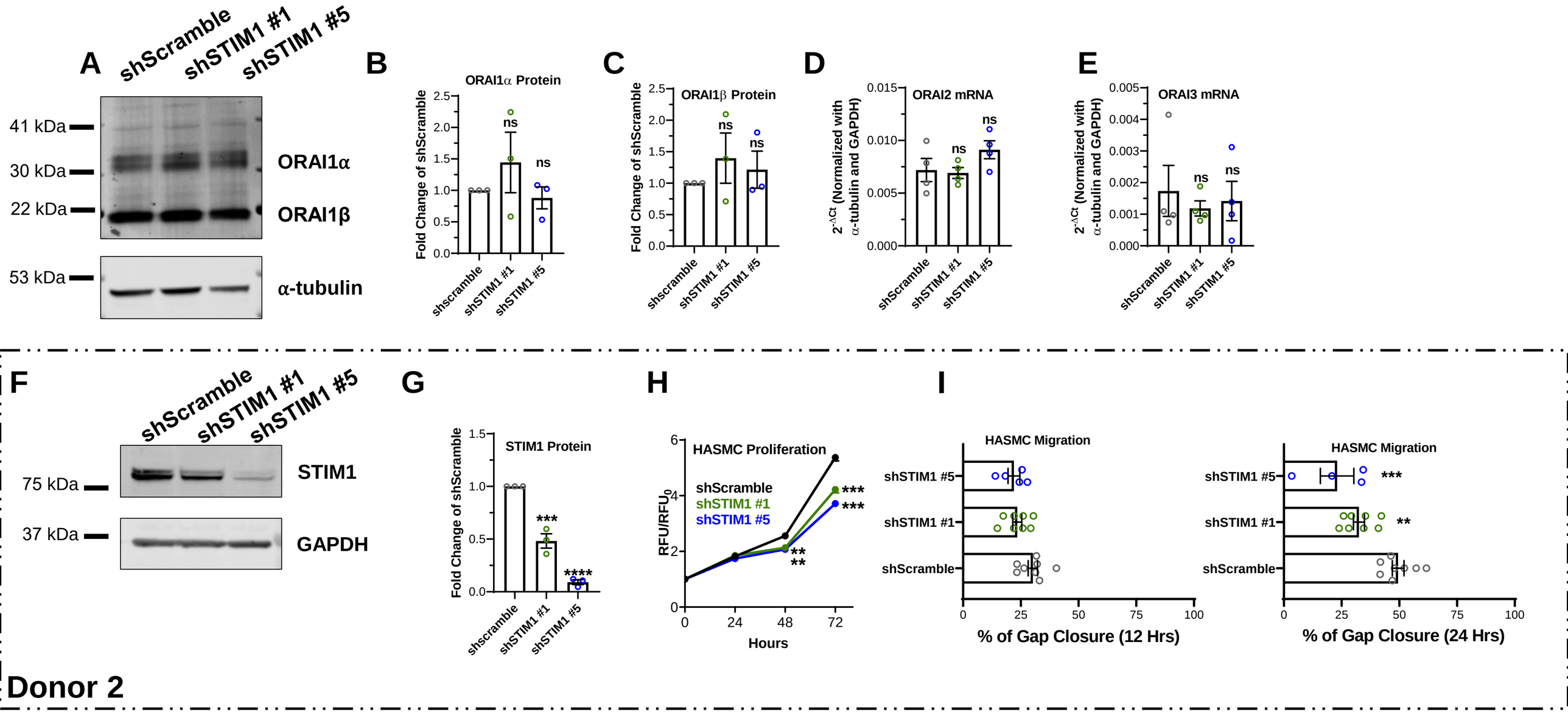
Supplemental Figure 1

A

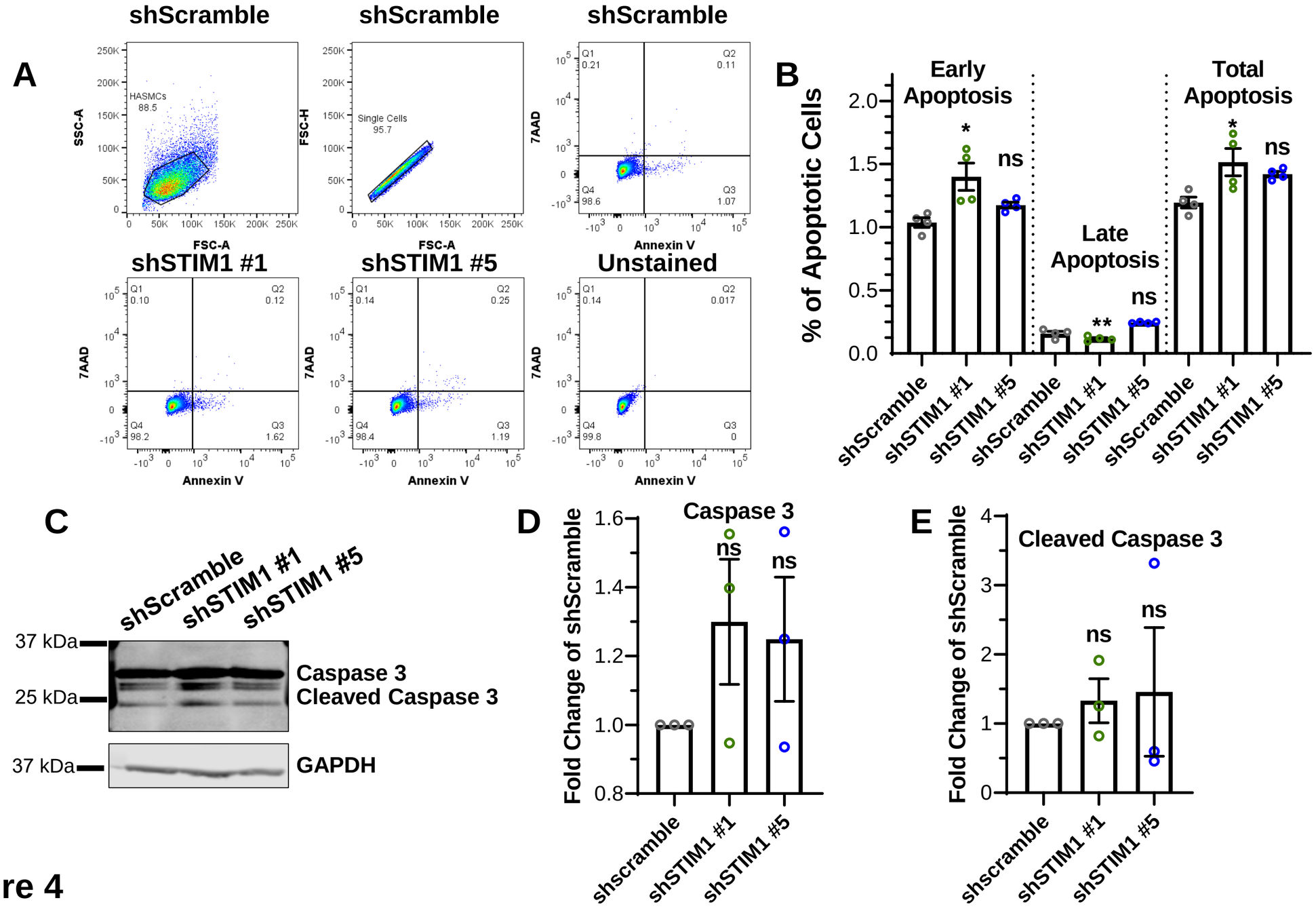
		Race	Age	Gender
Normal	Donor 1	Caucasian	52	F
	Donor 2	Hispanic	19	F
	Donor 3	Caucasian	29	F
	Donor 4	Caucasian	49	F
Asthmatic	Donor 1	Caucasian	46	F
	Donor 2	Hispanic	44	F
	Donor 3	Caucasian	48	F
	Donor 4	Caucasian	29	F



