

SUPPLEMENTAL MATERIAL

Cholesterol-Induced Phenotypic Modulation of Smooth Muscle Cells to Macrophage/Fibroblast-like Cells is Driven by an Unfolded Protein Response

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SUPPLEMENTAL METHODS

SMCs explant and culture conditions and cholesterol exposure

Mouse aortic SMCs were explanted from the ascending aortas of WT C57BL/6J (The Jackson Laboratory, Bar Harbor, ME) mice as described earlier and maintained in smooth muscle basal medium (Promo Cell) supplemented with 10% fetal bovine serum (FBS, Gibco), insulin, epidermal growth factor, fibroblast growth factor (Promo Cell), HEPES (Millipore Sigma), sodium pyruvate (Millipore Sigma), L-glutamine (Millipore Sigma), and antibiotic/anti-mycotic (Millipore Sigma).³⁴ Aortas from male and female mice were pooled to obtain enough viable cells for maintenance and studies were performed using this pooled population. This has been a standard practice in our laboratory. The SMCs were immortalized by human telomerase reverse transcriptase (hTERT) overexpression using conditioned medium containing retrovirus from *pBabe.hTERTpuro-pA317* packaging cell line. Infected cells were selected using 0.5 μ g/mL puromycin for one week, then switched back to regular SMC maintenance medium.

Free cholesterol complexed to methyl- β -cyclodextrin (MBD-Chol), containing ~40mg cholesterol per gram of product, was purchased from Millipore Sigma and dissolved in deionized water. All calculations for cholesterol treatment were based on the weight of cholesterol in the MBD-Chol complex. POVPC (Cayman Chemical) was obtained as a solution in ethanol and ethanol was used for the no-treatment controls. Human LDL (ThermoFisher Scientific) was aggregated by vortexing at the highest speed for 1 minute, centrifuging and removing the supernatant.³⁵ Immortalized SMCs were treated with 0 and 10 μ g/mL MBD-Chol (as described by Rong and colleagues) or 0 and 20 μ g/mL POVPC or 0 and 25 μ g/mL aggregated LDL in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose (Cellgro), 10% FBS (Gibco), 1% antibiotic/anti-mycotic (Millipore Sigma) and 0.2% bovine serum albumin (BSA, Fisher Scientific) for 72 hours at 37 $^{\circ}$ C and 5% CO₂.¹³

RNA extraction, quantitative real time PCR and immunoblot analyses

Total RNA was isolated from cultured immortalized SMCs, quantified by Nanodrop (Thermo Fisher Scientific), and used for cDNA synthesis using QScript reagent (Quantabio). mRNA expression for macrophage-specific and ER stress-specific genes was estimated using SYBR green chemistry (primers were obtained from Millipore Sigma and reaction master mix from Quantabio) and for SMC contractile genes using Taqman probes (Taqman probes were purchased from Applied Biosystems and qPCR master mix obtained from Takara Biosciences). Reactions were performed in triplicate and *18S rRNA* and *Gapdh* were used as endogenous controls for SYBR and Taqman reactions respectively.

For protein level analysis, cultured SMCs were lysed in cold lysis buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1% Triton X-100 and 1mM EDTA) supplemented with protease inhibitor cocktail (Millipore Sigma) and phosphatase inhibitor cocktails (Millipore Sigma) for 15 minutes on ice and then sonicated on ice for 15 seconds. Crude lysates were cleared by centrifugation at top speed for 10 minutes at 4 $^{\circ}$ C. Protein amounts in the samples were quantified by Bradford assay (Bio-Rad Laboratories). 5-15 μ g total protein was used to resolve on 4-20% TGX gels (Bio-Rad Laboratories), transferred to PVDF membranes (Millipore Sigma), blocked with 5% dry milk (or 5% BSA for detecting phosphorylated proteins and Atf4) in Tris-buffered saline (TBS), containing 0.05% Tween-20 (TBST) and probed with different antibodies. Bands were visualized by chemiluminescent substrate (GE Healthcare). Band intensities on the immunoblots were quantified using ImageJ software.

Klf4 transactivation assays

Klf4 transactivation assay was performed using a Cignal Reporter Plasmid (Qiagen) and the luciferase activity was measured using a Dual Luciferase Reporter Assay Kit (Promega) according to manufacturer's instructions. SMCs (25000) were plated in triplicates on wells of 24-well plates and transfected with 250ng Klf4 Cignal Reporter Plasmid (Qiagen) using Lipofectamine reagent (Life Technologies) for 4 hours, following which the cells were treated with or without cholesterol, aggregated LDL or POVPC in combination with the various drugs for 72 hours, or for 5 and 8 hours when treated with tunicamycin and thapsigargin respectively. Following incubation, the cells were subjected to luciferase assay using a Dual Luciferase Reporter Assay Kit (Promega) according to manufacturer's instructions. Klf4 transcriptional activity was quantified as the ratio between the luminescences from firefly luciferase and Renilla luciferase.

Analysis of phagocytosis:

SMCs (10^4 cells) were plated on coverslips and then treated with indicated compounds as described. Forty-eight hours later, Fluoresbrite™ Plain YG 1 Micron Microspheres (Polysciences) were added to the media and the cells were incubated for 24 hours. The plasma membrane was stained with CytoPainter Cell Plasma Membrane Staining Solution (Abcam) protected from light and fixed with 4% paraformaldehyde solution. The cover slips were then mounted on glass slides using Vectashield with DAPI (Vector Laboratories) imaged using a Leica DMi8 SPE confocal microscope. At least five images were obtained for each treatment and the number of green dots within the cellular boundaries was counted in each of the acquired images in a blinded fashion. The average number of dots per cell was calculated by dividing the number of dots in each image by the number of nuclei in the image. The images shown in the figures are representative of a set of at least five images taken at random fields.

Apoptosis assay

Effects of cholesterol and various UPR inhibitor drugs on SMC apoptosis were determined by flow cytometry, using a GFP-CERTIFIED® Apoptosis/Necrosis Detection Kit (Enzo Life Sciences), as per manufacturer's instructions. Briefly, SMCs (10^4 cells) were plated on wells of 6-well plates and allowed to attach overnight. The cells were then treated with $10\mu\text{g}/\text{mL}$ MBD-Chol and indicated amounts of various UPR inhibitor drugs for 72 hours. The cells were washed with PBS, trypsinized, incubated in binding buffer containing apoptosis detection reagent for 5 minutes and then immediately subjected to flow cytometry, using filters for annexin V.

Evaluation of foam cell formation:

SMCs (10^3 cells) were plated on coverslips and allowed to attach overnight, and then treated with cholesterol, aggregated LDL or POVPC with or without various drugs for 72 hours. The cells were then washed with PBS and fixed with 4% paraformaldehyde solution for 30 minutes and washed three times with PBS again. The cells were then incubated with Oil Red O working solution for 4 hours, washed with PBS three times, counterstained with hematoxylin for 90 seconds and finally washed with PBS. The coverslips were mounted on glass slides using Permount (ThermoFisher Scientific) and imaged using light microscopy.

Cholesteryl ester formation assay

Cholesteryl ester formation was quantified using Cholesterol/Cholesteryl Ester Quantitation Assay kit (Colorimetric/Fluorometric) from Abcam (ab65359), as per manufacturer's

instructions. Briefly, cells were treated with or without cholesterol, aggregated LDL or POVPC in the presence or absence of 70nmol/L U18666A for 72 hours, following which they were washed with cold PBS. Lipids were extracted from the cells by resuspending the cell pellets in 200 μ L chloroform:isopropanol:NP-40 (7:11:0.1) and centrifuging at 15000g for 10 minutes. The organic phase was collected in fresh tubes and air dried at 50 $^{\circ}$ C to remove the chloroform. The resultant dried lipids were dissolved in 200 μ L of Assay Buffer. Reaction wells were set up in 96-well plates, with 50 μ L cholesterol standard dilutions or test samples for total and free cholesterol. Reaction mixes were prepared with or without cholesterol esterase enzyme to measure total and free cholesterol respectively and added to the appropriate wells containing the standards or test samples. The samples were mixed and incubated at 37 $^{\circ}$ C for 60 minutes, protected from light. Absorbances were measured at 570nm on a microplate reader and total and free cholesterol concentration in each well was calculated using the standard curve. The concentration of cholesteryl esters was the difference between the total cholesterol and the free cholesterol. The assays were performed three different times, using three independent biological replicates and all standards and samples were analyzed in duplicates in each assay.

SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

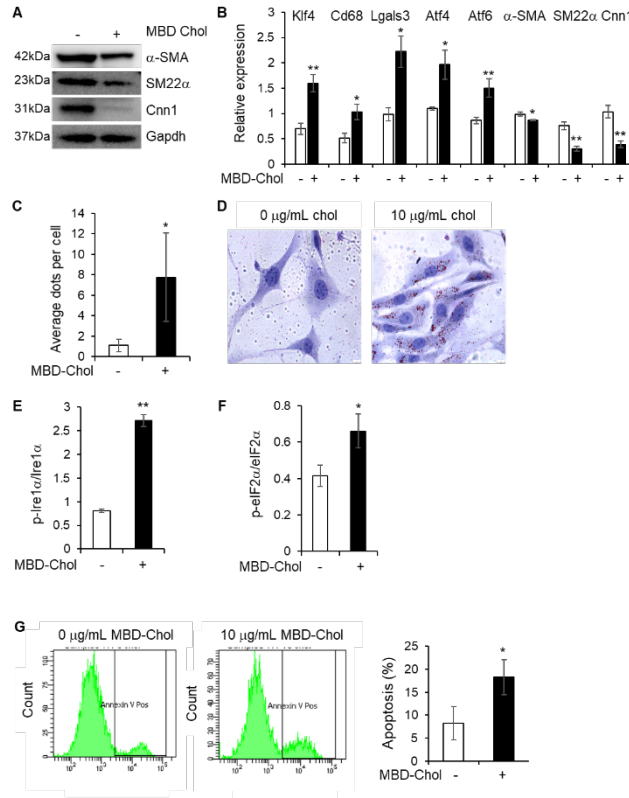


Figure I. Cholesterol induces phenotypic switching and ER stress in immortalized SMCs. (A) Immunoblot shows decreased contractile proteins upon treatment of cultured SMCs with 10 μ g/mL MBD-Chol for 72 hours. (B) Quantitation of immunoblots shows increased levels of macrophage markers and ER stress-related proteins with decreased levels of SMC contractile proteins with cholesterol treatment. (C) Quantification of fluorescent phagocytic beads taken up by cells shows increased phagocytic activity in immortalized SMCs after 72 hours treatment with 10 μ g/mL MBD-Chol. At least five images were obtained for each treatment and the number of green dots within the cellular boundaries was counted in each of the acquired images in a blinded fashion. The average number of dots per cell was calculated by dividing the number of dots in each image by the number of nuclei in the image. The images shown in the figures are representative of a set of at least five images taken at random fields (D) Oil Red O staining in immortalized SMCs treated with 10 μ g/mL MBD-Chol for 72 hours shows increased presence of lipid droplets stained in red, compared to untreated cells. (E,F) Quantification of phosphorylated Ire1 α (E) and phosphorylated eIF2 α (F) confirms increased phosphorylation of both Ire1 α and eIF2 α with cholesterol treatment. (G) Flow cytometry analysis shows increased apoptosis of SMCs on treatment with 10 μ g/mL MBD-Chol for 72 hours. For quantification of Western blots, the band intensities are normalized to that of Gapdh. For quantification of phosphorylation levels, the band intensities of the phosphorylated bands are normalized to the unphosphorylated bands. Each result displayed here is representative of at least three independent biological replicates. P-values were calculated using unpaired two-tailed Student's t-test. * indicates $p < 0.05$, ** - indicates $p < 0.01$ versus no cholesterol treatment.

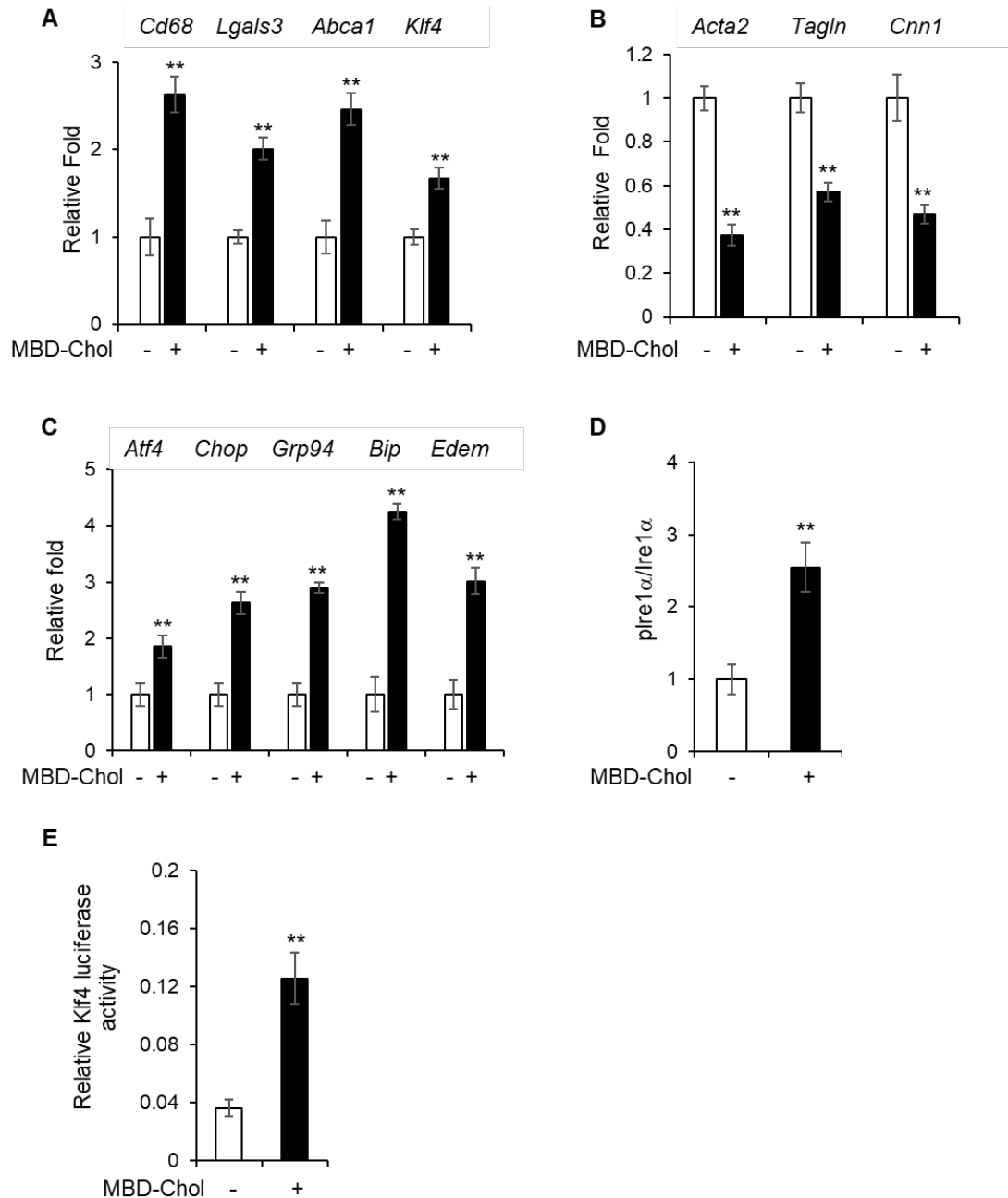


Figure II. Cholesterol induces phenotypic switching and ER stress in primary mouse SMCs. (A,B) qPCR shows increased *Cd68*, *Lgals3*, *Klf4*, and *Abca1* (A) and decreased contractile markers (B) upon treatment of cultured primary SMCs with 10 μ g/mL MBD-Chol for 72 hours. (C,D) 10 μ g/mL MBD-Chol for 72 hours also induces ER stress and activates all three arms of the UPR, as evident from the upregulation of the effectors *Atf4* and *Chop* and the chaperones *Bip*, *Grp94*, and *Edem* (C) and increased splicing of Xbp1 (D). (E) 10 μ g/mL MBD-Chol treatment for 72 hours also increases *Klf4* transcriptional activation in primary mouse SMCs. Each result displayed here is representative of at least three independent biological replicates. P-values were calculated using unpaired two-tailed Student's t-test. ** - indicates p < 0.01 versus no cholesterol treatment.

