Supplement Material

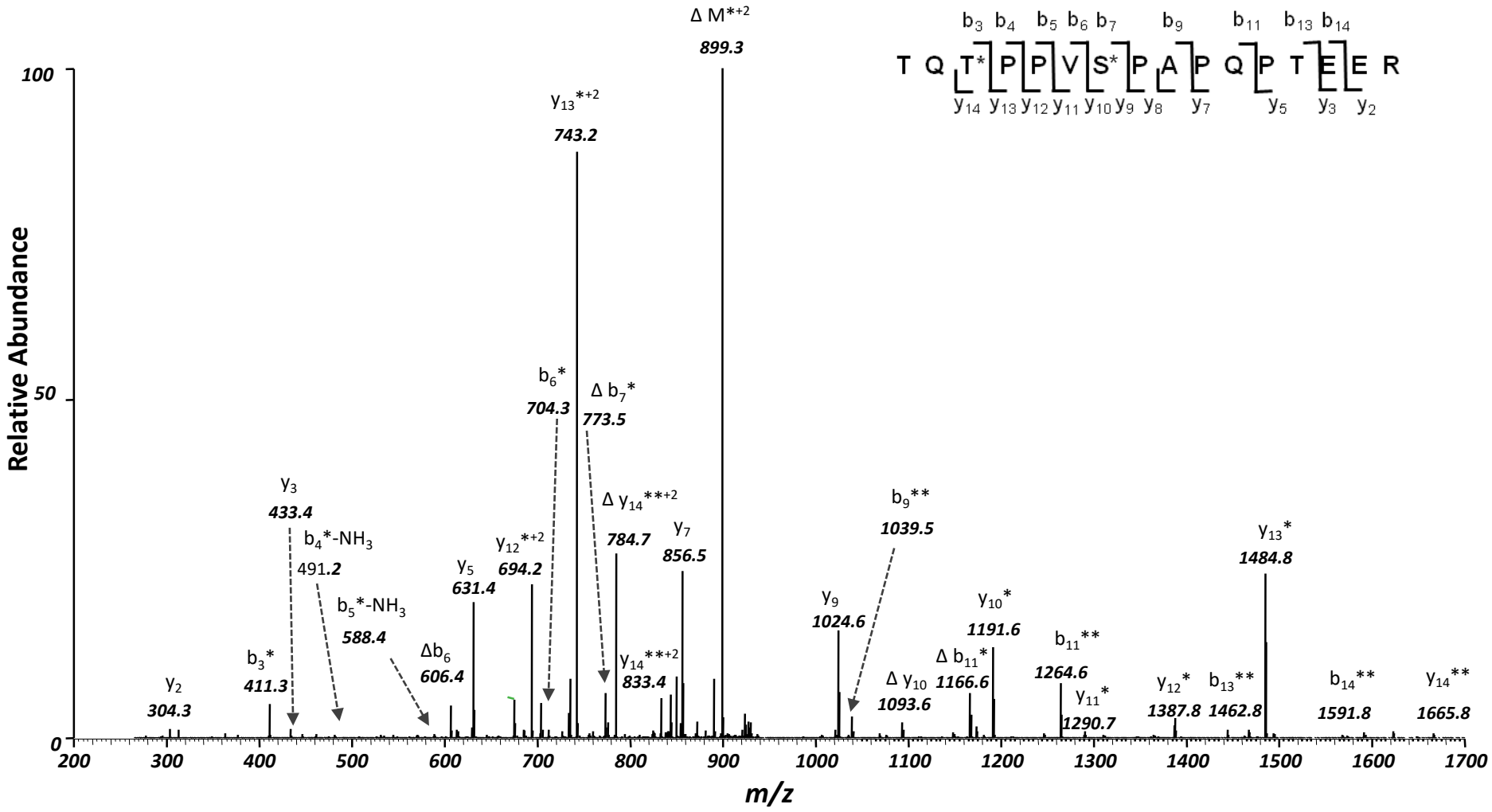
AMPK and SIRT1 Coregulation of Cortactin Contributes to Endothelial Function

