SUPPLEMENTAL MATERIAL

CaMKII (Ca^{2+}/calmodulin-dependent kinase II) in mitochondria of smooth muscle cells controls mitochondrial mobility, migration and neointima formation

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DETAILED METHODS

Materials

Mouse PDGF-BB (#PMG0044), MitoTracker green and far-red (#M7514, M22426), MitoSOX (#M36008), phalloidin 568 (#A12380) and Fura-2 AM (#F1201) were purchased from ThermoFisher. Tamoxifen (#T5648), aphidicolin (#A0781), CytochalasinD (C8273) and Nocodazole(M1404) were purchased from Sigma, Ru360 (#557440) from Calbiochem, MitoTEMPO (#ALX-430-150) was from Enzo Life Sciences. Mouse-on-mouse (MOM, #BMK-2202) and Vectashield kits (#H-1000) were purchased from Vector Labs. The streptavidin-conjugated fluorophores (#S11226) and TO-PRO-3 iodide (#T3605) were from Life Technologies. The transfection reagent DharmaFECT 4 (#T-2004-02) was purchased from Dharmacon, Lipofectamine 3000 (#L3000008) from ThermoFisher. Reverse transcription and real time PCR were performed with Micro RNeasy kit (#74004) from Qiagen, SuperScript III Reverse Transcriptase (#18080044) from ThermoFisher and Power SYBR Green PCR master mix (#4367659) from Applied Biosystems. The o-Cresolphthalein Calcium Assay Kit (#701220) was bought from Cayman Scientific.

The following antibodies were used: Anti-CaMKII antibody for immunoblots was purchased from EMD Millipore (#07-1496) and anti-CaMKII antibody for immunofluorescence was from LifeSpan Biosciences (LS-C100735/5122). Anti-CaMKIIN antibody was from Aviva Systems Biology (CaMK2N2 epitope, #OAAB07385). Antibodies against GAPDH (#2118), phosphorylated CaMKII (Thr-287, #12716, 0.5 µg/mL), COX IV (#4844), phosphorylated myosin light chain (MLC) (Ser19, #3671), Drp1 (#8570), PCNA (#13110), HA (#2367), phospho-FAK (Tyr577 #3281) and FAK (#13009) were purchased from Cell Signaling Technology. Anti-mitochondria antibody was from Abcam (MTC02 #ab3298). The antibody against total MCU was from Sigma Prestige (HPA016480). Anti-pSer92 MCU was a kind gift from Dr. Mark E. Anderson (Johns Hopkins University). Anti-rabbit IgG-HRP (#1706515) was from BioRad and Anti-mouse IgG-HRP was from Cell Signaling (#7076). The anti-Vinculin antibody (#MA5-11690) was purchased from ThermoFisher. The Anti-Miro-1 antibody (ABIN635090) was purchased from Antibodies Online. The rabbit anti-GFP antibody (#A6455) was from ThermoFisher. Detailed usage in Major Resource Tables

Adenoviral transduction

Generation of the mitochondrial-targeted CaMKII inhibitor peptide CaMKIIN has been previously described [1]. Recombination and amplification of adenoviruses expressing mtCaMKIIN (Ad-mtCaMKIIN), mtCaMKII (Ad-mtCaMKII), mitochondrial targeted EGFP (Ad-mtGFP), cre recombinase (Ad-Cre) or empty (Ad-control) were performed by the Gene Transfer Vector Core at the University of Iowa.

Immunoblotting

Whole cell lysates were generated by lysis in buffer containing 50 mM Tris/HCl, 150 mM NaCl, 0.012 M sodium deoxycholate, 0.1% SDS, and 1% NP-40 supplemented with protease and phosphatase inhibitors. Protein concentrations were determined by Pierce BCA protein assay (ThermoFisher). Equivalent amounts of 5-15 µg of protein (cell lysate, mitochondrial/cytoplasmic fractions, and mitoplasts) were separated by SDS/PAGE on 4-20% Tris/glycine precast gels (BioRad) and transferred to PVDF membranes (BioRad). Membranes were blocked in 5% BSA and incubated overnight at 4°C with primary antibodies at a dilution of 1:500 to 1:1000. Blots were washed 3 times for 10 minutes each with 0.05% Tween-20 in TBS and incubated for 1 hour at room temperature with the respective secondary antibodies at a dilution of 1:5000 to 1:15,000. After washing, blots were developed with ECL chemiluminescent substrate (Santa Cruz Biotechnology). Blots were scanned and analyzed using ImageJ software.

Determination of mitochondrial Ca^{2+} transients
Ratiometric Ca^{2+} measurements in mitochondria were performed using adenovirus expressing mtPericam (Ad-mtPericam), a fluorescent Ca^{2+} indicator protein targeted to the mitochondria by a COX IV targeting sequence[2]. VSMC were infected with Ad-mtPericam 48 hr prior to analysis. Pericam ratiometric fluorescent imaging with the Leica SP8 STED imaging system was used to determine fluorescence signal intensity, with an excitation at 405nm/480nm and acquisition at 510nm. Real-time shifts in Pericam fluorescence ratio were recorded before, during and after acute addition of PDGF (20 ng/mL). Peak amplitude (R) was calculated by subtracting the baseline fluorescence ratio from the highest fluorescence ratio. The area under the curve (AUC) was determined using GraphPad Prism and normalized by subtracting the AUC at baseline. Summary data represent the average difference in basal and peak mitochondrial [Ca^{2+}].

Determination of mitochondrial Ca^{2+} concentrations with o-Cresolphthalein
Isolated mitochondria from thoracic aortas or cultured VSMC were resuspended in Ca^{2+}-free PBS. Mitochondrial Ca^{2+} was then assessed using the o-Cresolphthalein conjugation colorimetric plate assay per the manufacturer's protocol (Cayman Scientific). Ca^{2+} concentration was normalized to total mitochondrial protein concentration determined by Pierce BCA protein assay.

Determination of cytosolic Ca^{2+} transients
Cells were loaded with Fura-2 acetoxymethyl ester (Fura-2AM) by incubating cells with 2 μM Fura-2AM in Ca^{2+}-free HBSS for 20 min at room temperature. Cells were then washed twice with Ca^{2+}-containing HBSS and incubated at 37º for 20 min to esterify the stain. Cells were excited alternatively at 340 and 380 nm. Fluorescence signal intensity was acquired at 510 nm. Real-time shifts in Fura-2AM fluorescence ratio were recorded before, during and after acute addition of PDGF (20 ng/mL) using a custom-built Olympus IX81 Inverted Light Microscope. Peak amplitude (R) was calculated by subtracting the baseline fluorescence ratio from the highest fluorescence ratio. The area under the curve (AUC) was determined using GraphPad Prism and normalized by subtracting the AUC at baseline. Summary data represent the average difference in the basal and peak increase in cytoplasmic [Ca^{2+}].