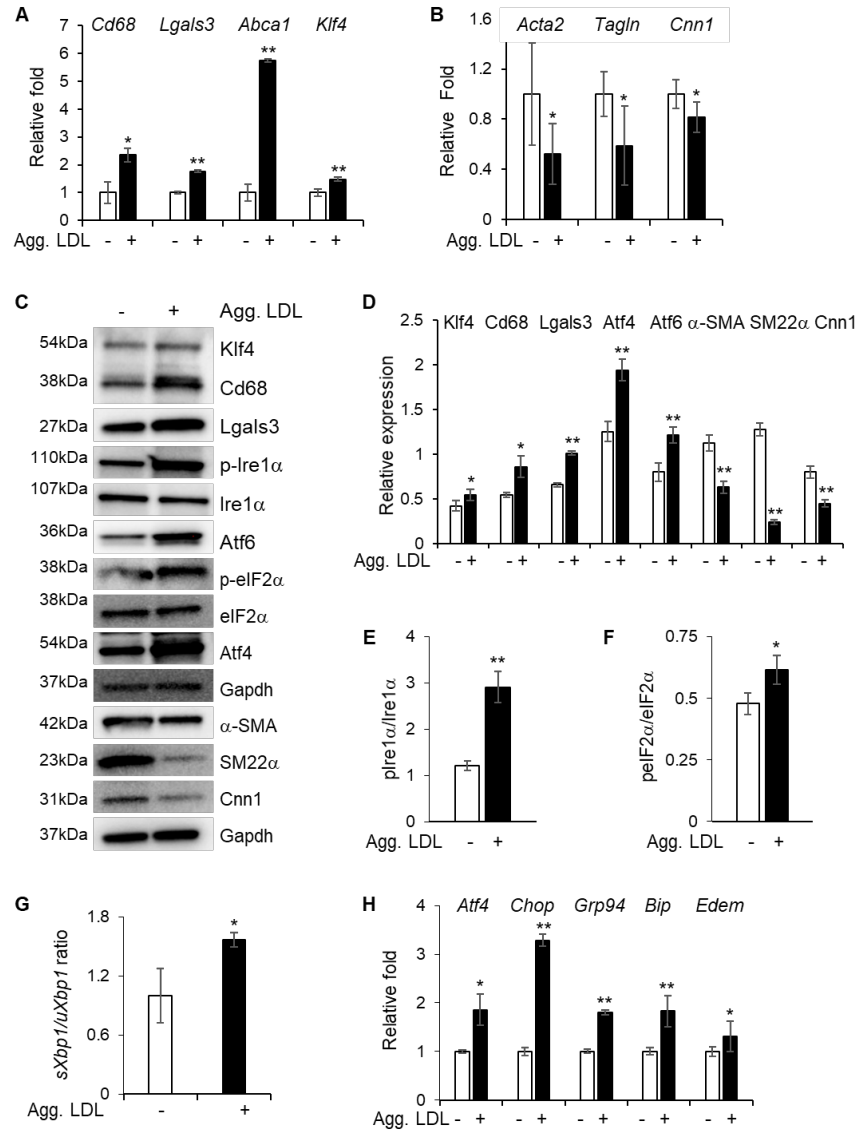


Figure III. Aggregated LDL induces phenotypic switching and ER stress in immortalized SMCs. (A,B) mRNAs for macrophage markers, *Klf4*, and *Abca1* are upregulated with exposure to 25 μ g/mL aggregated LDL for 72 hours (A) while contractile marker genes are downregulated (B). (C) Immunoblots confirm upregulation of macrophage markers, downregulation of contractile proteins and induction of ER stress including phosphorylation of Ire1 α and eIF2 α , induction of Atf4 and increased cleavage of Atf6. (D-F) Quantification of the immunoblots confirm increased phenotypic switching (D) and quantification of phosphorylation of Ire1 α (E) and eIF2 α (F) confirm activation of both with aggregated LDL treatment. (G) Aggregated LDL-induced activation of the Ire1 α pathway is confirmed by increased *Xbp1* splicing. (H) mRNA expression of the chaperones *Grp94*, *Bip*, and *Edem* and ER stress effectors *Atf4* and *Chop* are increased by 72 hours of aggregated LDL treatment. For quantification of Western blots, the band intensities are normalized to that of Gapdh. For quantification of phosphorylation levels, the band intensities of the phosphorylated bands are normalized to the unphosphorylated bands. Each result displayed here is representative of at least three independent biological replicates. P-values were calculated using unpaired two-tailed Student's t-test. * or ** - indicates $p < 0.05$ or $p < 0.01$ respectively, versus no aggregated LDL treatment.

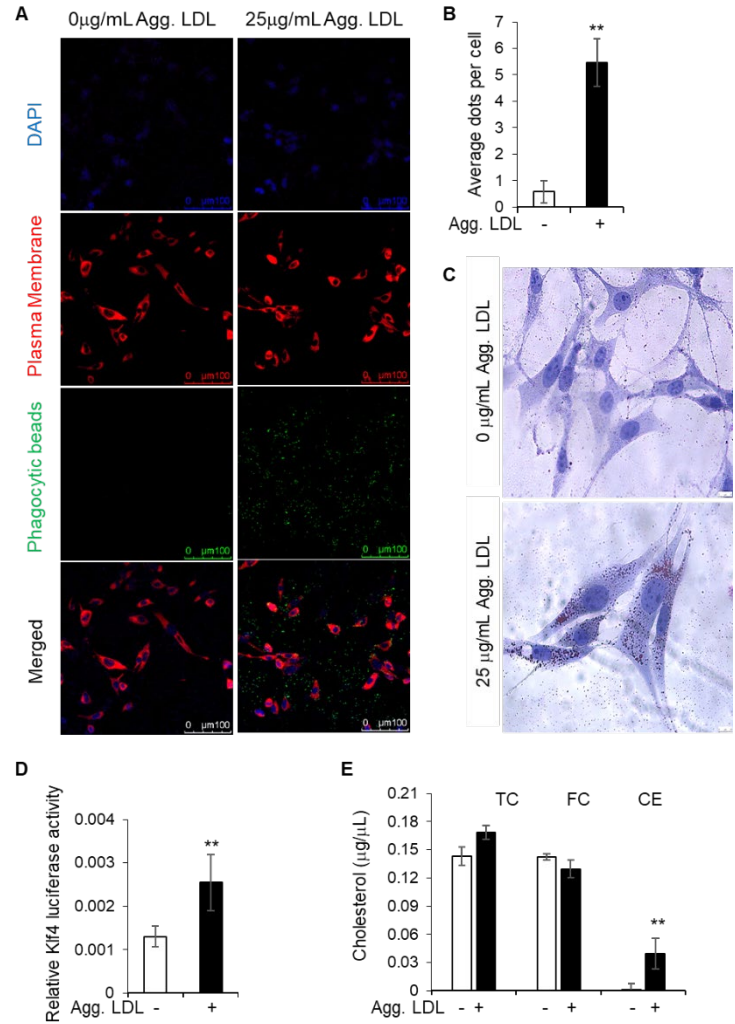


Figure IV. Aggregated LDL increases phagocytosis, foam cell formation, Klf4 activation, and cholesteryl ester formation in immortalized SMCs. (A,B) Increased uptake of green fluorescent phagocytic beads by immortalized mouse SMCs exposed to 25µg/mL aggregated LDL for 72 hours (A) is quantified in (B). At least five images were obtained for each treatment and the number of green dots within the cellular boundaries was counted in each of the acquired images in a blinded fashion. The average number of dots per cell was calculated by dividing the number of dots in each image by the number of nuclei in the image. The images shown in the figures are representative of a set of at least five images taken at random fields (C) Oil Red O staining in immortalized SMCs treated with 25µg/mL aggregated LDL for 72 hours shows increased presence of lipid droplets stained in red compared to untreated cells. (D) 25µg/mL aggregated LDL treatment for 72 hours also increases Klf4 transcriptional activation in immortalized mouse SMCs. (E) Exposure to 25µg/mL aggregated LDL for 72 hours increases cholesteryl ester formation. Each result displayed here is representative of at least three independent biological replicates. P-values were calculated using unpaired two-tailed Student's t-test. ** - indicates $p < 0.01$ versus no aggregated LDL treatment.