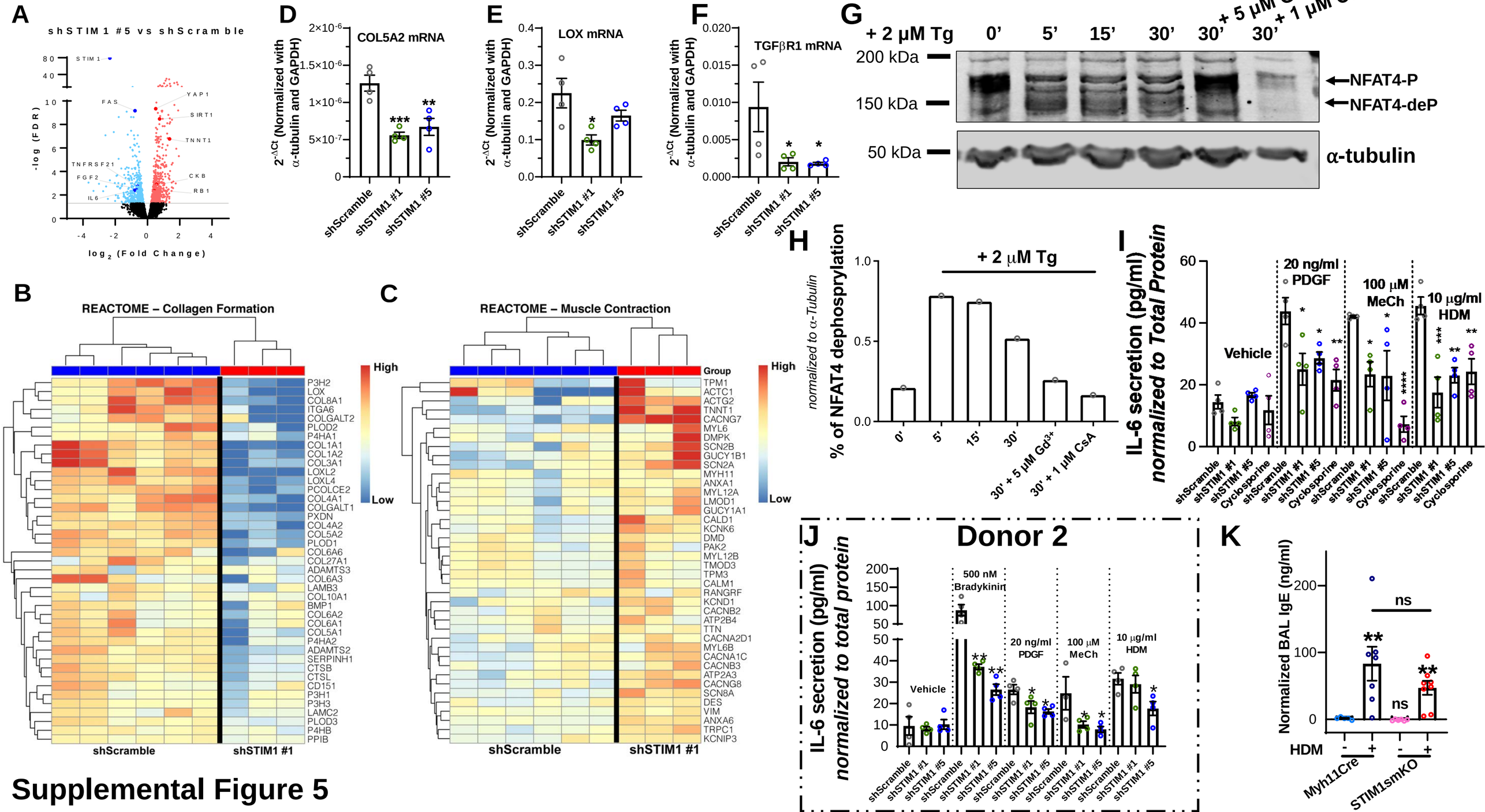
Supplemental Figure 2



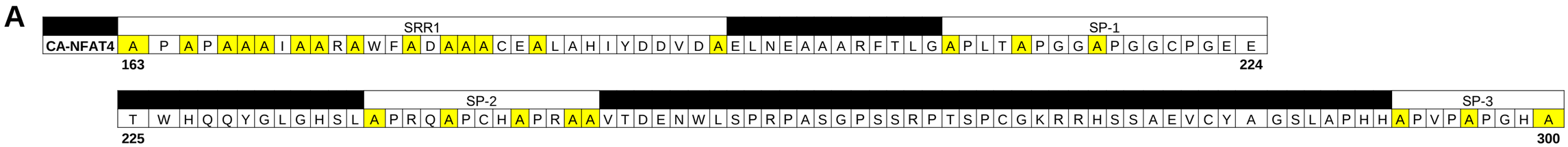
Supplemental Figure 3