Migration assays
Boyden chamber: The modified Boyden chamber method was performed using transwell cell-culture chambers with a collagen polycarbonate membrane with 8-μm pores. VSMC expressing Ad-mtCaMKII, Ad-mtCaMKIIN or Ad-control were added to the upper chamber (50,000 cells/well) of the transwell and allowed to attach for 30 min in DMEM with 0.1% FBS. Migration of VSMC was induced by the addition of 20 ng/ml PDGF in DMEM with 10% FBS to the lower compartment. After 6 hr, cells were scraped from the upper chamber. VSMC migrating to the lower surface of the membrane were fixed in 4% PFA-PBS, stained with DAPI and imaged with 10X objective using an epifluorescent microscope (Olympus BX-51). Nuclei were quantified in at least four fields per condition using ImageJ software.
Scratch wound assays: The serum-starved VSMC monolayer was disrupted with a sterile cell scraper to create a cell-free zone. Media was removed and replaced with DMEM containing 10% FBS. To inhibit cell proliferation during the scratch wound assay, aphidicolin, a specific inhibitor of DNA polymerase A,D, was added at a concentration of 100 μM. Images were taken 24 hr after injury using a microscope equipped with a digital camera. The area of the scratch wound was measured using ImageJ and calculated relative to the initial area at the time of injury.

Proliferation assays

VSMC were grown to 80% confluency on 10-cm dishes and infected with Ad-control or Ad-mtCaMKIIN. The next day (time 0), VSMC were seeded in triplicate into 12-well plates at 15,000 cells per well in a volume of 2 ml DMEM/10% FBS, supplemented with 20 ng/mL PDGF or vehicle control. Cells were counted daily for four consecutive days using a Beckman Coulter automated cell counter. Cell counts were normalized to the number of cells at time 0 and expressed as fold change.

Detailed quantification of mitochondrial distribution

To visualize mitochondrial distribution in migrating cells, VSMC were simultaneously infected with Ad-mtGFP and Ad-control or Ad-mtCaMKIIN. Cells were then grown to confluence on glass coverslips, and wounded using a sterile pipette tip. Cells were allowed to migrate in DMEM with 10% FBS and 20 ng/mL PDGF for indicated time points. VSMC were then fixed and stained with phalloidin 568 to visualize the actin cytoskeleton. Images were acquired using a Zeiss 510 confocal microscope with a 40X objective. Mitochondrial distribution was quantified by two methods using NIH ImageJ. 1) The first protocol was adapted from Zhao, et al. [3]. The leading edge region was defined as the area delimited by the leading cell edge and the half-distance between the nucleus and leading edge (indicated by dashed lines in images). The relative amount of mitochondria accumulating in the leading edge area of migrating cells was determined by calculating the mtGFP signal in the leading edge region relative to the total GFP intensity in the cell area between the nucleus and the leading edge (Supplemental Figure VA). Data are presented as the mean from 50 randomly selected cells in each treatment group. 2) Each cell along the wound edge was subject to five radially distributed line scans from the nucleus to the leading edge of the cell (Supplemental Figure VB). The leading part of the cell (L) was defined as the line segment from the leading cell edge to the half-distance to the nucleus. The fluorescence intensity (GFP) was plotted along each line using ImageJ (Supplemental Figure VC). The AUC of the GFP intensity for the leading part of the cell was normalized to the total AUC along the line. For both methods, data are expressed as percent of mitochondria in the leading edge area or segment. The image analysis was performed independently by two scientists who were blinded to the treatment group.

Immunofluorescent imaging

Immunofluorescent staining was performed using the following procedure unless otherwise noted: VSMC or murine tissue samples were fixed in 4% PFA for 10 min. Tissue sections were fixed and paraffin-embedded as described above. Samples were permeabilized in 0.1% Triton-X 100 for 10 min, followed by blocking for 1 hour using either MOM kit (when mouse primary antibodies were used) or normal goat serum (for rabbit primary antibodies). Samples were then incubated in mouse primary antibodies for 1 hour or rabbit primary antibody overnight and AlexaFluor 488-, 568-, or 647-conjugated secondary antibodies (Invitrogen) for 1 hour. Samples were counterstained with TO-PRO-3 to visualize nuclei, mounted onto slides using Vectashield H-1000 and visualized using Zeiss 510 LSM confocal microscope.

To analyze proliferation in the neointima and media of murine arteries, 5-μm carotid artery sections (5 μm) were subjected to antigen retrieval in citrate buffer and immunofluorescent staining with PCNA primary antibody (Cell Signaling) and 488-conjugated rabbit secondary antibody. Nuclei were counterstained with TO-PRO-3. Images were acquired using a Zeiss LSM 510 confocal microscope. Data were quantified in ImageJ by counting PCNA-positive nuclei normalized to total nuclei count. This analysis was performed for four sections per mouse, eight mice per genotype.

For human sections, 10 μm sections of fixed and paraffin-embedded coronary arteries were deparaffinized and subjected to immunohistochemistry using anti-CaMKII with streptavidin

amplification, α-smooth muscle actin to label smooth muscle cells, and the mitochondrial marker MTC02. Negative controls without primary antibody were included in every experiment. Images were acquired with a Zeiss 710 LSM in the University of Iowa Central Microscopy Research Facilities that was purchased with funding from the NIH SIG grant 1 S10 RR025439-01. The imaging parameters were set based on the negative control samples and all images were obtained with the same parameters.

Phosphorylated myosin light chain immunostaining and analysis

VSMC were seeded onto gelatin-coated glass coverslips and grown to confluence. A scratch was made with a sterile pipette tip and cells were washed twice with warm HBSS and replaced with DMEM containing 20 ng/mL PDGF. After 6 hr, cells were washed and fixed in 4% PFA. Cells were subject to the staining protocol described above. The primary antibodies used were anti-pMLC, and anti-vinculin. Cells were mounted onto microscope slide with mounting medium Vectashield and images acquired using the Zeiss LSM 510 confocal microscope.

To quantify the pMLC colocalization with FA, confocal images were processed through a Cell Profiler pipeline adapted from related objects. Region of interest (ROI) was selected at the leading edge of every cell. FA were identified using "primary objects" function with automatic thresholding by object intensity. Colocalization of pMLC signal within FA objects was analyzed and expressed as Mander's coefficient. This analysis was performed in 30 cells from 3 separate experiments by two scientists who were blinded to the treatment group.

Mitochondrial ROS detection

Mitochondrial ROS were measured in live cells using the dihydroethidium derivative, mitoSOX red (1 μM). Cells were treated with PDGF or vehicle for 1 hr, followed by loading with mitoSox or mitoTracker for 20 min, washed once in HBSS and imaged using a confocal microscope with a 20X objective. All images were acquired using the same imaging settings. Analysis was performed by tracing a region of interest around the cell using NIH Image J. Mean fluorescence intensity of mitoSOX was normalized to mitoTracker. Data were presented as relative fluorescent units.

Analysis of mitochondrial morphology

VSMC infected with either Ad-Ctrl or Ad-mtCaMKIIN were seeded on 3-cm coverslip dishes (Matek) in DMEM supplemented with 10% FBS. Cells were then simultaneously treated with 20 ng/mL PDGF or vehicle and 500 nM mitoTracker Green or mitoTracker FarRed for 30 min at 37ºC. For automated morphometry, images were processed using NIH ImageJ software with the plugins involving either "rolling ball" background subtraction or deblurring by 2-D deconvolution with a computed point spread function. Using a custom-written NIH ImageJ macro provided by Dr. Stefan Strack (University of Iowa), processed images were converted to binary (black and white) images by auto-thresholding, and mitochondrial particles were analyzed for length [4].

qRT-PCR

DNA-free total RNA was isolated from mouse arteries using the Micro RNeasy kit (Qiagen), and reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen). cDNA was amplified with primers for CaMKIIN and acidic ribosomal phosphoprotein P1 (ARP) with SYBR Green PCR master mix. CaMKIIN forward primer was 5'-ATC CTA CCC TAC GGC GAG GAC AAG -3', CaMKIIN reverse primer was 5'-ATC CTC GAT CAC CAC TCT CTT GGC -3'. ARP forward primer was 5'-CAT CCA GCA GGT GTT TGA CAA -3' and ARP reverse primer was 5'-ATT GCG GAC ACC CTC TAG GAA G-3'.