Supplementary Figure I-IX
Supplementary Table I-III

Supplemental Figure I

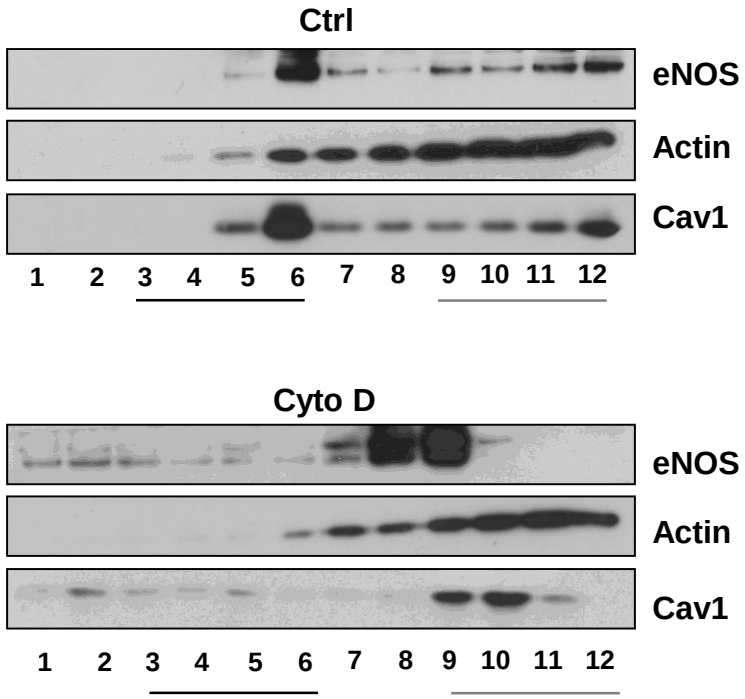
Supplemental Figure I. Conserved phosphorylation/acetylation site among species. Phosphorylation sites at Ser-348, Thr-401 and Ser-432 are conserved among mice, rat and human. Acetylation and phosphorylation sites are marked in bold red.