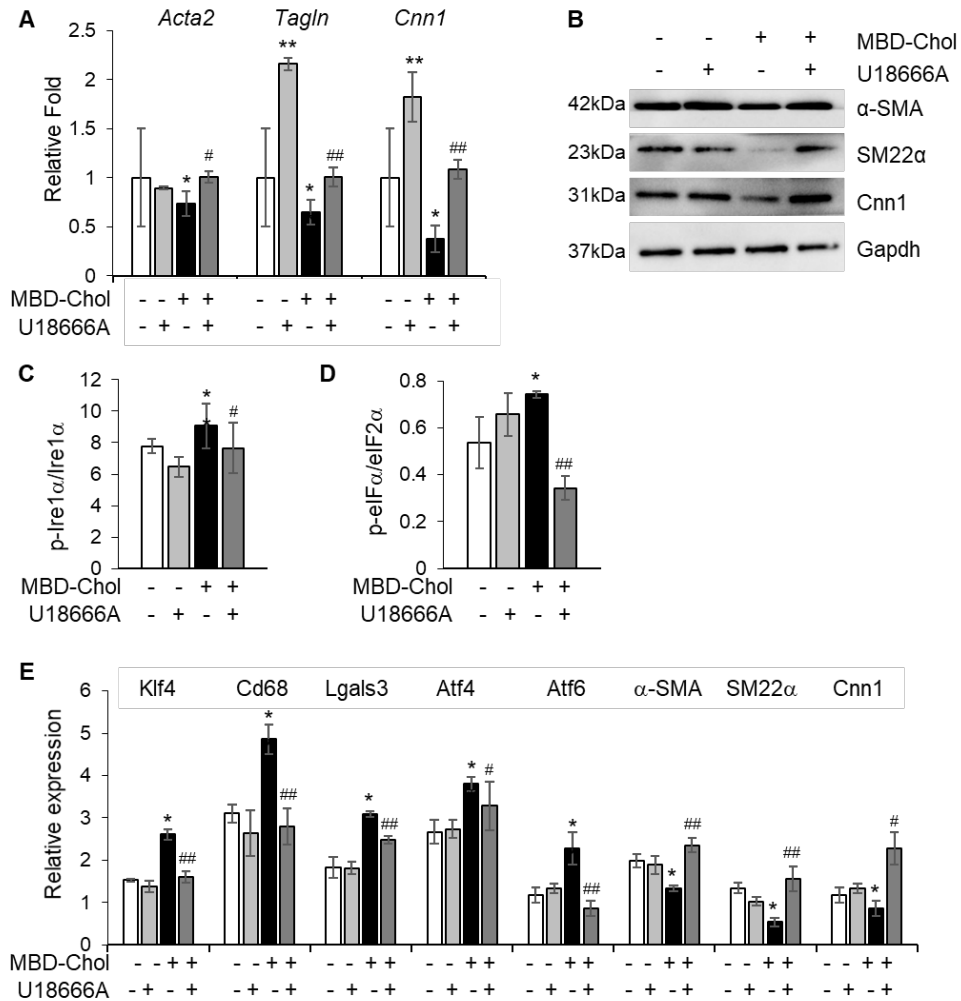


Figure V. Inhibition of cholesterol transport from the plasma membrane to the ER inhibits cholesterol-induced phenotypic switching and ER stress in SMCs. (A) q-RT-PCR data shows that cholesterol-induced decrease in contractile gene expression is rescued by U18886A when the cells are treated with 10mg/mL MBD-Chol with or without 70nmol/L U18666A. (B) Immunoblot analysis confirms decreased levels of SMC contractile proteins following cholesterol treatment are rescued by U18666A cotreatment. (C,D) Quantification of phosphorylated Ire1 α (C) and phosphorylated eIF2 α (D) also confirms decreased phosphorylation and activation of both Ire1 α and eIF2 α upon concurrent treatment with cholesterol and U18666A. (E) Quantification of immunoblots shows that U18666A rescues cholesterol-induced increases in macrophage marker proteins, decreases in SMC contractile proteins, and increases in ER stress-related proteins. For quantification of the blots, the band intensities are normalized to Gapdh; for Ire1 α and eIF2 α phosphorylation, the band intensities of the phosphorylated bands are normalized to the unphosphorylated bands. For quantification of Western blots, the band intensities are normalized to that of Gapdh. For quantification of phosphorylation levels, the band intensities of the phosphorylated bands are normalized to the unphosphorylated bands. Each result displayed here is representative of at least three independent biological replicates. P-values were calculated using two-way ANOVA followed by Tukey's Honest Significant Difference post-hoc t-test. * or ** - indicates p < 0.05 or p < 0.01 respectively, versus no cholesterol treatment. For the U18666A-treated samples, # or ## indicates p < 0.05 or p < 0.01 respectively for U18666A treatment versus DMSO.

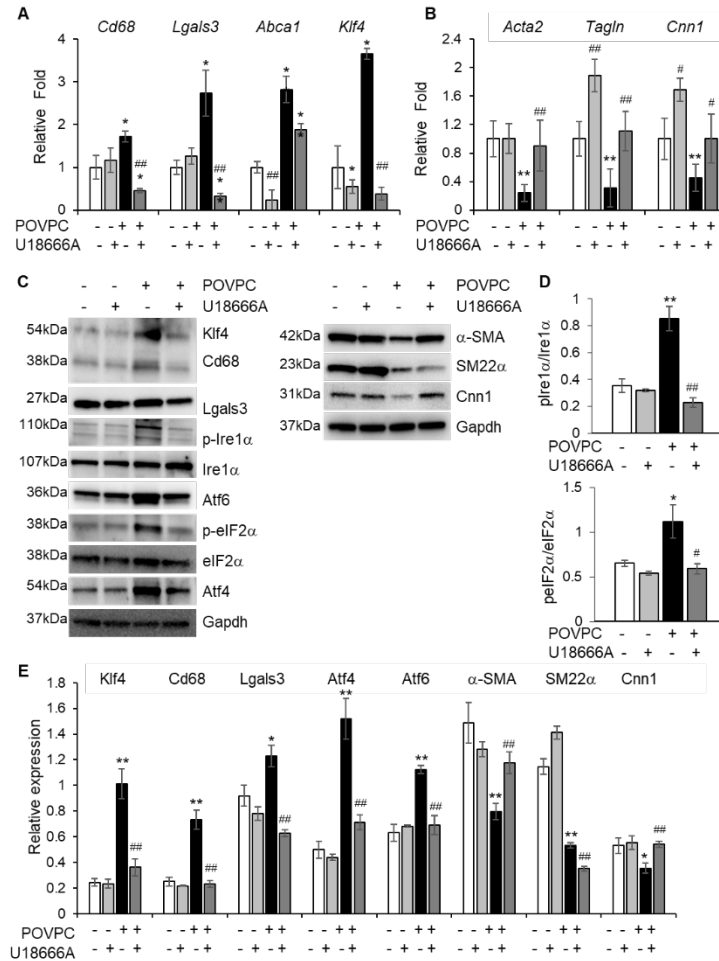


Figure VI. The oxidized phospholipid POVPC induces phenotypic switching and ER stress in immortalized SMCs and this effect can be reversed by blocking movement of cholesterol from the plasma membrane to the ER. (A,B) qRT-PCR data show that 20 μ g/mL POVPC for 72 hours induces macrophage markers *Cd68* and *Lgals3*, along with *Klf4* and *Abca1* (A) and decreases contractile gene expression (B), both of which are reversed by concurrent treatment with 70nmol/L U18666A. (C) Immunoblot analysis confirms increased levels of macrophage markers, decreased levels of SMC contractile proteins and upregulation of all three arms of UPR, following POVPC treatment, all of which are rescued by U18666A cotreatment. (D,E) Quantification of phosphorylated Ire1 α and phosphorylated eIF2 α also confirms decreased phosphorylation and activation of both Ire1 α and eIF2 α upon concurrent treatment with POVPC and U18666A. (E) Quantification of immunoblots shows that U18666A rescues POVPC-induced increases in macrophage marker proteins, decreases in SMC contractile proteins, and increases in ER stress-related proteins. For quantification of the blots, the band intensities are normalized to Gapdh; for Ire1 α and eIF2 α phosphorylation, the band intensities of the phosphorylated bands are normalized to the unphosphorylated bands. Each result displayed here is representative of at least three independent biological replicates. P-values were calculated using two-way ANOVA followed by Tukey's Honest Significant Difference post-hoc t-test. * or ** - indicates p < 0.05 or p < 0.01 respectively, versus no cholesterol treatment. For the U18666A-treated samples, # or ## indicates p < 0.05 or p < 0.01 respectively for U18666A treatment versus DMSO.

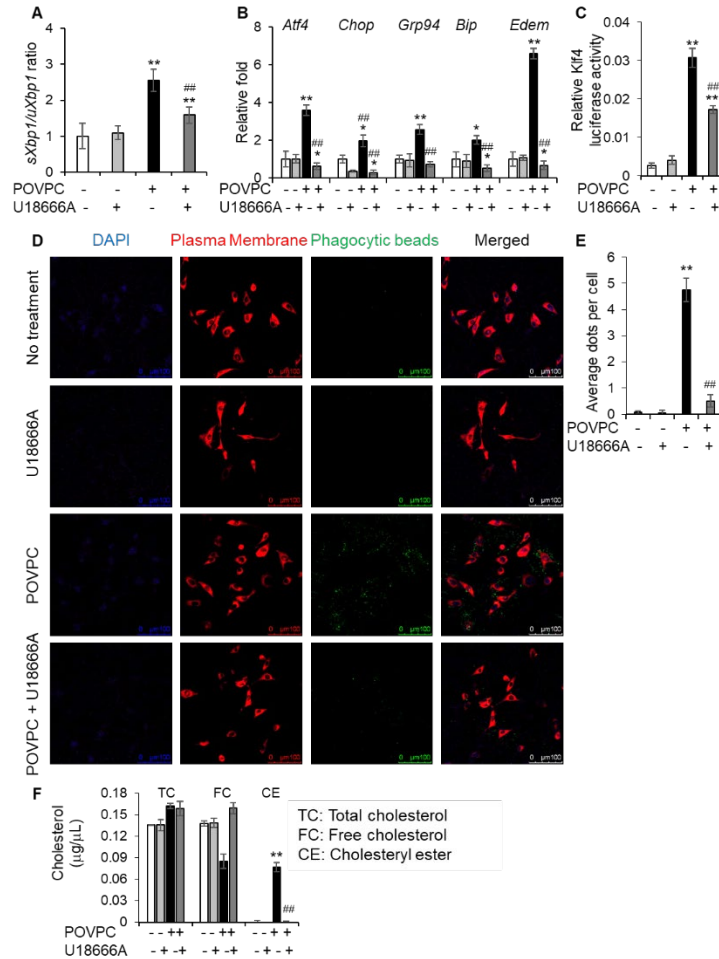


Figure VII. The oxidized phospholipid POVPC induces ER stress, increases phagocytic activity and cholesteryl ester formation in immortalized SMCs, all of which can be reversed by blocking movement of cholesterol from the plasma membrane to the ER. (A,B) qRT-PCR data show that 20 μ g/mL POVPC for 72 hours induces activation of all three arms of UPR – increased splicing of *Xbp1* (A) and expression of the ER stress effectors and ER-resident chaperones (B) are all reversed by concurrent treatment with U18666A. (C) POVPC treatment also increases Klf4 transcriptional activation, which again can be prevented by U18666A co-treatment. (D,E) 20 μ g/mL POVPC treatment for 72 hours increases uptake of green fluorescent phagocytic beads and this phagocytic activity is successfully reversed by U18666A (D), which is quantified in (E). At least five images were obtained for each treatment and the number of green dots within the cellular boundaries was counted in each of the acquired images in a blinded fashion. The average number of dots per cell was calculated by dividing the number of dots in each image by the number of nuclei in the image. The images shown in the figures are representative of a set of at least five images taken at random fields. Each result displayed here is representative of at least three independent biological replicates. P-values were calculated using two-way ANOVA followed by Tukey’s Honest Significant Difference post-hoc t-test. * or ** - indicates $p < 0.05$ or $p < 0.01$ respectively, versus no POVPC treatment (ethanol). For the U18666A-treated samples, # or ## indicates $p < 0.05$ or $p < 0.01$ respectively for U18666A treatment versus no treatment (DMSO).