Detailed Carotid injury model
Male mice (10-12 weeks-old) were subject to carotid injury by endothelial denudation using a resin-bead coated suture as previously described [5]. The left carotid artery was exposed after midline incision. A distal ligature was placed on the internal branch of the carotid artery and a loose ligature just proximal. Arteriotomy was performed and a 3-0 suture coated with resin beads was advanced towards the aorta 5-7 times. The proximal ligature was tightened and blood flow restored through the external branch. At 28 days after injury, all animals were anesthetized and perfused at physiological pressure with PBS followed by 4% PFA for 3 min. The carotid arteries were excised and paraffin-embedded for subsequent analyses.

For morphometric analysis with Verhoeff's Van Gieson staining, 5 μm cross sections were obtained at 50 μm intervals from the carotid bifurcation site for up to 500 μm. Total vessel, luminal and intimal areas were determined after tracing the circumference of the external and internal elastic lamina (EEL, IEL), and the luminal area using NIH Image J. The vessel wall area was calculated by subtracting the luminal area from the area defined by the EEL, the neointimal area by subtracting the luminal area from the area defined by the IEL.

Human coronary arteries
Human tissue samples from autopsies were procured from the University of Iowa Decedent Center in accordance with guidelines established by the University of Iowa Institutional Review Board. Upon submission of a full application, it was determined by the IRB that the sample collection was exempt from federal regulations (University of Iowa IRB# 201210793). The exemption was granted on the basis that the research involved the collection or study of existing data and pathological specimens and that the information was recorded in such a manner that subjects could not be identified, directly or through identifiers linked to the subjects.
Autopsy samples were procured from subjects without a history of hypertension, diabetes, or cardiovascular disease. The absence of atherosclerosis was confirmed by gross pathology. Samples were fixed in formalin, processed and paraffin-embedded for immunohistochemistry.

Nucleofection
Cells were nucleofected, following manufacturer's protocol (Lonza #VPI-1004). Briefly, 600,000 cells were nucleofected in the presence of 5 μg plasmid DNA and plated onto 3 cm glass coverslip dishes. For focal adhesion turnover assays, VSMC were infected with either Ad-mtCaMKIIN or Ad-Control the next day and allowed to express for 48 hour.

siRNA transfection.
500,000 VSMC were seeded in a T75 flask and grown overnight. The next day, small interfering RNA (siRNA) duplexes purchased from IDT (Integrated DNA Technologies) were transfected at 5 nM using 20 μl DharmaFECT 4 reagent (Dharmacon). Knockdown efficiency was determined after 72 hr by immunoblotting for MCU. The MCU band intensity was normalized to COXIV signal. The knockdown efficiency was consistently greater than 75%.

HEK293T cell plasmid transfection
HEK 293T cells were transfected with DNA plasmid expressing a fusion protein of GFP and WT MCU or mutated MCU at Ser92 (Ser to Ala), a kind gift from Dr. Mark E. Anderson, (Johns Hopkins University) [6]. Cells were seeded at 80% confluency and transfected using Lipofectamine 3000 (ThermoFisher) as recommended by the manufacturers' protocol. Transfection was verified by visualizing GFP expression and by immunoblot for GFP and MCU.

Detailed Statistical Analyses

Data are expressed as mean±SEM and analyzed with GraphPad Prism 7.0 software. All data sets were analyzed for normality and equal variance. Kruskall-Wallis test with Dunn's post hoc test was used for data sets where normal distribution could not be assumed (Figure 1A, Supplemental Figure XB). Two-tailed unpaired Student's T-test (Figure 1E; Figure 2E; Figure 4F; Figure 6C, and D; Supplemental Figure XD) and 1-way ANOVA followed by Tukey's multiple comparison test (Figure 2D; Figure 3A, E; Figure 4A, C and D; Figure 5A; Supplemental Figure IVB and C, Supplemental Figure VIIC and D; Supplemental Figure VIIIB) were used for data sets with normal distribution. Two-way ANOVA followed by Tukey's multiple comparison test was used for grouped data sets (Figure 1B-D; Figure 2A,B, and F; Figure 3C-D; Figure 5B and C; Supplemental Figure IA; Supplemental Figure II; Supplemental Figure IIIB; Supplemental Figure 4D; Supplemental Figure VI, Supplemental Figure IXB). A p-value < 0.05 was considered significant.

Author Contributions

EKN, OMK and IMG conceived of the project and contributed to study design; EKN, OMK, KB and CA performed data collection; EKN, OMK, PN, KB, MW and IMG analyzed and interpreted the data; MW assisted with high throughput imaging; SS provided conceptual input and data analysis software; WHT conceived of the high throughput migration assay. EKN and IMG wrote the manuscript with input from all authors; all authors read and approved the final version of the manuscript.