Supplemental Figure II



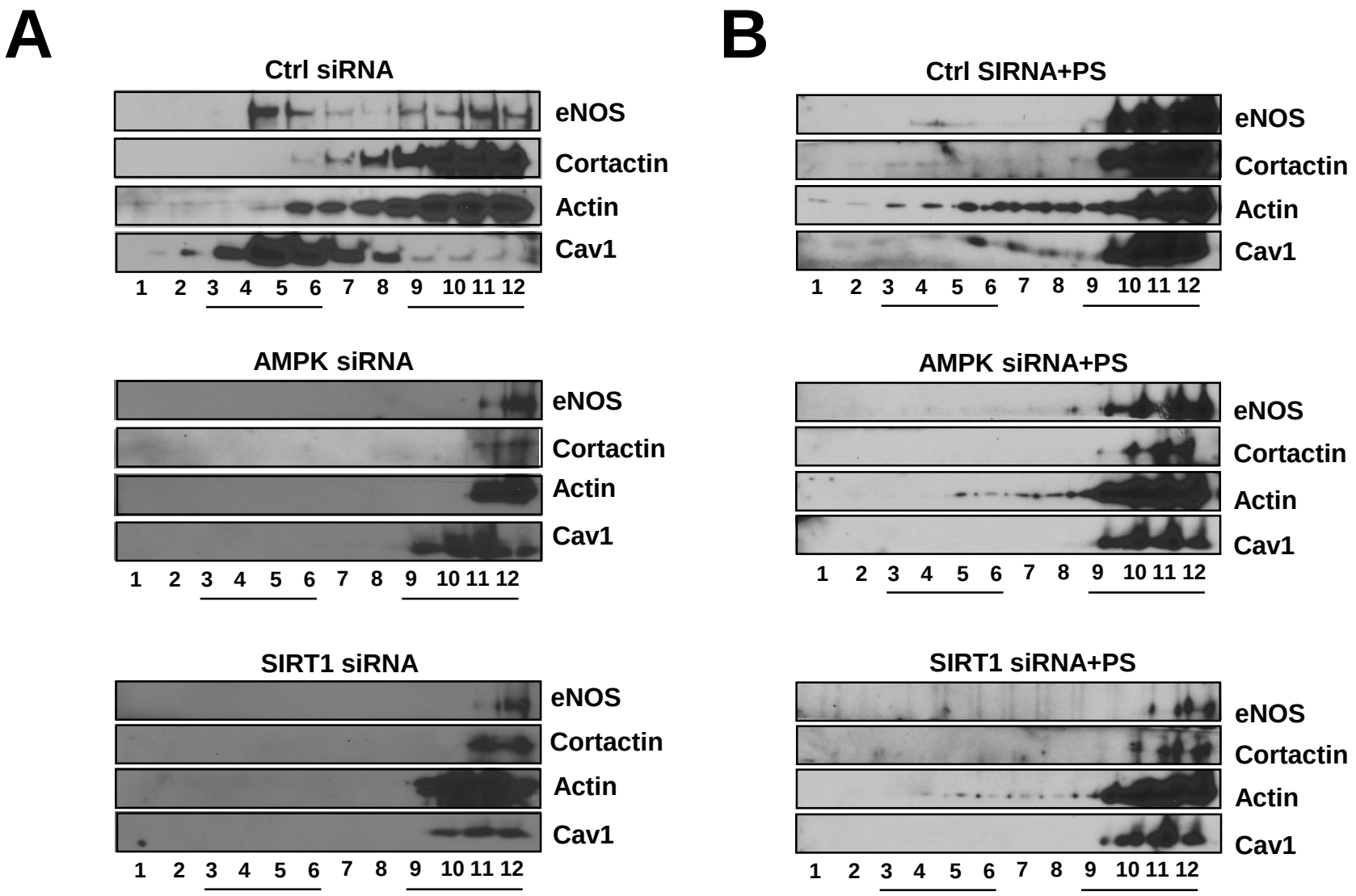
Supplemental Figure II. Identification of pT401 by nano-LC-MS/MS tandem mass spectrometry. Human umbilical vein endothelial cells (HUVECs) were treated with AICAR and cortactin was immunoprecipitated for tandem mass spectrometry. MS/MS of phosphorylated cortactin tryptic peptides corresponding to residues 399-414 (TQTPPVSPAPQPTEER) obtained from the immunprepicated mixtures and analyzed by LC-MS/MS. * indicates that an ion bears a phosphate group, and Δ indicates neutral loss of an H_3PO_4.