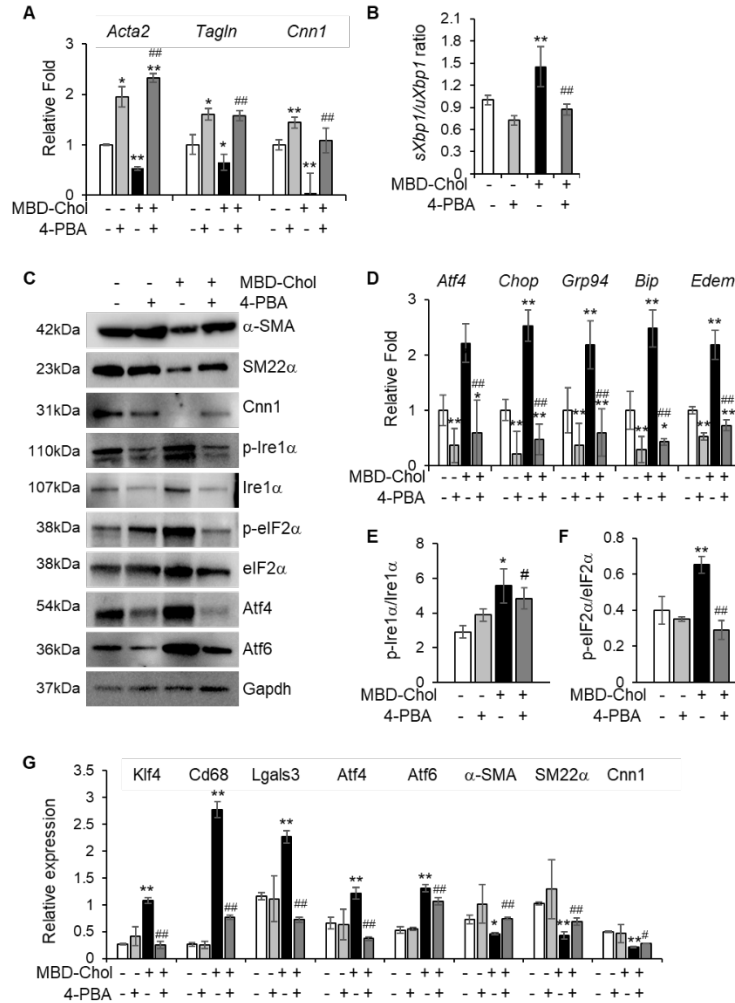


Figure VIII. Inhibition of ER stress abrogates phenotypic switching of SMCs. (A)

Cholesterol-induced downregulation of contractile genes is reduced to baseline upon concurrent exposure to 5mmol/L 4-PBA. **(B)** 4-PBA reduces cholesterol-induced splicing of *Xbp1*, a marker for *Ire1α* activation, as evident from a lower spliced *Xbp1* (*sXbp1*) to unspliced *Xbp1* (*uXbp1*) ratio. **(C)** Immunoblots demonstrate that exposure to 4-PBA also reduces cholesterol-induced increases in ER stress marker proteins and decreases in contractile proteins. **(D)** Exposure to 4-PBA also reduces cholesterol-induced increase in ER stress marker genes. **(E)** Quantification of phosphorylated *Ire1α* and, **(F)** phosphorylated *eIF2α* proteins also confirms decreased phosphorylation and activation of both *Ire1α* and *eIF2α* upon concurrent treatment with cholesterol and 4-PBA. **(G)** Quantification of macrophage, contractile, and ER-stress related proteins confirms 4-PBA treatment rescues the cholesterol-induced upregulation of macrophage markers and ER stress proteins and the downregulation of contractile marker proteins. For quantification of the blots, the band intensities are normalized to *Gapdh*; for *Ire1α* and *eIF2α* phosphorylation, the band intensities of the phosphorylated bands are normalized to the unphosphorylated bands. Each result displayed here is representative of at least three independent biological replicates. P-values were calculated using two-way ANOVA followed by Tukey's Honest Significant Difference post-hoc t-test. * or ** - indicates $p < 0.05$ or $p < 0.01$ respectively, versus no cholesterol treatment. For the 4-PBA-treated samples, # or ## indicates $p < 0.05$ or $p < 0.01$ respectively for 4-PBA treatment versus no treatment (DMSO).

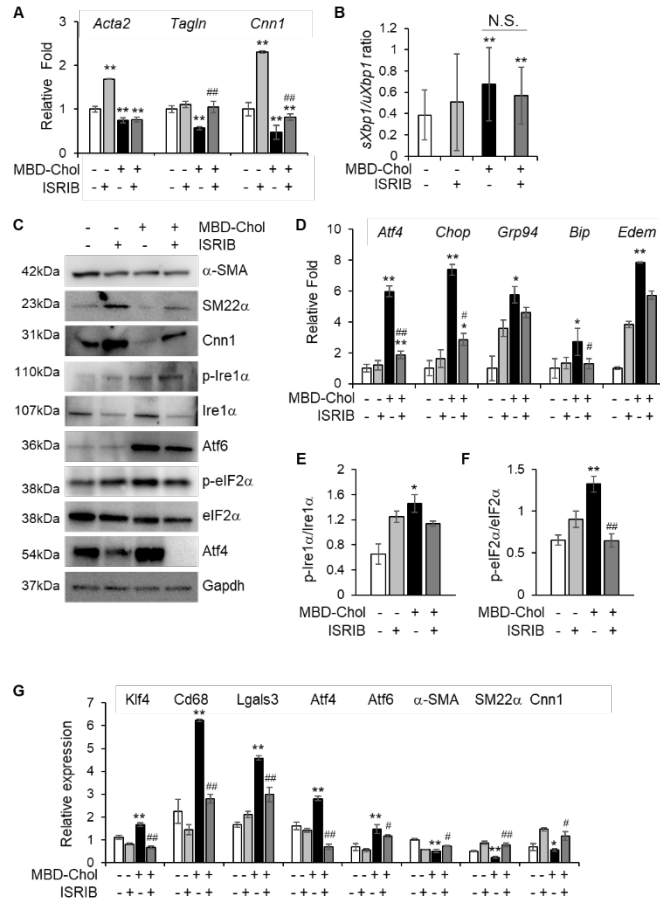


Figure IX. The Perk arm of UPR is responsible for phenotypic switching of SMCs. (A)

Downregulation of contractile genes following 10μg/mL MBD-Chol treatment is reduced to baseline upon concurrent exposure to 200nmol/L ISIRIB for 72 hours. (B) ISIRIB does not affect cholesterol-induced splicing of *Xbp1*, a marker for *Ire1α* activation, as there is no significant difference in spliced *Xbp1* (*sXbp1*) to unspliced *Xbp1* (*uXbp1*) ratio. (C) Immunoblots demonstrate that exposure to ISIRIB prevents cholesterol-induced decreases in contractile proteins. There is no effect on phospho-*Ire1α* or *Atf6*, but cholesterol-induced *Atf4* activation is abrogated with ISIRIB treatment. This indicates specificity of the drug to targeting the PERK arm of the UPR. (D) ISIRIB successfully reverses the upregulation of *Atf4* and its downstream target *Chop* as shown by quantitative RT-PCR. (E,F) Quantification of phosphorylated *Ire1α* (E) and phosphorylated *eIF2α* (F) demonstrates no significant changes in phosphorylation of both *Ire1α* and *eIF2α* upon concurrent treatment with cholesterol and ISIRIB. (G) Quantification of immunoblots confirms that ISIRIB prevents cholesterol-induced increases in macrophage-related proteins and decreases in contractile proteins. ISIRIB also inhibits the induction of *Atf4* but does not affect cleavage of *Atf6*. For quantification of the blots, the band intensities are normalized to *Gapdh*; for *Ire1α* and *eIF2α* phosphorylation, the band intensities of the phosphorylated bands are normalized to the unphosphorylated bands. Each result displayed here is representative of at least three independent biological replicates. P-values were calculated using two-way ANOVA followed by Tukey's Honest Significant Difference post-hoc test. * or ** - indicates p < 0.05 or p < 0.01 respectively, versus no cholesterol treatment. For the ISIRIB-treated samples, # or ## indicates p < 0.05 or p < 0.01 respectively for ISIRIB treatment versus no treatment (DMSO).