SUPPLEMENTAL REFERENCES

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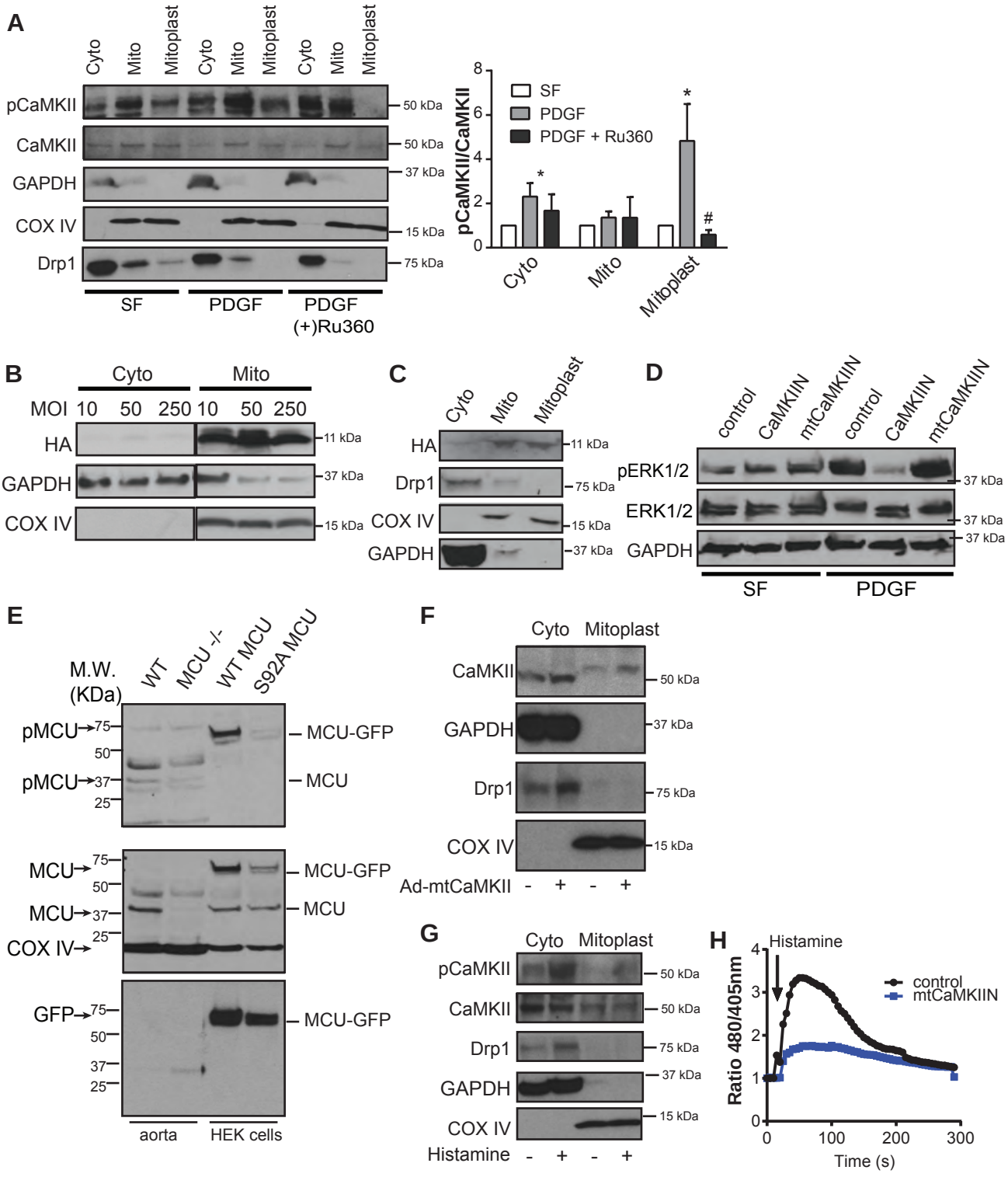
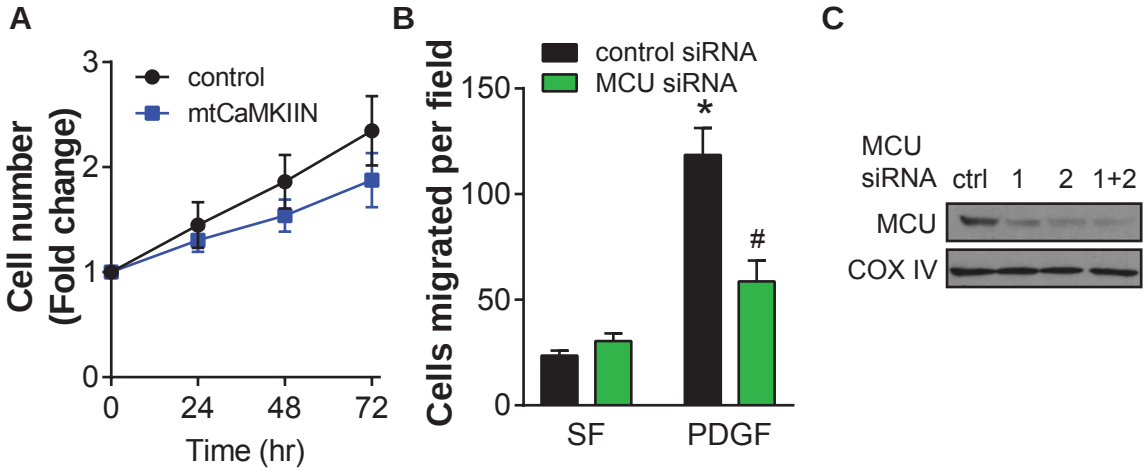
Supplemental Figure I



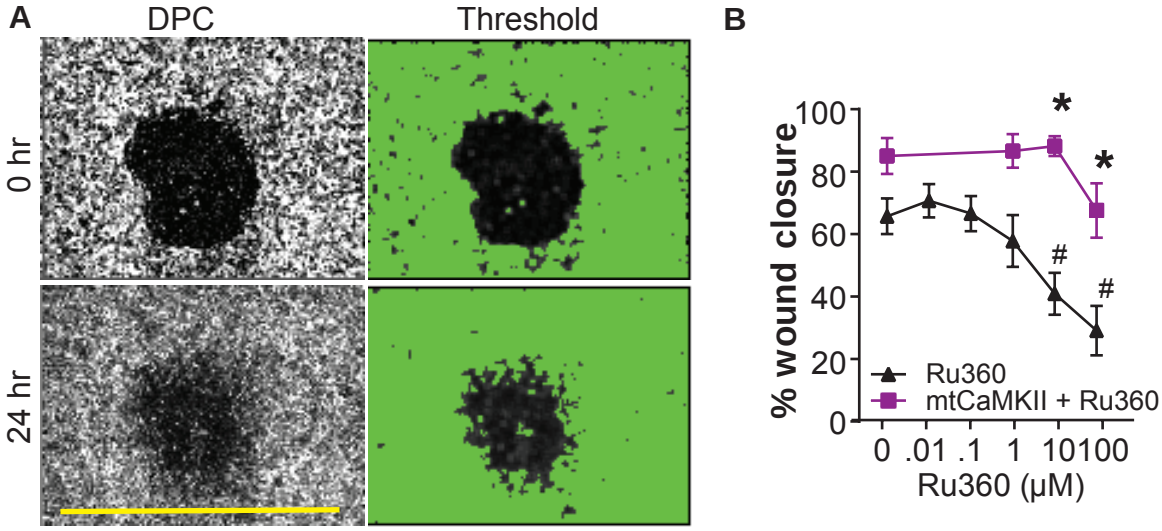
Supplemental Figure I. mtCaMKIIN localizes to the mitochondria and has no significant effect on canonical cytoplasmic CaMKII signaling. A) Immunoblot from one representative experiment for active (phosphorylated, pCaMKII) and total CaMKII in VSMC pretreated overnight with 1 μM Ru360 in serum-free (SF) conditions or after 15 minutes treatment with PDGF (20ng/mL) in cytosolic, mitochondrial, and mitoplast fractions and normalized to pCaMKII/CaMKII in SF conditions for each fraction. *p<0.05 versus control SF, #p<0.05 versus PDGF-treated control by 2-way ANOVA (data quantified from 4 independent experiments). B) Representative immunoblot for HA-tagged mtCaMKIIN in cytoplasm (Cyto) and mitochondria (Mito) fractions from VSMC infected with mtCaMKIIN at 10, 50, and 250 MOI for 48 hr. Three independent experiments were performed. C) Representative immunoblot for HA-tagged mtCaMKIIN in Cyto and Mito fractions and mitoplasts from VSMC infected with Ad-mtCaMKIIN (MOI 50 for 48 hr). Four independent experiments were performed. D) Representative immunoblot for total and phosphorylated (p)-ERK-1/2 in whole cell lysates from VSMC infected with adenovirus expressing untargeted CaMKIIN (CaMKIIN), mitochondria-targeted CaMKIIN (mtCaMKIIN) or control at MOI 50 for 48 hr and treatment with 20 ng/ml PDGF for 15 minutes in serum-free (SF) media. Three independent experiments were performed. E) Representative immunoblot for active (phosphorylated, pMCU, at Ser92) and total MCU and GFP in mitochondria isolated from aortas of WT and $MCU^{-/-}$ mice, and HEK cells transfected with GFP-tagged WT MCU or MCU with a Serine 92 to Alanine mutation. COX IV as loading control. Four independent experiments were performed. F) Immunoblot from one representative experiment for CaMKII in cytoplasmic and mitoplast fractions from VSMC infected with Ad-mtCaMKII overexpression (MOI 50 for 48 hr). Four independent experiments were performed. G) Representative immunoblot for active CaMKII in isolated mitoplasts and cytoplasm in VSMC treated with 20μM Histamine for 5 minutes. Four independent experiments were performed. Drp1, outer mitochondrial membrane marker; COX IV, mitochondrial matrix marker; GAPDH, cytoplasmic marker for all subcellular fractionation immunoblots. H) mtPericam tracing in VSMC expressing mtCaMKIIN or control for 48 hr; arrow indicates addition of Histamine (20 μM). The tracing from 20 cells over 3 independent experiments were averaged.