Supplemental Figure III



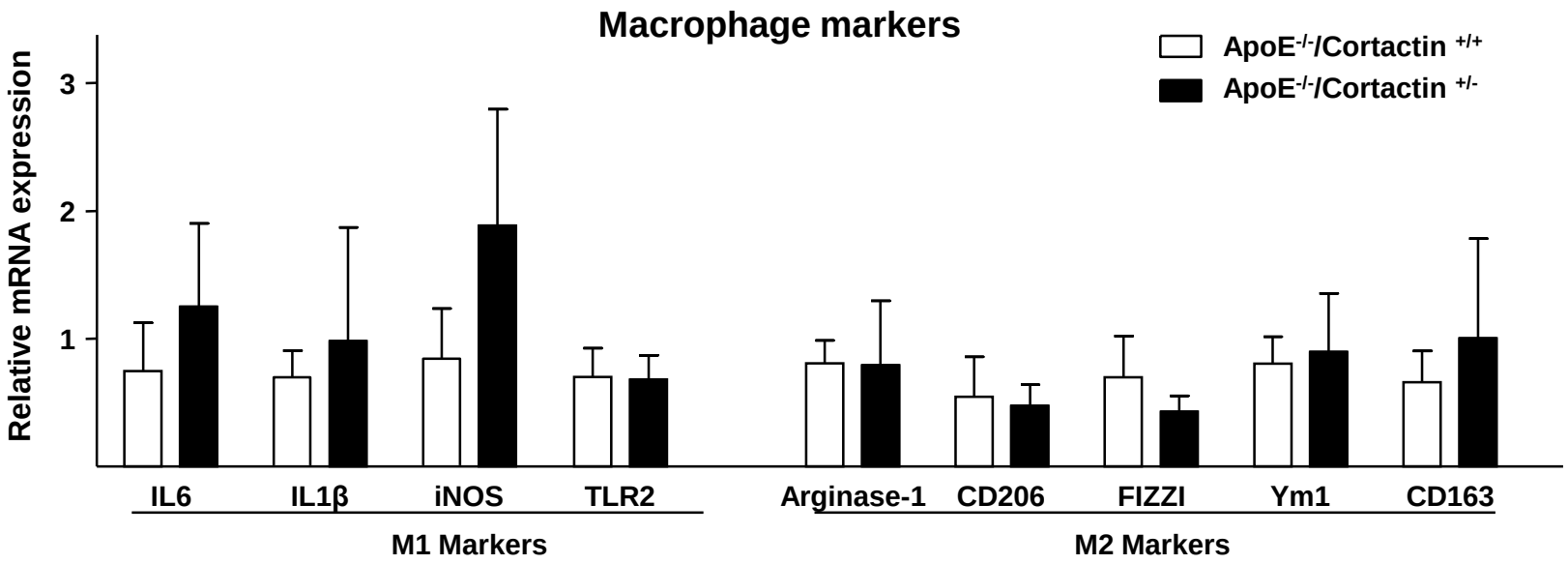
Supplemental Figure III. Lipid raft distribution under cytochalasin D treatment.
Cytochalasin D (cytoD) treatment caused a shift of eNOS, Cav1 and actin from lipid raft fractions to non-lipid raft fractions.

Supplemental Figure IV



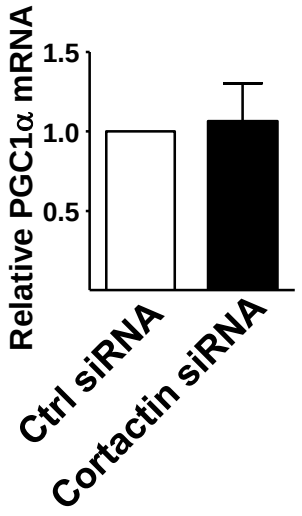
Supplemental Figure IV. AMPK or SIRT1 knockdown prohibited molecular trafficking. (A) HUVECs were transfected with scramble control, AMPK or SIRT1 siRNA and then kept under static conditions or (B) subjected to PS. Cells were then fractionized by sucrose gradient ultracentrifugation to observe the distribution of eNOS, cortactin, actin and Cav1 in the lipid raft fraction (3-6) or non-lipid raft fractions (9-12). The distribution of cortactin, actin, eNOS, and Cav-1 was revealed by immunoblotting with antibodies against cortactin, actin, eNOS, and Cav-1.