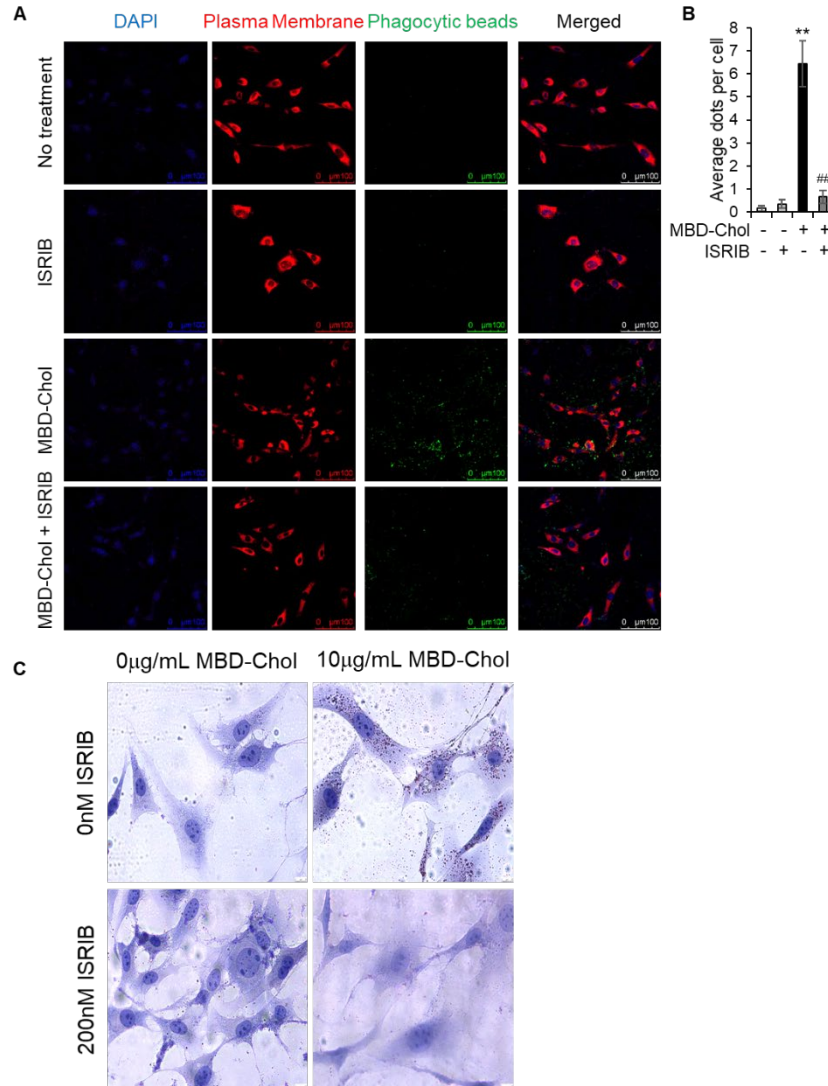


Figure X. Inhibition of the Perk arm of UPR reduces phagocytosis and foam cell formation in immortalized SMCs. (A,B) Treatment with 10 μg/mL MBD-Chol for 72 hours increases phagocytic activity of immortalized SMCs as evidenced by the increased number of green fluorescent dots, while co-treatment with 200 nmol/L ISRIB reverses the phagocytic activity (**A**). (**B**) Reversal of MBD-Chol-induced phagocytic activity is confirmed by the quantification of fluorescent beads per cell. At least five images were obtained for each treatment and the number of green dots within the cellular boundaries was counted in each of the acquired images in a blinded fashion. The average number of dots per cell was calculated by dividing the number of dots in each image by the number of nuclei in the image. The images shown in the figures are representative of a set of at least five images taken at random fields. (**C**) 10 μg/mL MBD-Chol treatment for 72 hours also increases foam cell formation as evident from the lipid droplets stained in red inside the cells, which is reversed by concurrent treatment with 200 nmol/L ISRIB for 72 hours. Each result displayed here is representative of at least three independent biological replicates. P-values were calculated using two-way ANOVA followed by Tukey's Honest Significant Difference post-hoc test. * or ** - indicates $p < 0.05$ or $p < 0.01$ respectively, versus no cholesterol treatment. For the ISRIB-treated samples, # or ## indicates $p < 0.05$ or $p < 0.01$ respectively for ISRIB treatment versus no treatment (DMSO).

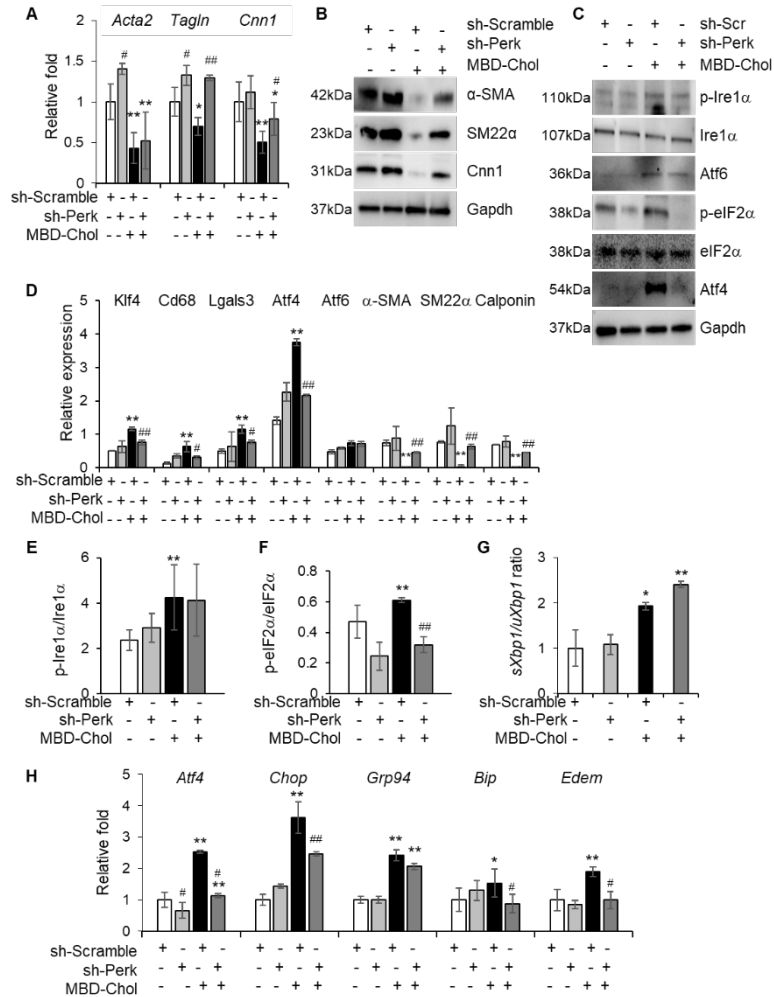


Figure XI. Downregulation of Perk abrogates phenotypic switching of SMCs. (A,B) Cholesterol-induced downregulation of contractile genes (A) and proteins (B) is reduced to baseline upon downregulation of Perk. (C) Immunoblots demonstrate downregulation of eIF2 α phosphorylation and reduction of Atf4 levels occur when Perk is downregulated, without affecting Ire1 α phosphorylation or Atf6 cleavage. (D) Quantification of immunoblots confirms that Perk knockdown prevents cholesterol-induced increases in macrophage-related proteins and decreases in contractile proteins. It also inhibits the induction of Atf4 but does not affect cleavage of Atf6. (E,F) Quantification of immunoblots shows Perk knockdown does not affect Ire1 α phosphorylation (E), but significantly reduces eIF2a phosphorylation (F). (G) Knocking down Perk also does not affect cholesterol-induced splicing of *Xbp1*, a marker for Ire1 α activation, as there is no significant difference in spliced *Xbp1* (*sXbp1*) to unspliced *Xbp1* (*uXbp1*) ratio. (H) Perk knockdown successfully reverses the upregulation of *Atf4* and its downstream target *Chop* as shown by quantitative RT-PCR. For quantification of the blots, the band intensities are normalized to Gapdh; for Ire1 α and eIF2 α phosphorylation, the band intensities of the phosphorylated bands are normalized to the unphosphorylated bands. Each result displayed here is representative of at least three independent biological replicates. P-values were calculated using two-way ANOVA followed by Tukey's Honest Significant Difference post-hoc test. * or ** - indicates $p < 0.05$ or $p < 0.01$ respectively, versus no cholesterol treatment. For the shRNA-treated samples, # or ## indicates $p < 0.05$ or $p < 0.01$ respectively for comparison between sh-Scramble and sh-Perk.