Supplemental Figure II



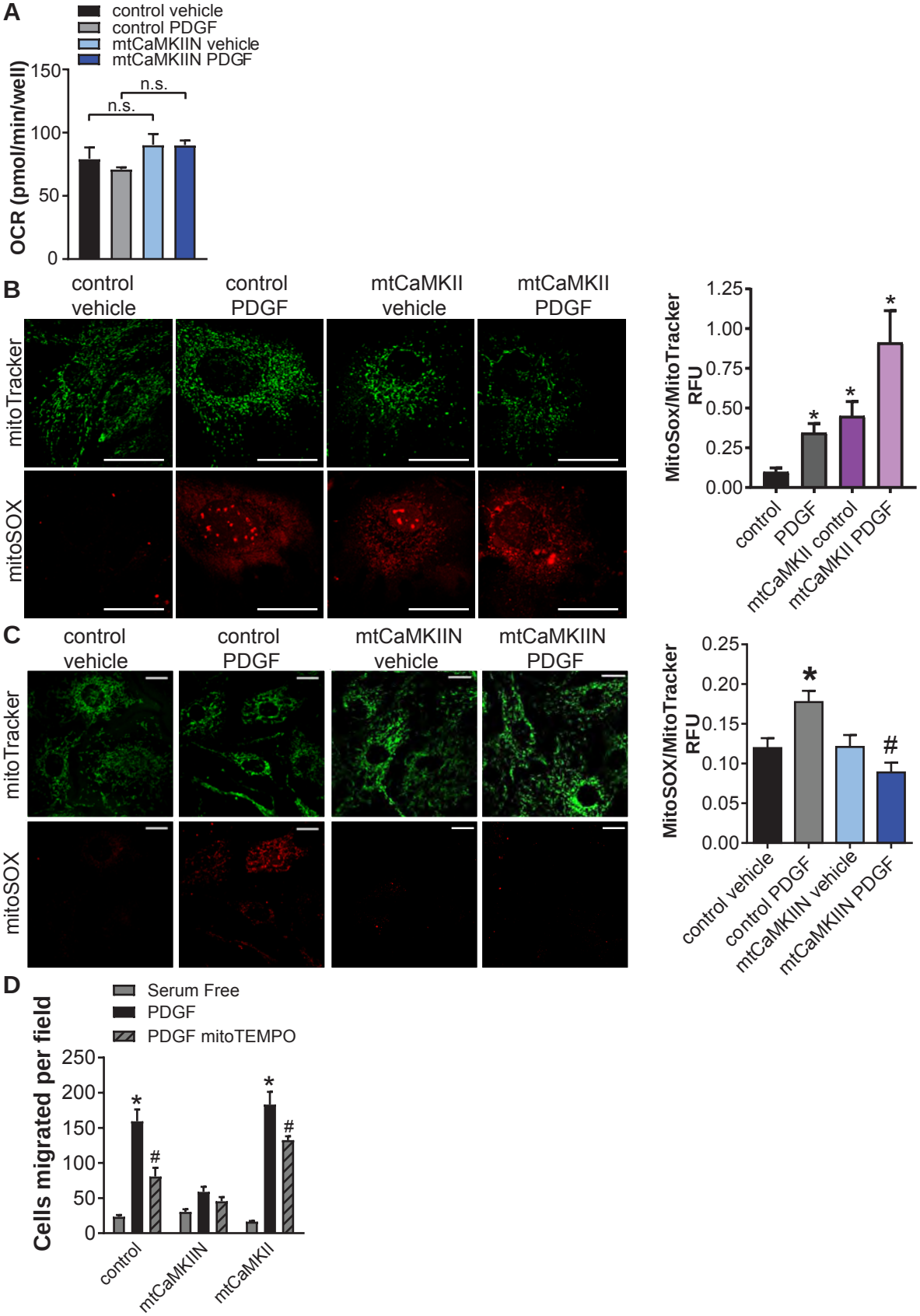
Supplemental Figure II. VSMC migration and proliferation with mtCaMKIIN and MCU inhibition. A) Cell counts in VSMC expressing mtCaMKIIN or control. Cells were transduced with adenovirus 24 hr before plating (time 0 hr), treated with PDGF (20ng/mL) and counted at indicated times. Data were calculated as fold change relative to the cells counts at 0 hr (n=6 independent experiments). B) Boyden chamber migration assays in VSMC transfected with control siRNA or siRNA against MCU treated in serum-free media (SF) or with 20ng/mL PDGF for 6 hr (n=5 independent experiments). C) Representative immunoblot from one representative experiment of total MCU for knockdown of MCU with siRNA. Three independent experiments were performed.

Supplemental Figure III



Supplemental Figure III. High throughput wound scratch assay and analysis in a 96-well plate format. A) Representative DPC and threshold images of an untreated well immediately after scratch (0 hr) and 24 hr later. B) High throughput scratch wound assay in VSMC with or without mtCaMKII overexpression in the presence of increasing concentrations of Ru360 for 24 hr, conducted in quadruplicate, (n=3 independent experiments). *p<0.05 versus SF, #p<0.05 versus control PDGF by 2-way ANOVA.

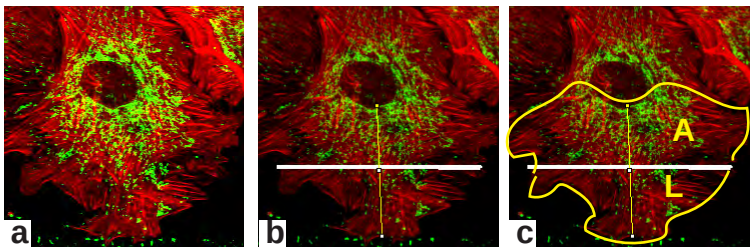
Supplemental Figure IV



Supplemental Figure IV. mtCaMKIIN reduces mitochondrial ROS. A) Oxygen Consumption Rate (OCR) using Seahorse in VSMC expressing control or mtCaMKIIN treated with vehicle or PDGF for 1 hr (n=3 independent experiments). B) Mitochondrial ROS production after PDGF treatment for 1 hr in VSMC overexpressing mtCaMKII or control using mitoSOX fluorescence (n=3 independent experiments). Quantification of mitoSOX mean fluorescent intensity in relative fluorescence units (RFU) normalized to MitoTracker RFU per cell. 50 cells were analyzed per condition. MitoTracker Green was used to visualize mitochondria. C) Mitochondrial ROS production after PDGF treatment for 1 hr in VSMC expressing mtCaMKIIN or control using mitoSOX fluorescence (n=4 independent experiments). D) Boyden chamber migration assays in VSMC expressing control, mtCaMKIIN or mtCaMKII and treated with mitoTEMPO or vehicle in serum-free media or after incubation with 20ng/mL PDGF for 6 hr (n=3 independent experiments). Scale bar = 20μm. For B and C, *$p<0.05$ vs. control vehicle, #$p<0.05$ vs control PDGF by 1-way ANOVA. For D, *$p<0.05$ vs. Serum Free, #$p<0.05$ vs PDGF by 2-way ANOVA