Supplemental Figure V



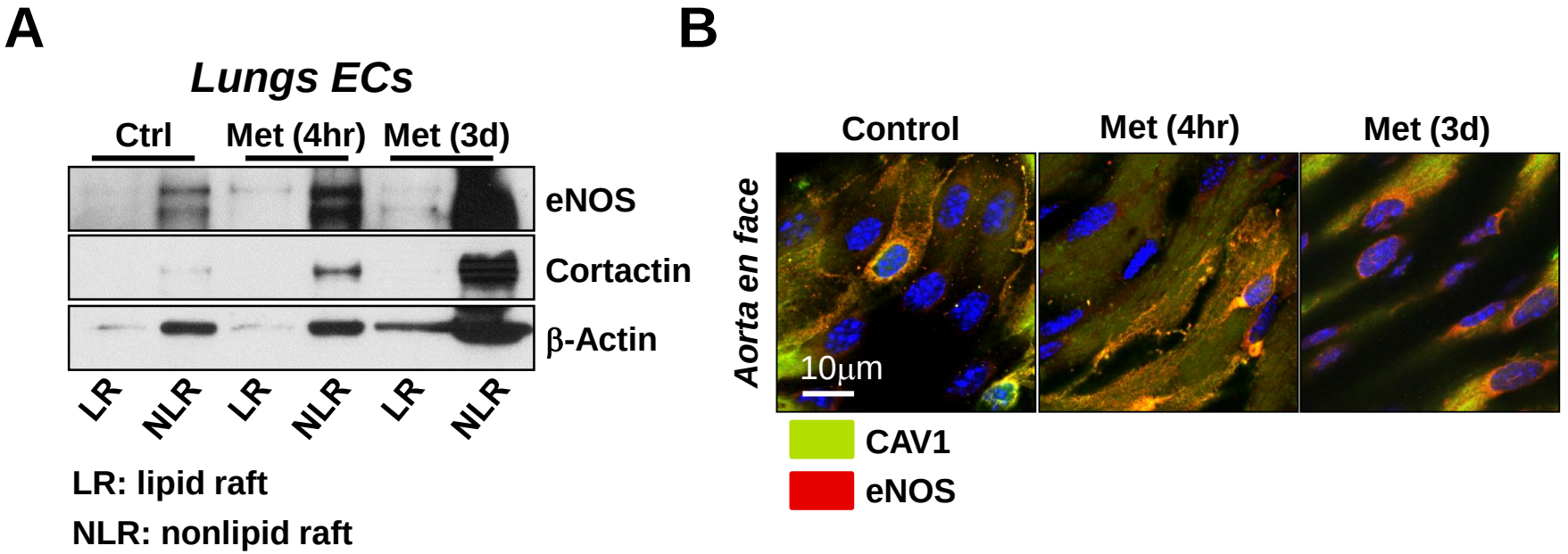
Supplemental Figure V. Comparable expression of M1 and M2 markers between ApoE$^{-/-}$/cortactin$^{+/-}$ mice and their wild-type littermates. Total mRNA was extracted from murine peritoneal macrophages elicited by thioglycollate for 3 days. The mRNA levels of M1 macrophage markers IL-6, IL1b, iNOS and TLR-2 vs M2 macrophage markers Arginase-1, CD206, FIZZI, Ym1 and CD163 in ApoE$^{-/-}$/cortactin$^{+/+}$ and ApoE$^{-/-}$/cortactin$^{+/-}$ mice. Data are mean ± SEM from 4 mice in each group.