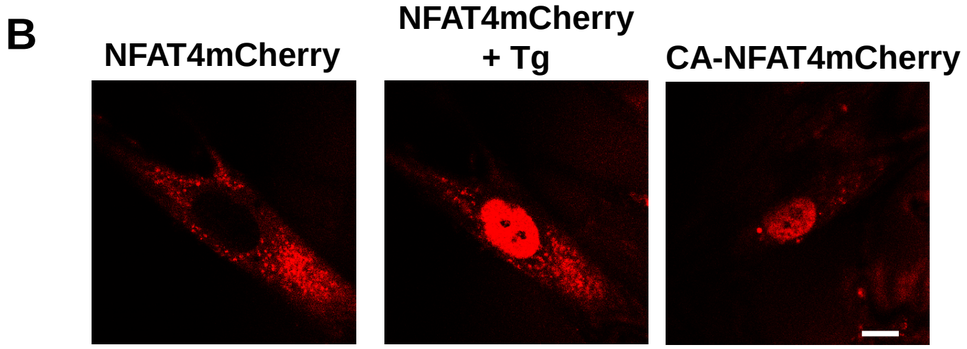


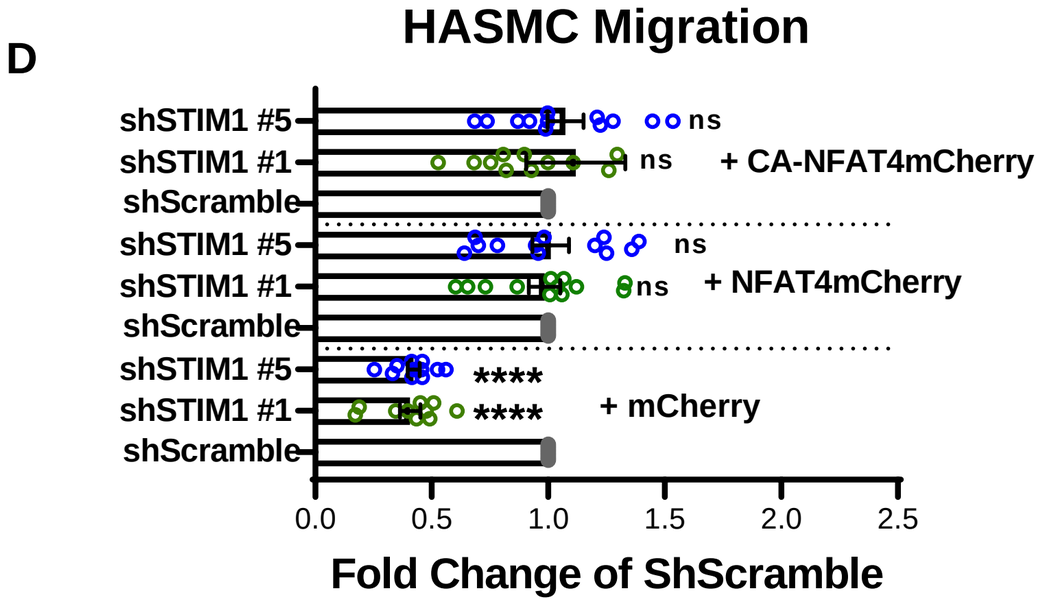
Supplemental Figure 4

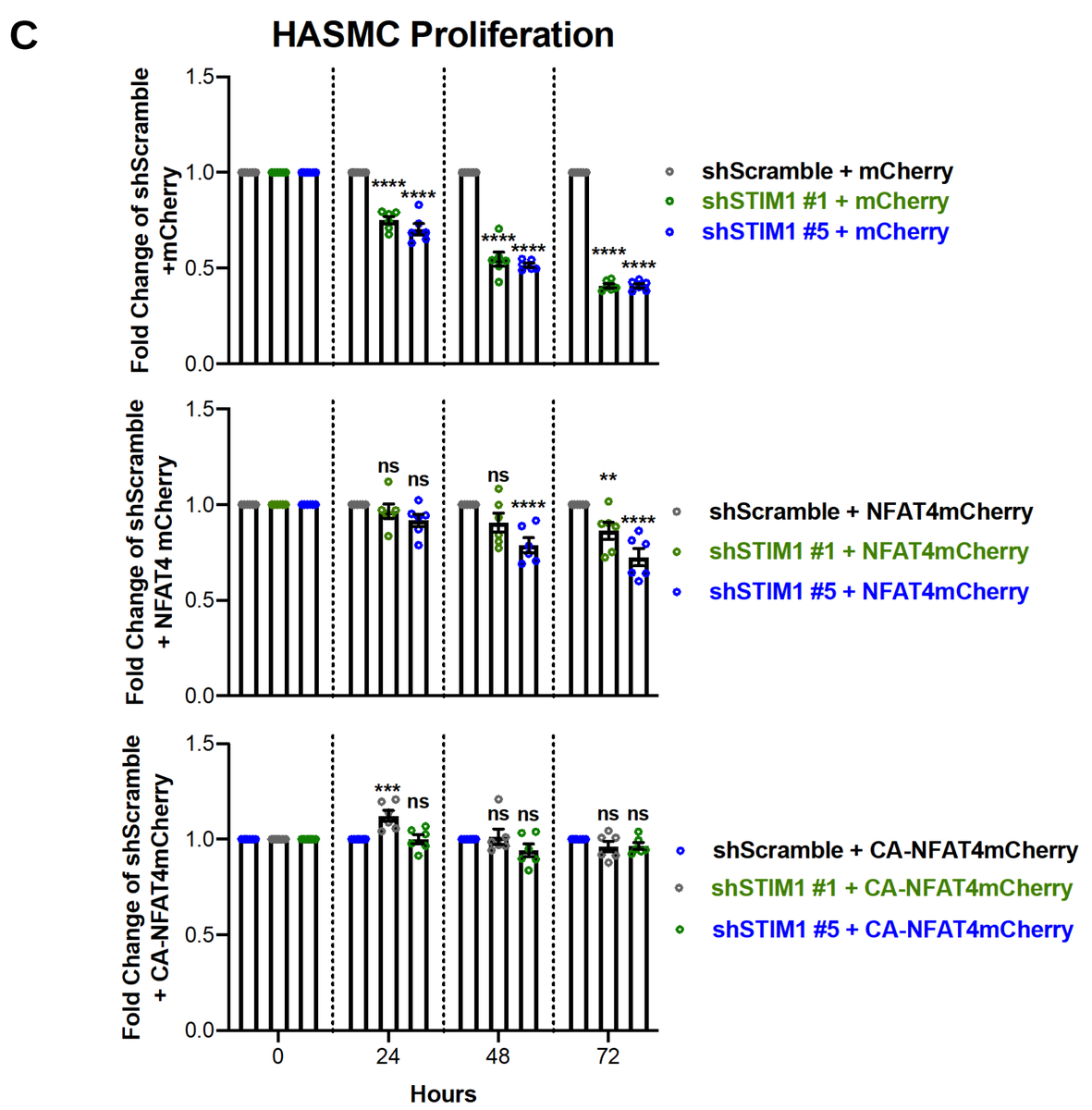