Supplemental Figure V

A



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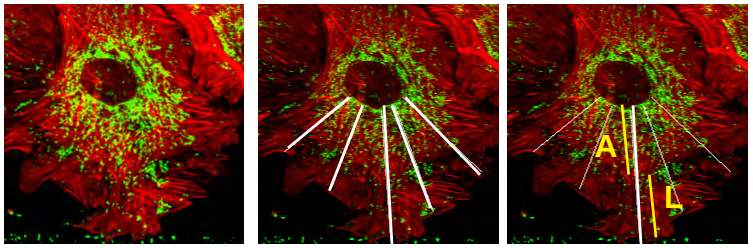
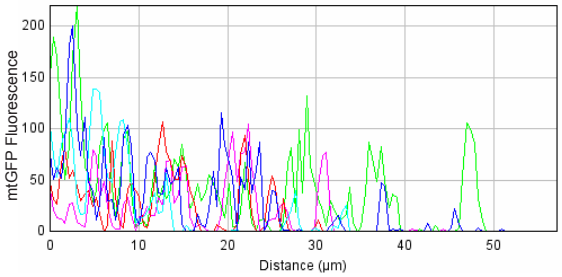


C

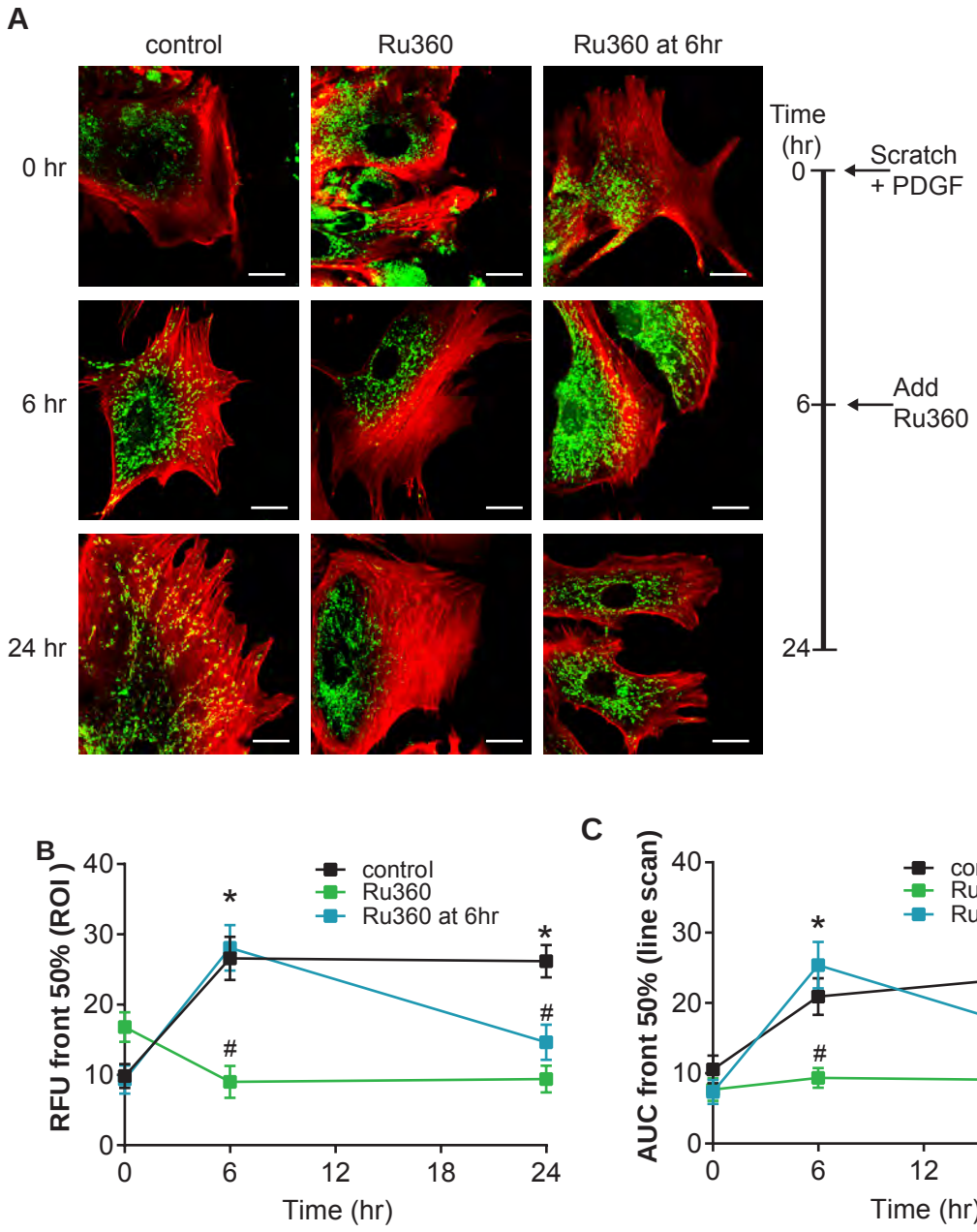


D

$$\text{\% Anterior mitochondrial Distribution} = \frac{L}{L+A}$$

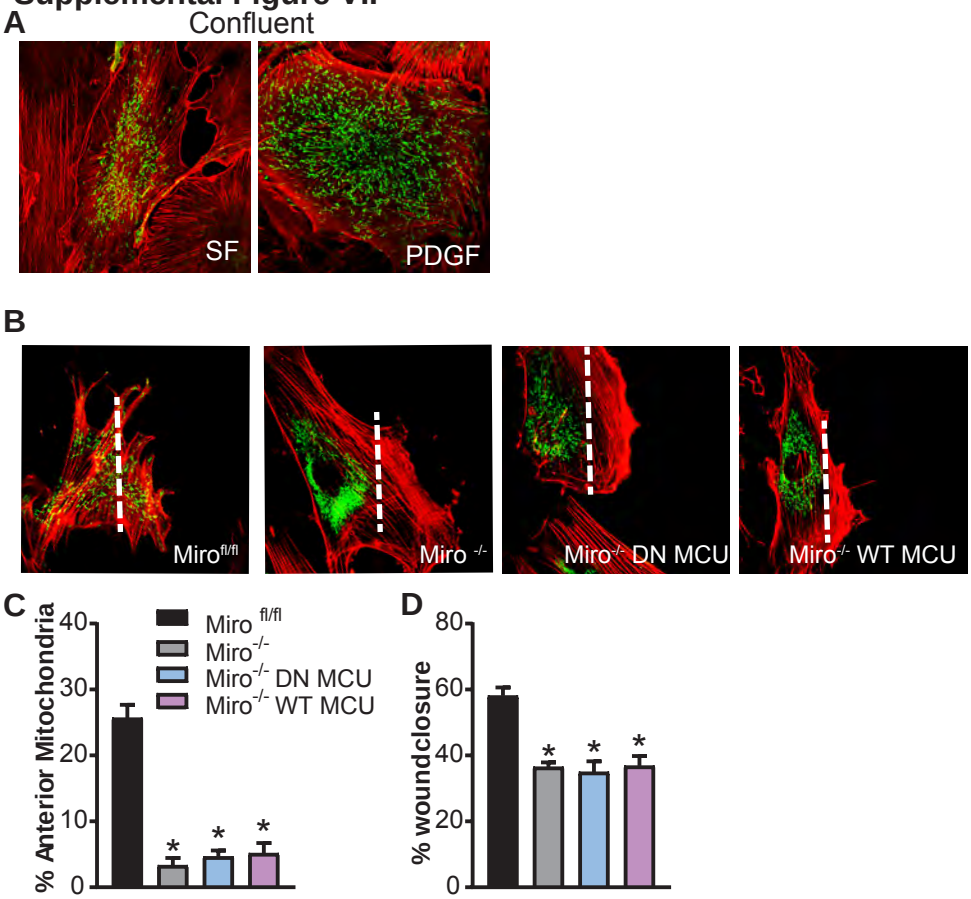
Supplemental Figure V. Methods for quantification of intracellular mitochondrial distribution. A) Mitochondrial distribution by area using methods adapted from Zhao, et al. a) Confocal immunofluorescent images for mitochondria (labeled with mtGFP) and F-actin (labeled with Phalloidin-568) were taken at 40X. b) Definition of the leading area in migrating VSMC. A line (yellow) was drawn from the center of the nucleus to the furthest edge of the migrating cell as labeled by F-actin staining. A second line (white) was placed orthogonal at the half-way point. c) The leading (L) and the anterior areas (A) were determined by tracing the cell border anterior to the center of the nucleus. B) Method to analyze mitochondrial distribution using AUC of mitochondrial signal intensity by line scan. a) Confocal immunofluorescent images for mtGFP and Phalloidin-568 were taken at 40x; note that this is the same image used in the first method. b) Five radially distributed lines (white) were drawn from the nucleus at equal distances to the leading edge of the cell. The lines were divided into two segments of equal size: the leading part (L), defined as the segment from the leading cell edge to the half-distance to the nucleus, and the anterior segment from the half-way point to the nucleus (A). C) Plot Profiler was used to determine the intensity of the mtGFP signal over the distance of the line. The AUC in each segment was determined in Prism 7.0. D) For both methods, the data were expressed as AUC of signal in L over AUC in L + A. This analysis was performed in at least 40 cells per condition.