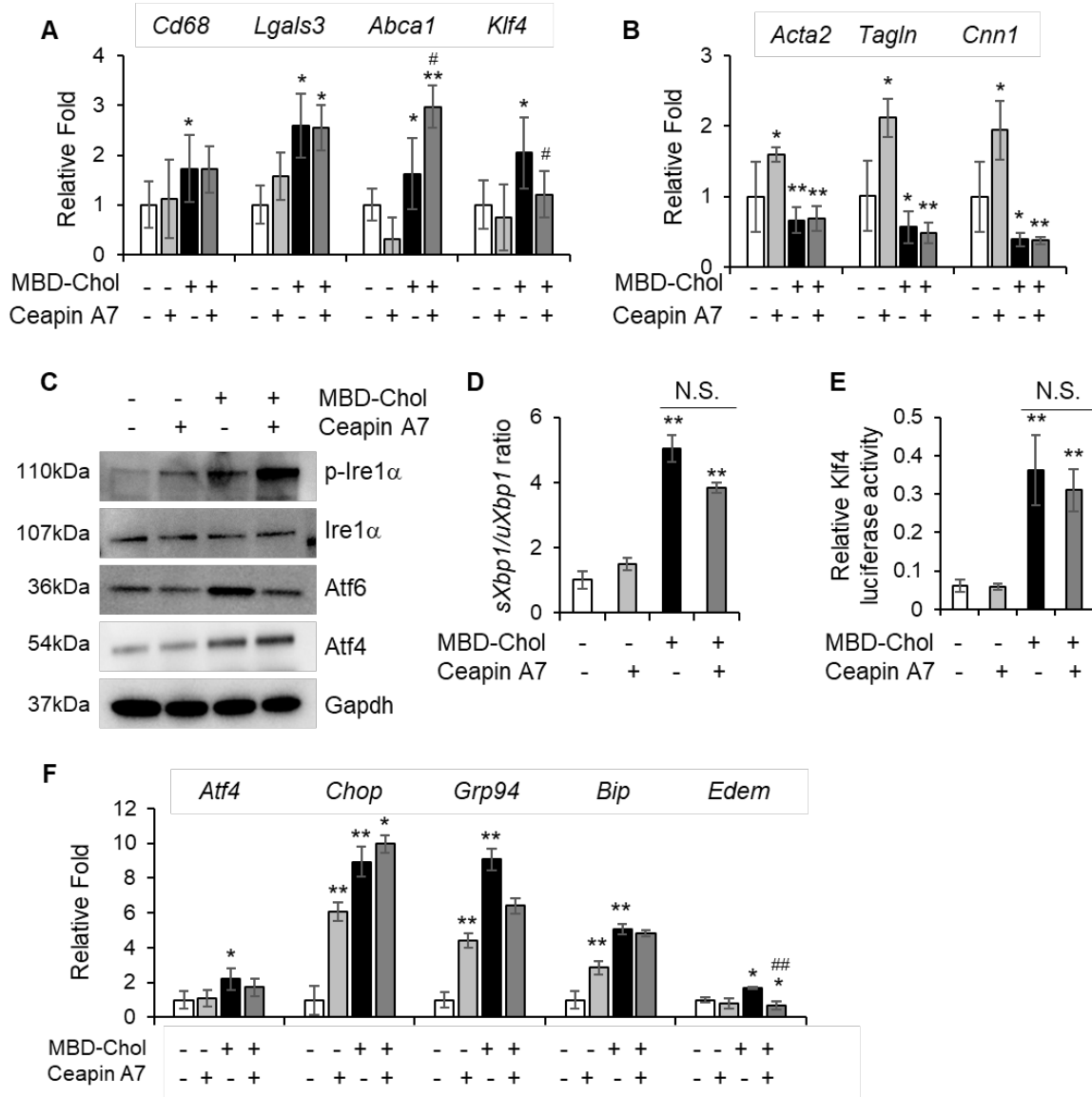


Figure XII. Atf6 pathway is not responsible for cholesterol-induced phenotypic switching of SMCs. (A) Ceapin A7 treatment fails to reverse cholesterol-induced upregulation of macrophage markers. (B) Ceapin A7 treatment fails to reverse cholesterol-induced reduction of contractile marker levels. (C) Immunoblot showing efficacy and specificity of ceapin A7 treatment: ceapin A7 lowers Atf6 cleavage, but does not affect Atf4 levels or Ire1 α phosphorylation. (D) Exposure to ceapin A7 does not affect cholesterol-induced *Xbp1* splicing as indicated by no significant change in the spliced (*sXbp1*) to unspliced (*uXbp1*) ratio, when compared to cholesterol treatment only, confirming its specificity. (E) Ceapin A7 treatment does not affect the cholesterol-induced increase in the transcriptional activity of *Klf4*. (F) Ceapin A7 leaves most ER stress markers unaffected, except *Edem*. Each result displayed here is representative of at least three independent biological replicates. P-values were calculated using two-way ANOVA followed by Tukey's Honest Significant Difference post-hoc test. * or ** - indicates $p < 0.05$ or $p < 0.01$ respectively, versus no cholesterol treatment. * or ** - indicates $p < 0.05$ or $p < 0.01$ respectively, versus no cholesterol treatment. # or ## indicates $p < 0.05$ or $p < 0.01$ respectively for Ceapin A7 treatment versus no treatment (DMSO).

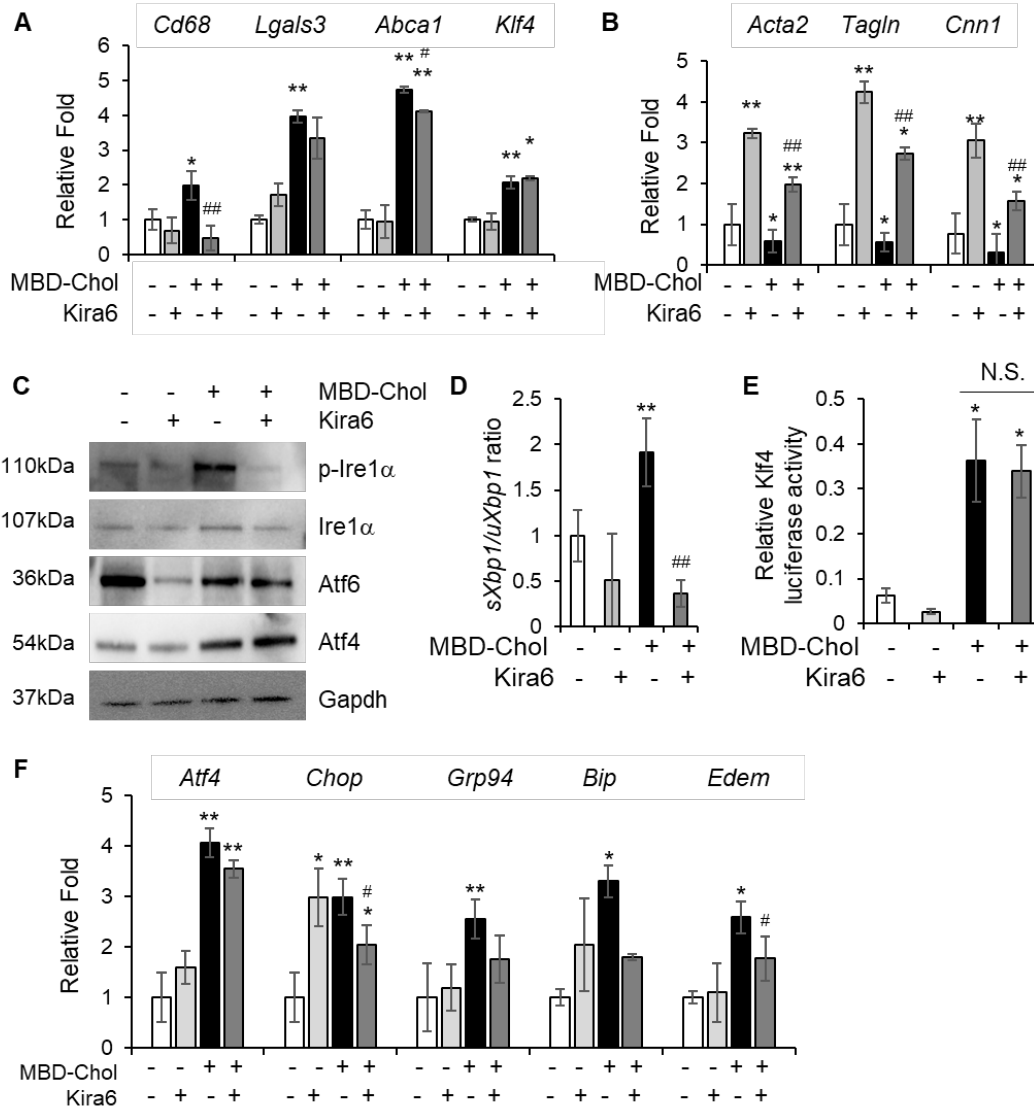


Figure XIII. Ire1 α pathway is possibly responsible for cholesterol-induced phenotypic switching of SMCs. (A) Kira6 treatment reverses cholesterol-induced upregulation of *Cd68* and *Abca1*, but not *Lgals3* and *Klf4*. (B) Kira6 treatment successfully reverses cholesterol-induced downregulation of contractile genes. (C) Immunoblot showing efficacy and specificity of Kira6 treatment: Kira6 lowers Ire1 α phosphorylation, but does not affect Atf4 levels or Atf6 cleavage. (D) Exposure to Kira6 reduces *Xbp1* splicing as indicated by reduction in the spliced (*sXbp1*) to unspliced (*uxbp1*) ratio, confirming its efficacy. (E) Kira6 treatment does not affect the cholesterol-induced increase in the transcriptional activity of Klf4. (F) Kira6 treatment leaves other ER stress markers unaffected, again confirming its specificity for the Ire1 α pathway. Each result displayed here is representative of at least three independent biological replicates. P-values were calculated using two-way ANOVA followed by Tukey's Honest Significant Difference post-hoc test. * or ** - indicates $p < 0.05$ or $p < 0.01$ respectively, versus no cholesterol treatment. # or ## indicates $p < 0.05$ or $p < 0.01$ respectively for Kira6 treatment versus no treatment (DMSO).