Supplemental Figure VI



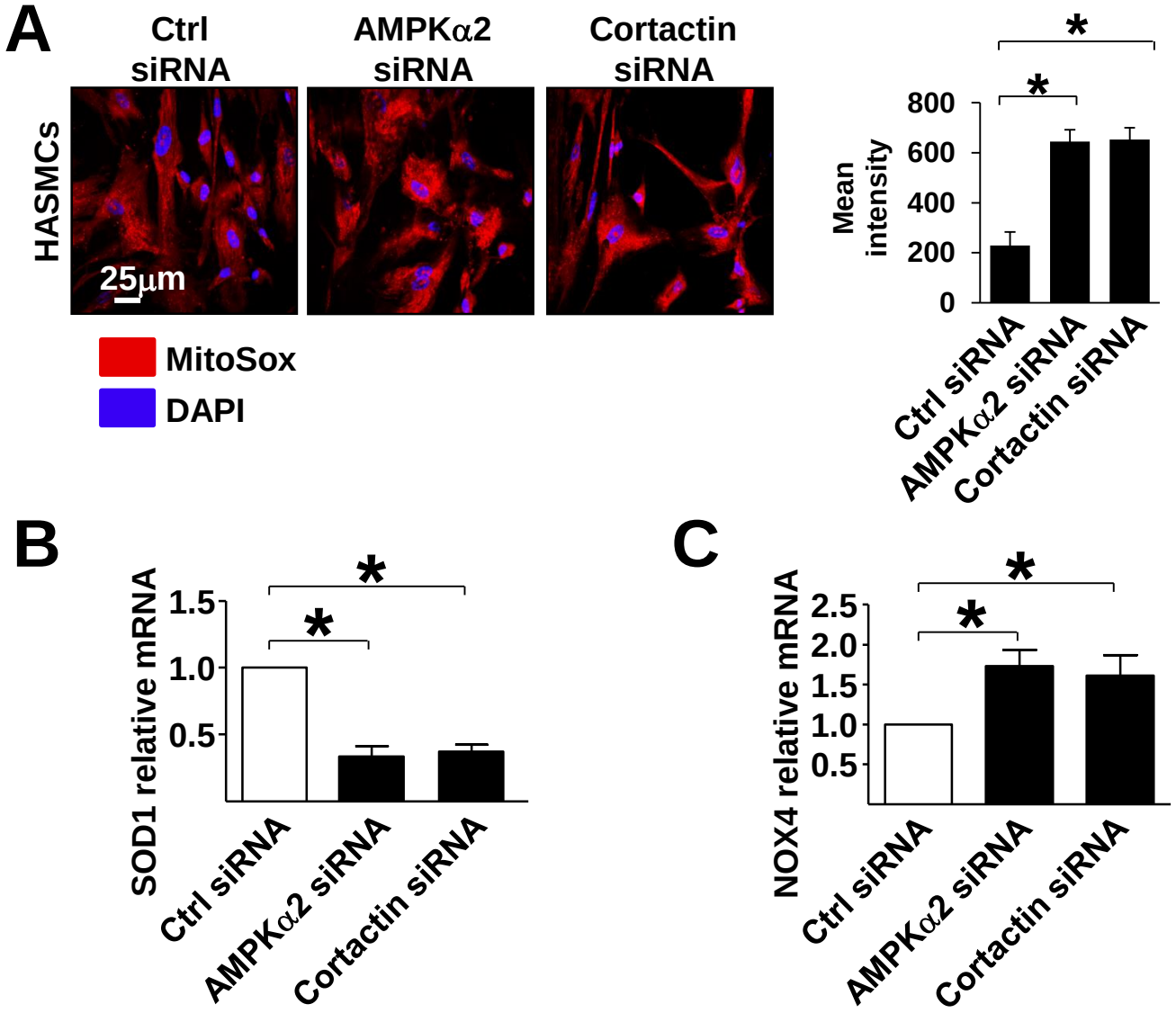
Supplemental Figure VI. PGC1α expression in cortactin knockdown. HUVECs were transfected with control or cortactin siRNA and the PGC1α mRNA level was assessed by RT-PCR. The results are mean±SEM from 3 independent experiments.