Supplemental Figure VI



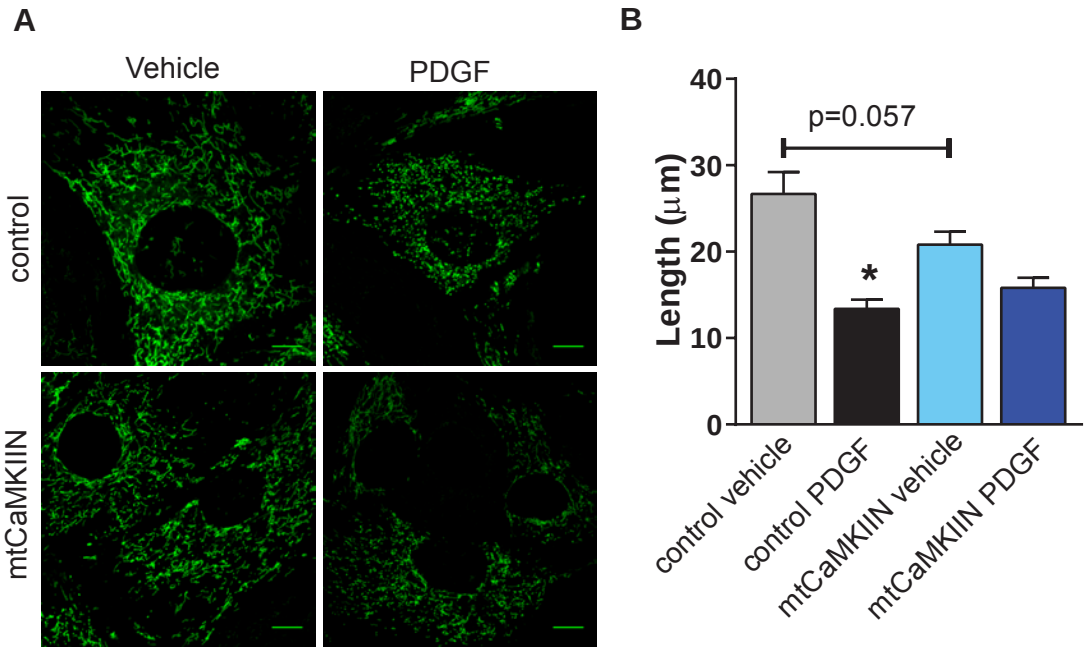
Supplemental Figure VI. Addition of Ru360 arrests mitochondrial localization to the leading edge in migrating VSMC. A) Representative images of mitochondrial localization (green: mtGFP; red: F-actin, Phalloidin-568) at indicated time points after scratch wound and PDGF treatment in VSMC treated with vehicle (left), Ru360 (1μM, center), or by addition of Ru360 after 6 hr of PDGF treatment (right). Quantification of mitochondrial distribution expressed as percent mtGFP signal in the leading cell area by ROI (B) and line scan (C) as defined in supplemental figure 5. *p<0.05 versus Time 0 hr, #p<0.05 versus control at same time point.

Supplemental Figure VII



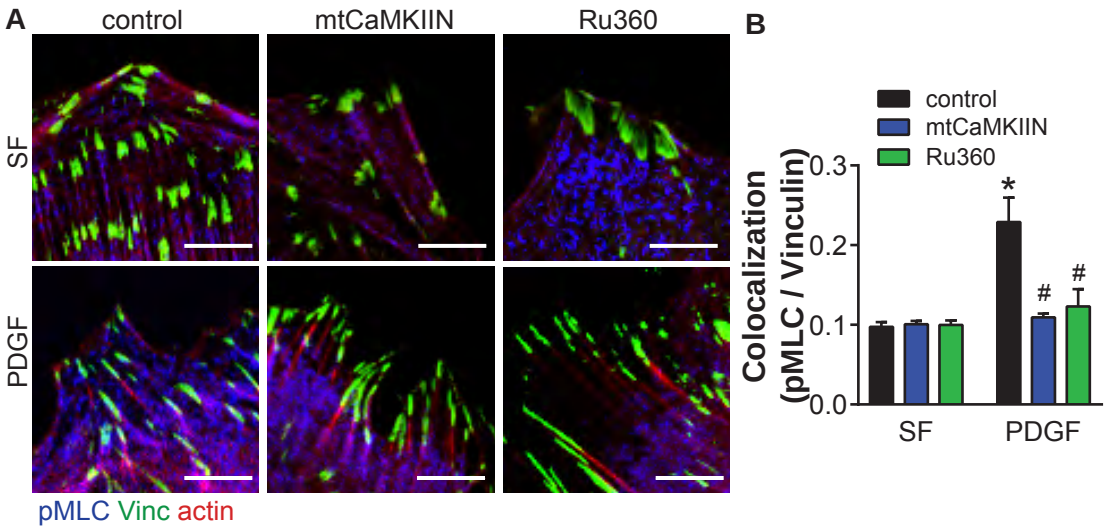
Supplemental Figure VII. Miro-1 is required for MCU-mediated active mitochondrial translocation in VSMC. A) Representative images from one experiment of mitochondrial localization in a confluent VSMC monolayer when serum starved (SF) or treated for 6 hr with PDGF, 30 cells were imaged over 3 independent experiments (green: mtGFP; red: F-actin, Phalloidin-568). B) Representative images from one experiment of mitochondrial localization in migrating VSMC during scratch wound assay in control (Miro$^{fl/fl}$) or Cre-infected Miro$^{fl/fl}$ (Miro$^{-/-}$) VSMC co-expressing dominant negative MCU (DN-MCU) or WT MCU. C) Quantification of mitochondrial distribution for (B) by line scan analysis in the leading to anterior cell areas expressed as percent mtGFP signal in the leading cell (30 cells over 3 independent experiments). D) VSMC migration quantified as percent wound closure at 24 hr as compared to 0 hr (n=3 independent experiments). *$p<0.05$ vs Miro$^{fl/fl}$ by 1-way ANOVA. Scale bar = 10 μm.