Supplemental Figure 5

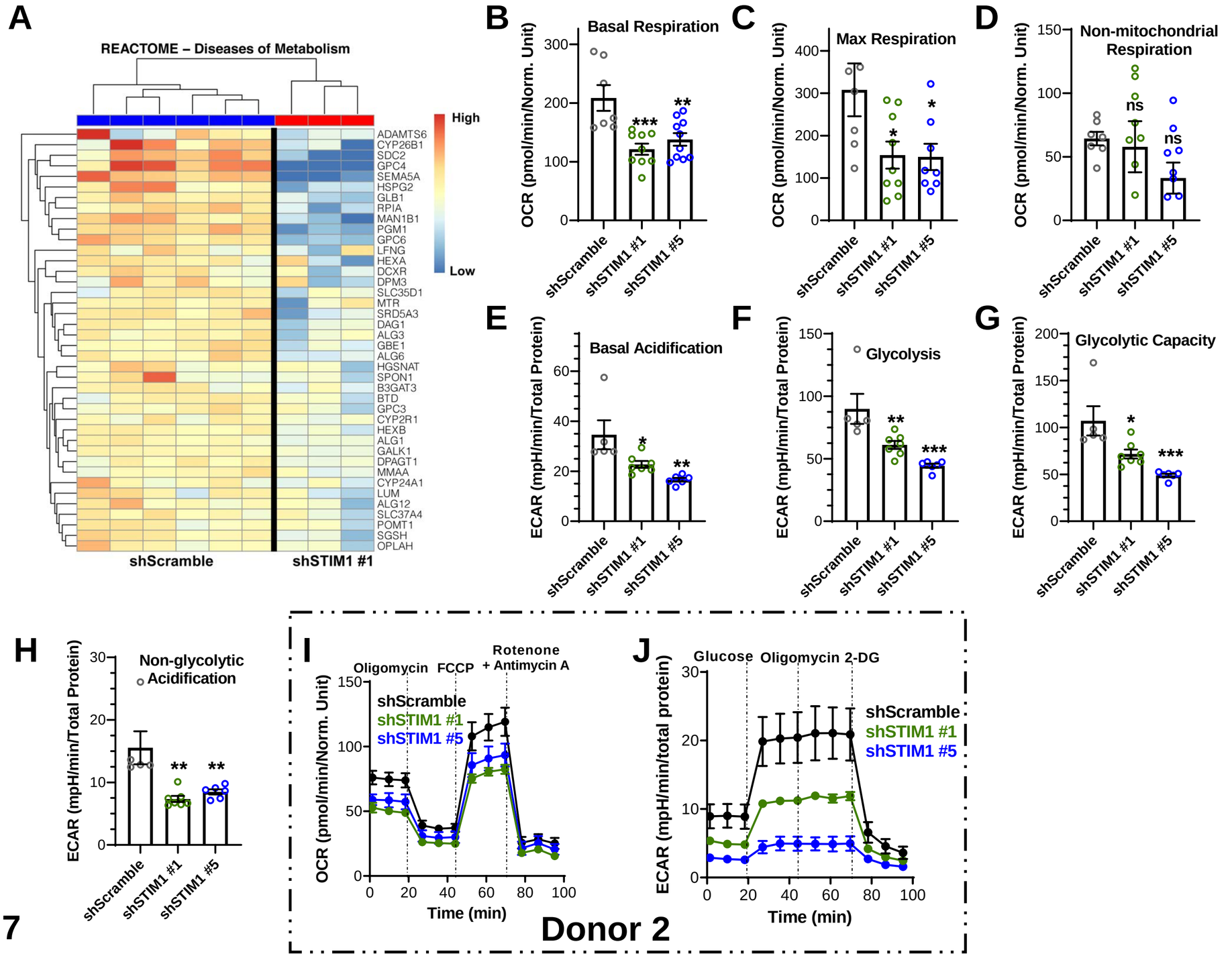




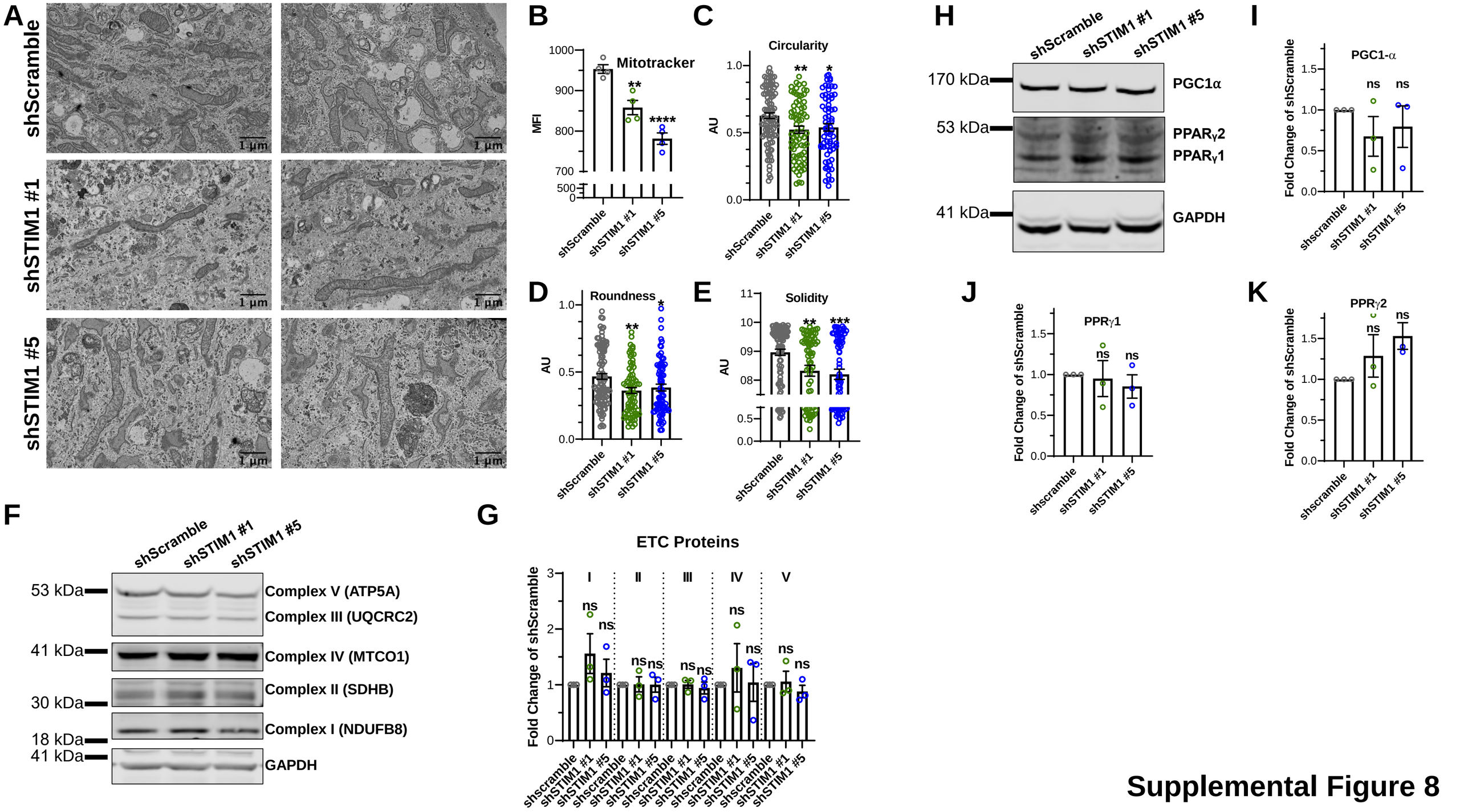