Supplemental Figure VII



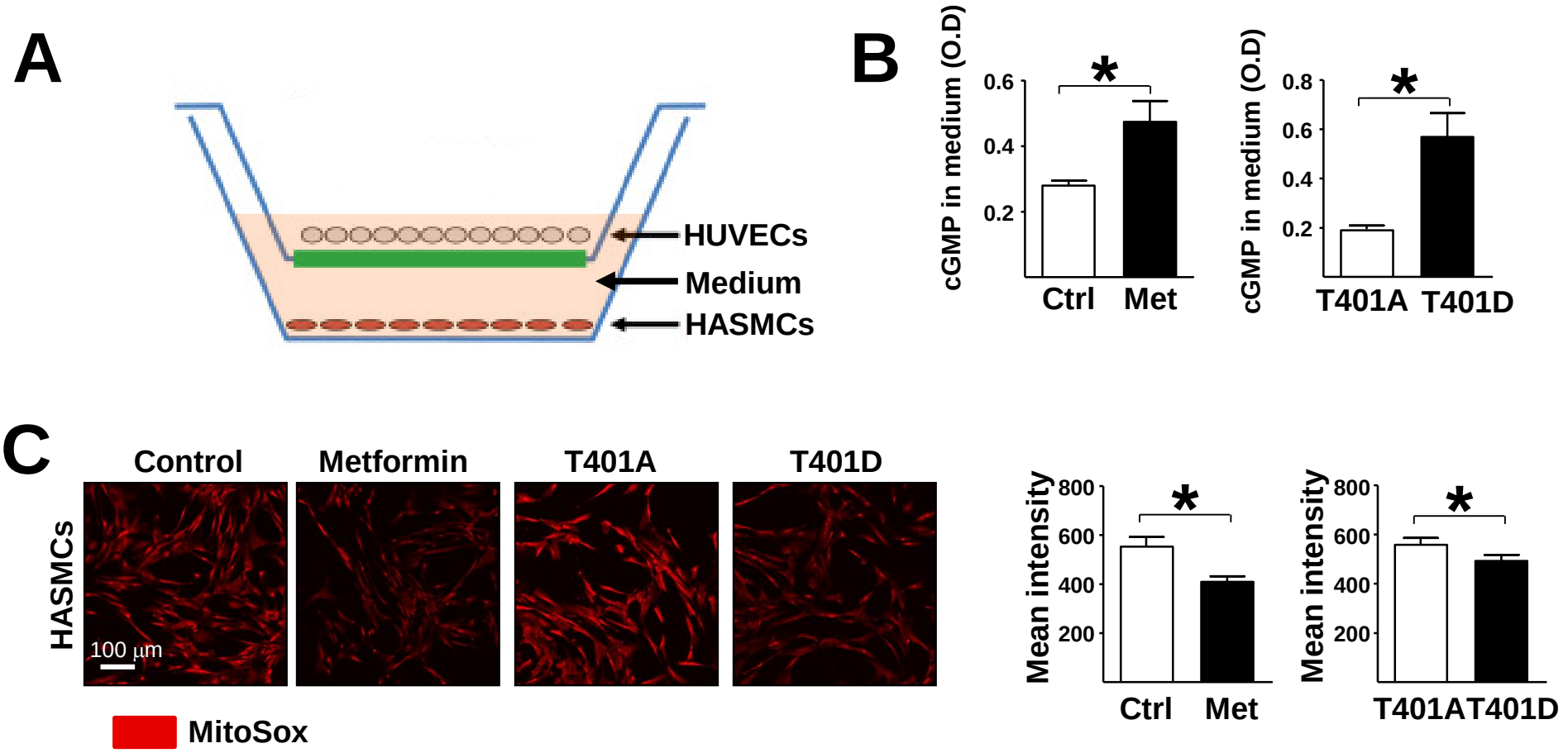
Supplemental Fig. VII. Metformin increases cortacin and eNOS translocation into nonlipid raft in mouse endothelium. C57BL/6 mice were administered metformin (200 mg/kg) via i.p. injection. Mice (n=3 per condition) were then sacrificed following 4 hr (acute) or 3 days (chronic). **(A)** Lung ECs were isolated and protein was separated into lipid raft and nonlipid raft fractions. Immunoblot was performed with the use of anti-eNOS, anti-cortactin, and anti-β-actin. (**B**) *En face* immunostaining of aortic endothelium with the use of anti-CAV1 and anti-eNOS, nuclei are stained blue with DAPI (n=3 mice each condition, 5-8 images for each mouse).

Supplemental Figure VIII



Supplemental Figure VIII. AMPK/SIRT1-cortactin signaling regulates human aortic smooth muscle cell (HASMC) redox state. HASMCs were transfected with control siRNA, AMPKα2, or cortactin siRNA. (**A**) Representative images of MitoSox staining revealing ROS status in HASMCs in which AMPKα2 or contactin was knocked down. (**B, C**) Total mRNA was extracted and the mRNA levels of SOD1 and NOX4 and compared with those from cells transfected with control siRNA. The results in (A) are representative from 3 independent experiments and in (B, C) are mean±SEM from 3 independent experiments.

Supplemental Figure IX



Supplemental Figure IX. Cortactin in ECs positively regulates HASMC phenotype. (**A**) Depiction of a HUVEC-HASMC co-culture system. Meformin treatment for 4 hr or HUVECs were transfected with cortactin T401A/D mutants (48 hr) were used to stimulate the AMPK/SIRT1-cortactin pathway. (**B**) cGMP level was measured from collected condition media with a kit from Cayman chemical. (**C**) Representative images of MitoSox staining revealing ROS status of HASMCs co-cultured with HUVECs treated with metformin or transfected with cortactin T401A/D.