Supplemental Figure VIII



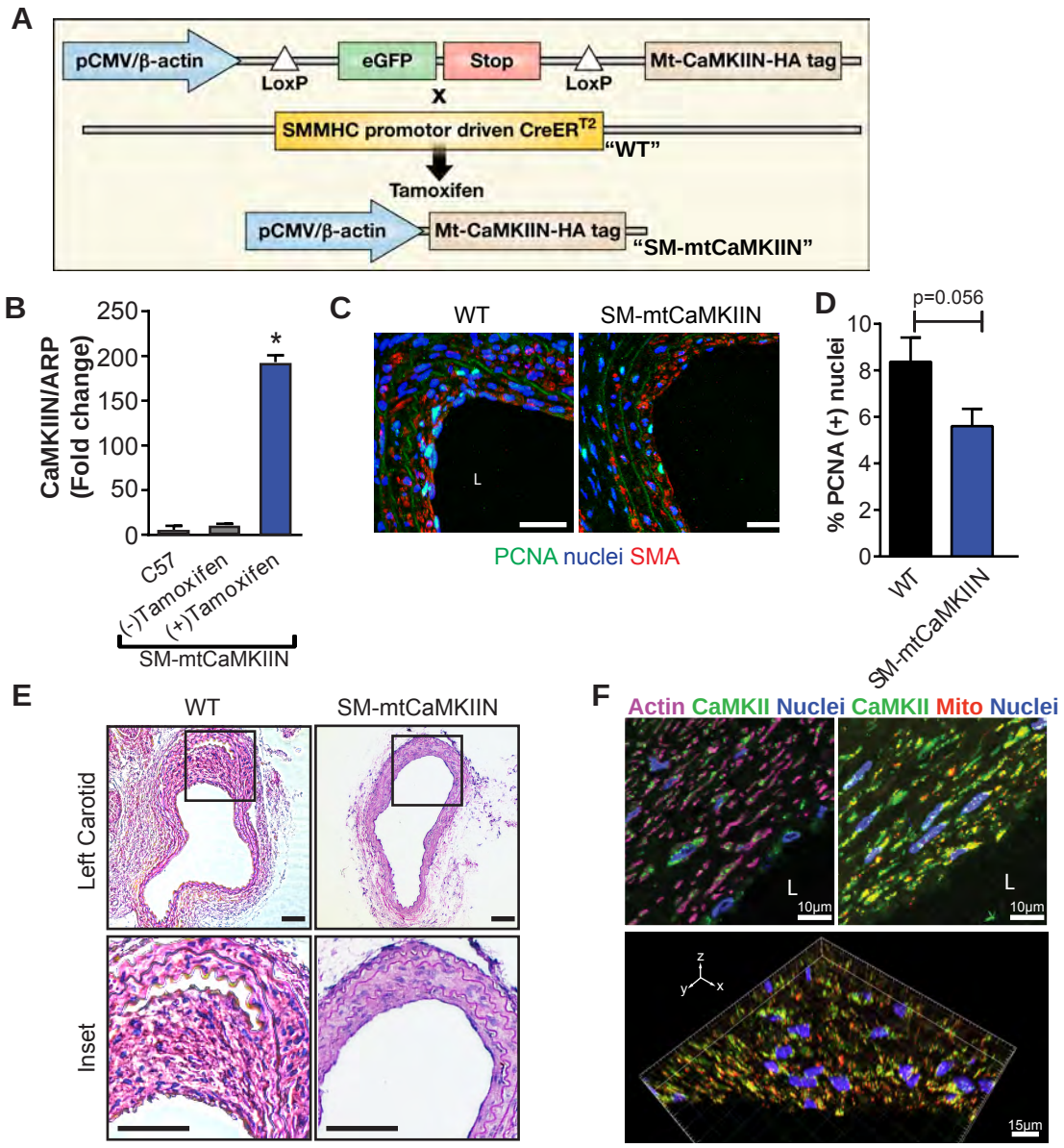
Supplemental Figure VIII. Effect of mtCaMKIIN on mitochondrial fission in VSMC.
A) Immunofluorescent images of mitochondria stained with mitoTracker Green in VSMC expressing mtCaMKIIN or control, before or after treatment with PDGF (20ng/mL) for 30 min. B) Mitochondrial length quantified using custom build ImageJ macro in 50 cells during three independent experiments, *p<0.05 versus control vehicle, scale bar = 10 μm.

Supplemental Figure IX



Supplemental Figure IX. MtCaMKIIN and Ru360 prevent phosphorylated (p)-MLC to colocalize with focal adhesions. A) Representative images for phosphorylated (p)-MLC at Ser19 (blue), Vinculin (green) and Phalloidin-568 (F-actin, red) at the leading edge after 6 hr of treatment with PDGF (20ng/mL) or SF media in VSMC expressing mtCaMKIIN or treated with RU360. B) Quantification of pMLC colocalization with FA at 6 hr using Cell Profiler software. n=3 independent experiments. * $p<0.05$ vs control SF, # $p<0.05$ vs control PDGF by 2-way ANOVA. Scale bar= 10 μm

Supplemental Figure X



Supplemental Figure X. Transgenic model to inhibit CaMKII selectively in mitochondria of VSMC. A) Schematic overview of generation of tamoxifen-inducible smooth muscle specific -mtCaMKIIN mice (termed "SM-mtCaMKIIN mice"). B) Quantitative rtPCR for CaMKIIN in aortas of C57Bl6/J mice and SM-mtCaMKIIN mice with and without cre recombination by tamoxifen injection. Aortas were harvested on day 14 after the first tamoxifen injection. Data were expressed as the fold change relative to C57Bl6/J mice. (n=3 per genotype); * $p < 0.05$ vs. (-) Tamoxifen by Kruskall-Wallis. C) One representative image per genotype of PCNA immunostaining of carotid arteries 28 days after injury. Nuclei: DAPI (blue); smooth muscle actin (red). PCNA (green), scale bar = 20 μm. D) Quantification of PCNA-positive nuclei in the intima of injured carotid arteries as percent of total number of nuclei; (n=8 per genotype). E) One representative image per genotype of Hematoxylin-Eosin staining of left common carotid arteries at 28 days after endothelial injury in WT or SM-mtCaMKIIN mice; scale bar= 50 μm. F) Representative image of staining for CaMKII (green), smooth muscle actin (magenta) and mitochondria (anti-MTC02, red) with 3D projection in human coronary arteries from autopsy samples (nuclei TO-PRO-3, blue). Coronary autopsy samples from four individuals without a history of or gross pathology consistent with coronary artery disease were imaged.