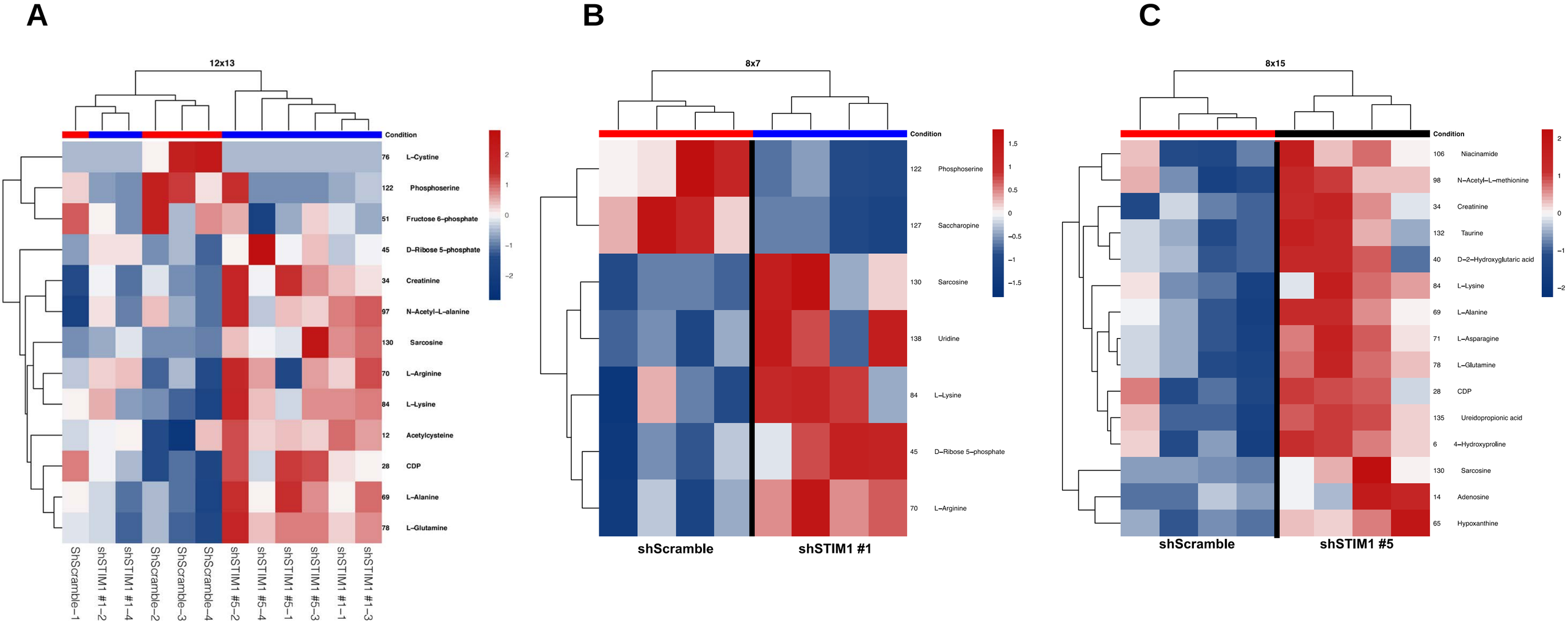
Supplemental Figure 6



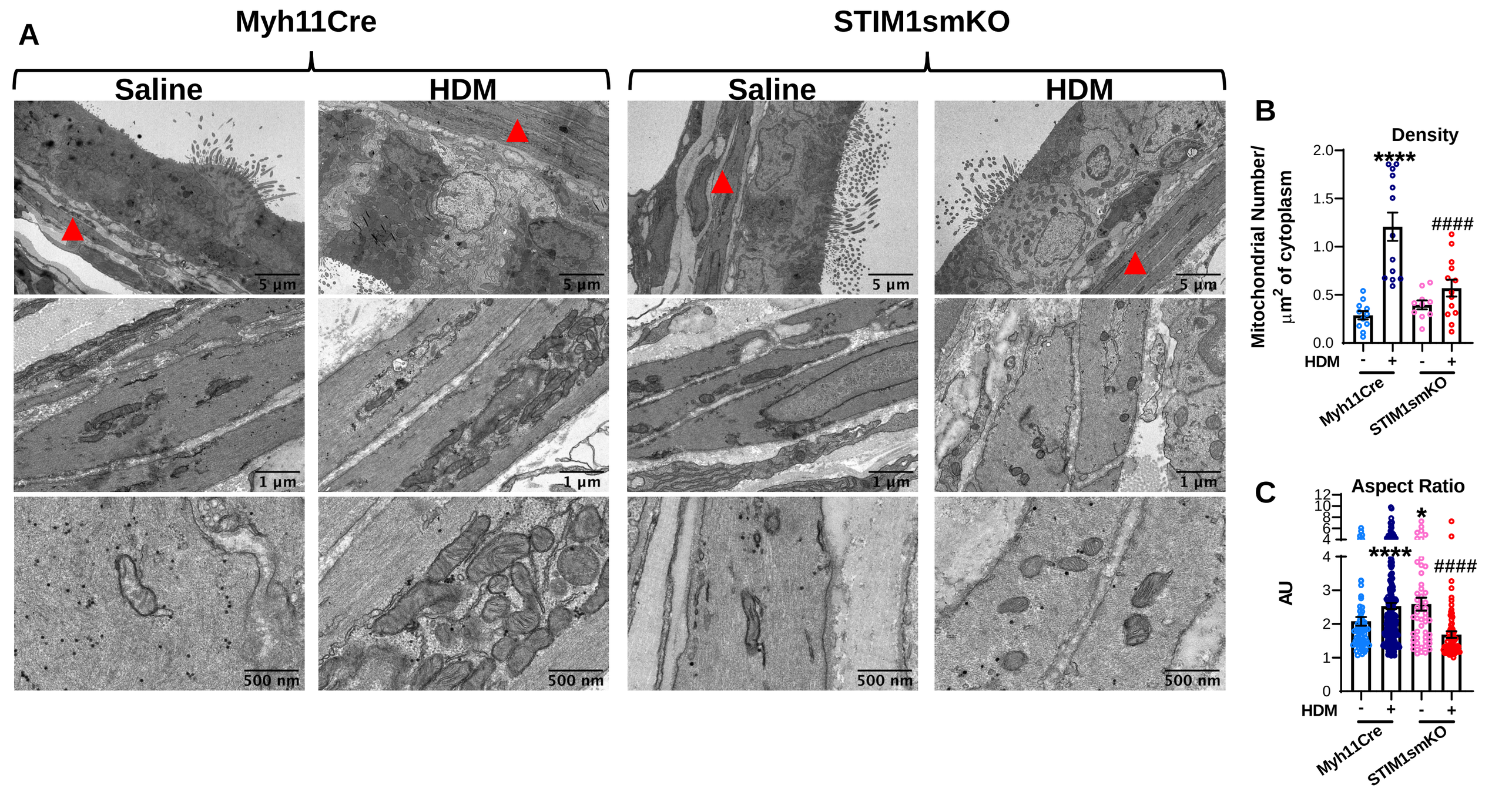
Supplemental Figure 7