Table I: Values of cortactin pThr-401 main sequence ions

pThr-401 main sequence ions	***m/z***
b ion	
b1	-
b2	230.1
b3	411.1
b4	508.2
b5	605.2
b6	704.3
b7	791.3
b8	888.4
b9	959.4
b10	1056.5
b11	1184.5
b12	1281.6
b13	1382.6
b14	1511.7
b15	1640.7
y ions	
y1	88.1
y2	152.6
y3	217.1
y4	267.6
y5	316.2
y6	380.2
y7	428.7
y8	464.2
y9	512.8
y10	556.3
y11	605.8
y12	654.3
y13	702.9
y14	793.4
y15	857.4

Low energy collision induced dissociation was used for fragmentation to generate b and y ions. *m/z* , mass to charge ratio.

Table II: Serum lipid profile of $CTTN^{+/+}/ApoE^{-/-}$ and their $CTTN^{+/-}/ApoE^{-/-}$ littermates

Serum lipids	$CTTN^{+/+}$, $ApoE^{-/-}$, mg/dL (n = 15)	$CTTN^{+/-}$, $ApoE^{-/-}$, mg/dL (n = 11)
TC	2651±85	2567±104
TG	277±23	209±25
LDL-C	1926±82	1765±88
HDL-C†	669±116	760±78
VLDL-V*	55±5	42±5

All values are expressed as means ± SEM averaged from indicated number animals in each group. HDL-C, HDL–cholesterol; LDL-C, LDL–cholesterol; TC, total cholesterol; TG, triglyceride; VLDL-C, very-low-density lipoprotein–cholesterol. *The TG value is divided by 5 to estimate VLDL-C levels. † The HDL-C value was determined by the following formula: HDL-C = TC − LDL-C – (TG/5).

Table III：Sequences of primers used in mRNA RT-qPCR

Gene	**Species**	**Sequence**	
E-selectin	Mouse	Forward	CCAATCTGAAACATTCACCGAGT
		Reverse	CGAGTCTTTGGTTCGTTGGATG
MCP-1	Mouse	Forward	TTAAAAACCTGGATCGGAACCAA
		Reverse	GCATTAGCTTCAGATTTACGGGT
VCAM-1	Mouse	Forward	AGTTGGGGATTCGGTTGTTCT
		Reverse	CCCCTCATTCCTTACCACCC
ICAM-1	Mouse	Forward	GCTACCATCACCGTGTATTCG
		Reverse	TAGCCAGCACCGTGAATGTG
IL6	Mouse	Forward	GGCGGATCGGATGTTGTGAT
		Reverse	GGACCCCAGACAATCGGTTG
IL1β	Mouse	Forward	GCAACTGTTCCTGAACTCAACT
		Reverse	ATCTTTTGGGGTCCGTCAACT
iNOS	Mouse	Forward	GTTCTCAGCCCAACAATACAAGA
		Reverse	GTGGACGGGTCGATGTCAC
TLR2	Mouse	Forward	GCAAACGCTGTTCTGCTCAG
		Reverse	AGGCGTCTCCCTCTATTGTATT
Arginase-1	Mouse	Forward	CTCCAAGCCAAAGTCCTTAGAG
		Reverse	AGGAGCTGTCATTAGGGACATC
CD206	Mouse	Forward	CTCTGTTCAGCTATTGGACGC
		Reverse	CGGAATTTCTGGGATTCAGCTTC
FIZZI	Mouse	Forward	CCAATCCAGCTAACTATCCCTCC
		Reverse	ACCCAGTAGCAGTCATCCCA
Ym1	Mouse	Forward	CAGGTCTGGCAATTCTTCTGAA
		Reverse	GTCTTGCTCATGTGTGTAAGTGA
CD163	Mouse	Forward	ATGGGTGGACACAGAATGGTT
		Reverse	CAGGAGCGTTAGTGACAGCAG