Supplemental Figure 8



Supplemental Figure 9



Supplemental Figure 10

A

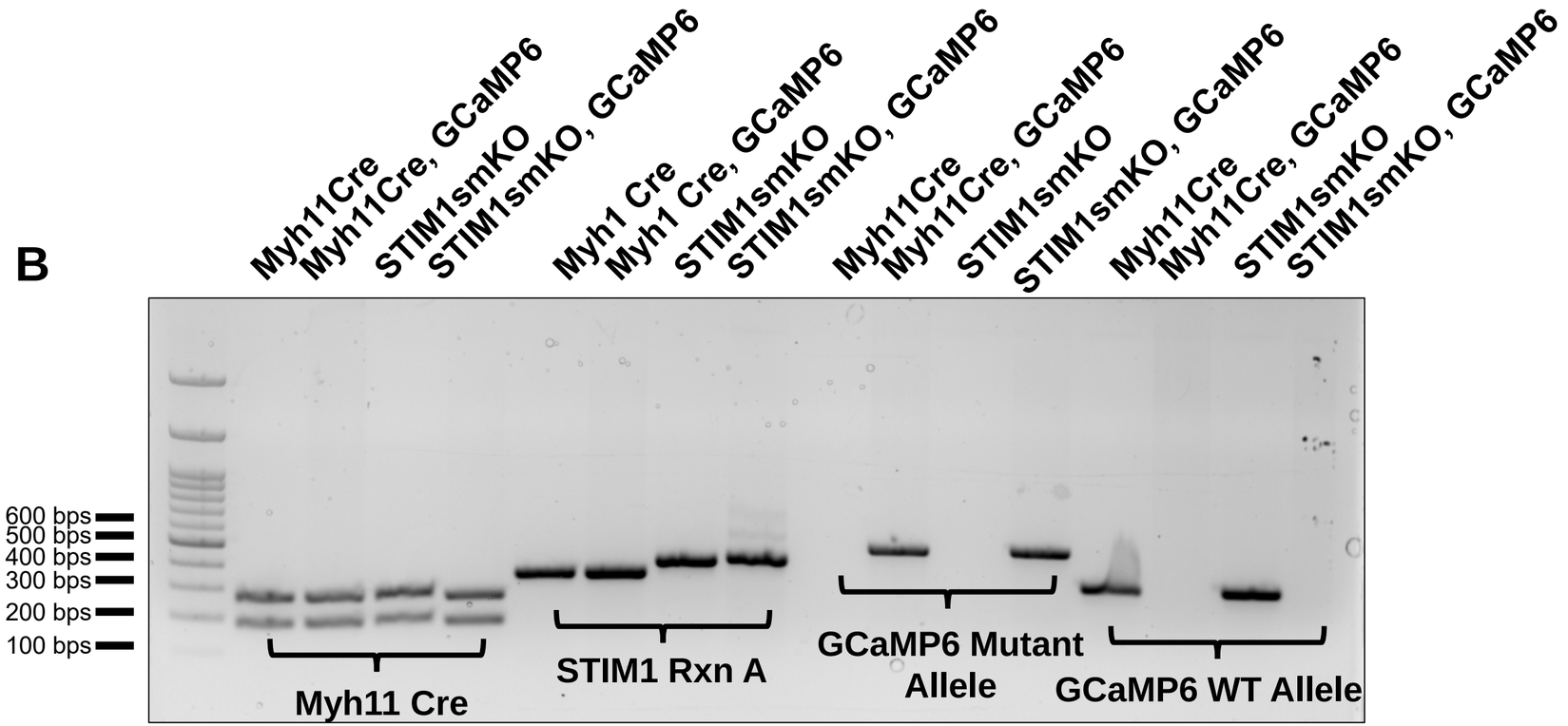
PCR Products

Myh11 Cre
Cre Transgene=287 bps
Internal Control= 180 bps

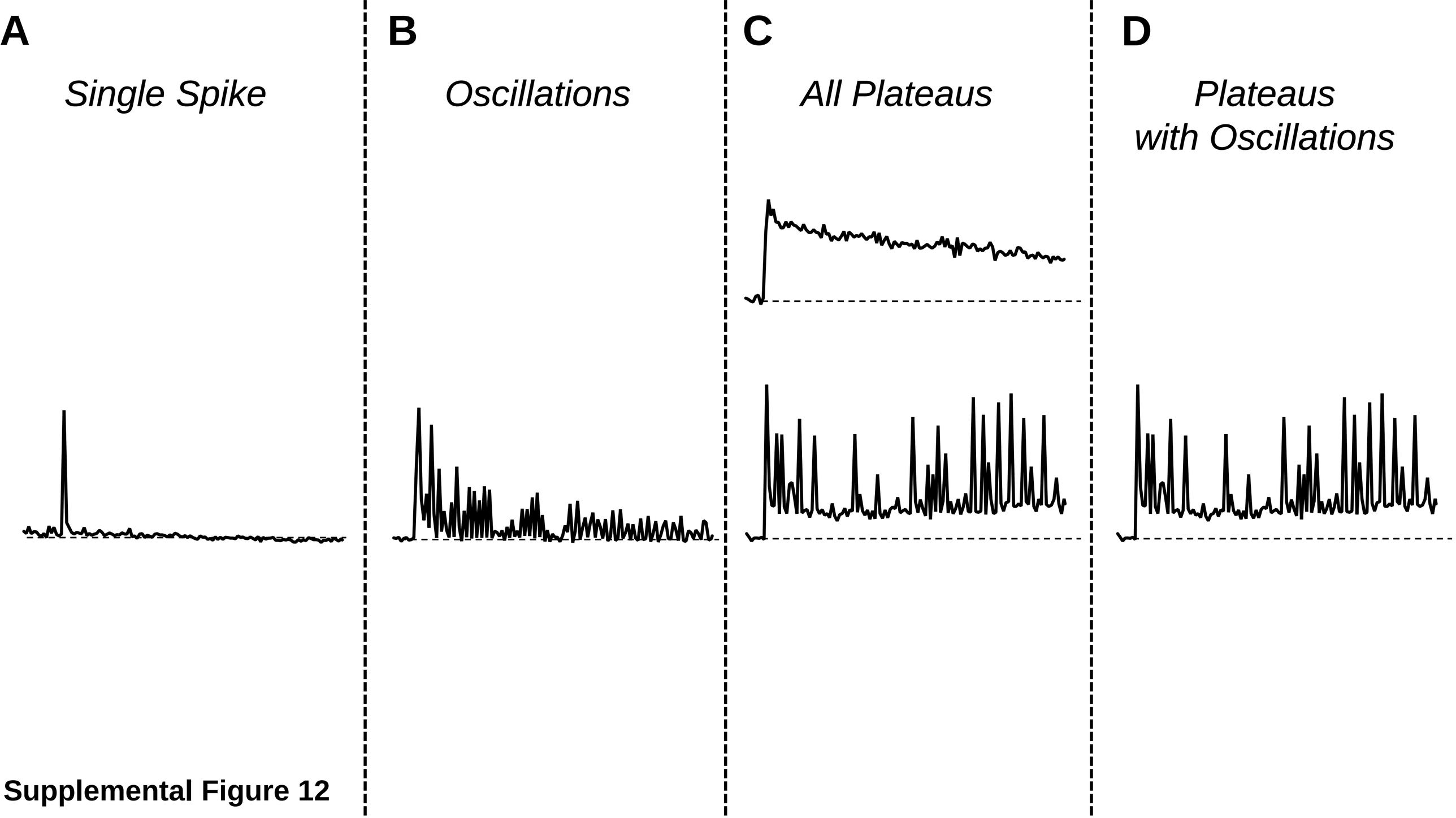
STIM1 Rxn A
WT Allele=348 bps
Flox Allele=399 bps

GCaMP6
Mutant Allele=450 bps
WT Allele=297 bps

B



Supplemental Figure 11



Supplemental Figure 12