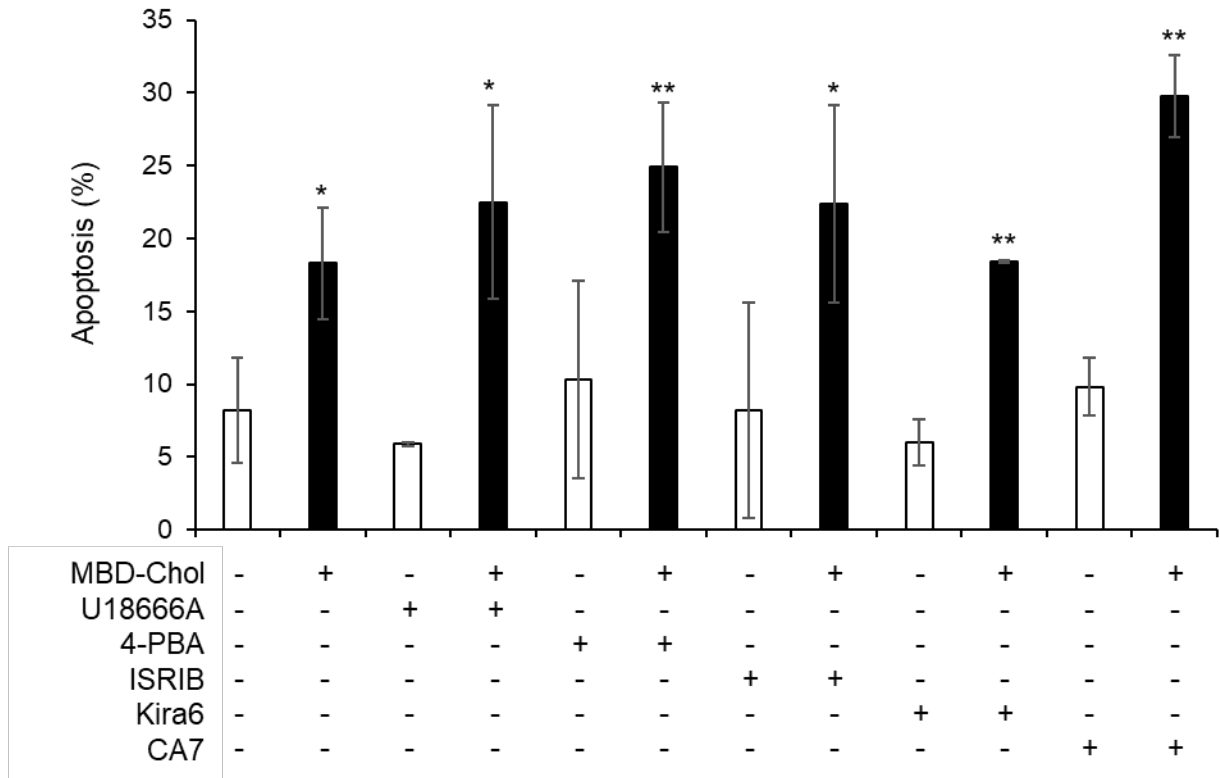


Figure XIV: Effect of cholesterol and various UPR inhibitors on apoptosis of SMCs: (A) Treatment of immortalized mouse SMCs with 10 μ g/mL MBD-Chol caused a significant increase in apoptosis compared to non-treatment. However, when MBD-Chol was used in combination with 70nmol/L U18666A or 5mmol/L 4-PBA or 200nmol/L ISRIB or 1 μ mol/L Kira6 or 6 μ mol/L CA7 for 72 hours, none of the drugs caused a statistically significant difference in apoptosis compared either vehicle only (DMSO) or MBD-Chol + vehicle. Each result displayed here is representative of at least three or more independent biological replicates. P-values were calculated using two-way ANOVA followed by Tukey's Honest Significant Difference post-hoc test. * or ** - indicates p < 0.05 or p < 0.01 respectively, versus no cholesterol treatment. # or ## indicates p < 0.05 or p < 0.01 respectively for drug treatment versus no treatment (DMSO).

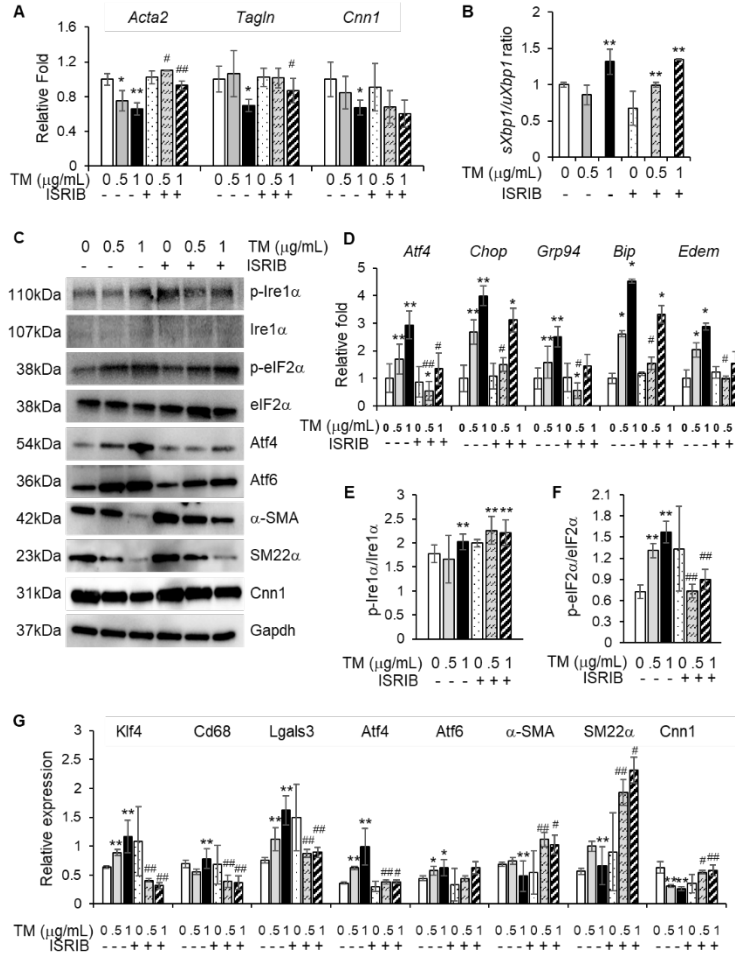


Figure XV. Tunicamycin induces phenotypic switching in SMCs independent of cholesterol, which is reversed by ISRIB treatment. (A) TM treatment reduces expression of SMC contractile genes, which is rescued by concurrent treatment with ISRIB. (B) TM treatment induces *Xbp1* splicing, which is unaffected by ISRIB. (C) Immunoblots confirm that TM treatment activates all three branches of UPR and that Atf4 induction is successfully reversed by ISRIB. Contractile proteins are also downregulated by TM treatment, which is rescued by ISRIB co-treatment. (D) TM induces ER stress, but only the induction of Atf4 is reversed by ISRIB, indicating its specificity. (E,F) Quantification of phosphorylated bands demonstrates increased phosphorylation and activation of both Ire1α (E) and eIF2α (F), upon TM treatment, which remains unaffected with ISRIB. (G) Quantification of band intensities confirms exposure to tunicamycin in culture upregulates macrophage markers, along with Klf4, which is successfully reversed by ISRIB treatment, while contractile proteins are decreased by TM and increased by ISRIB. Quantification of Atf4 and cleaved Atf6 levels confirms TM induces both, while only the induction of Atf4 is reversed by ISRIB. For quantification of the blots, the band intensities are normalized to Gapdh; for Ire1α and eIF2α phosphorylation, the band intensities of the phosphorylated bands are normalized to the unphosphorylated bands. Each result displayed here is representative of at least three independent biological replicates. P-values were calculated using two-way ANOVA followed by Tukey's Honest Significant Difference post-hoc test. * or ** - indicates p < 0.05 or p < 0.01 respectively, versus no tunicamycin treatment (DMSO). # or ## indicates p < 0.05 or p < 0.01 respectively for TM + ISRIB treatment versus TM treatment only.

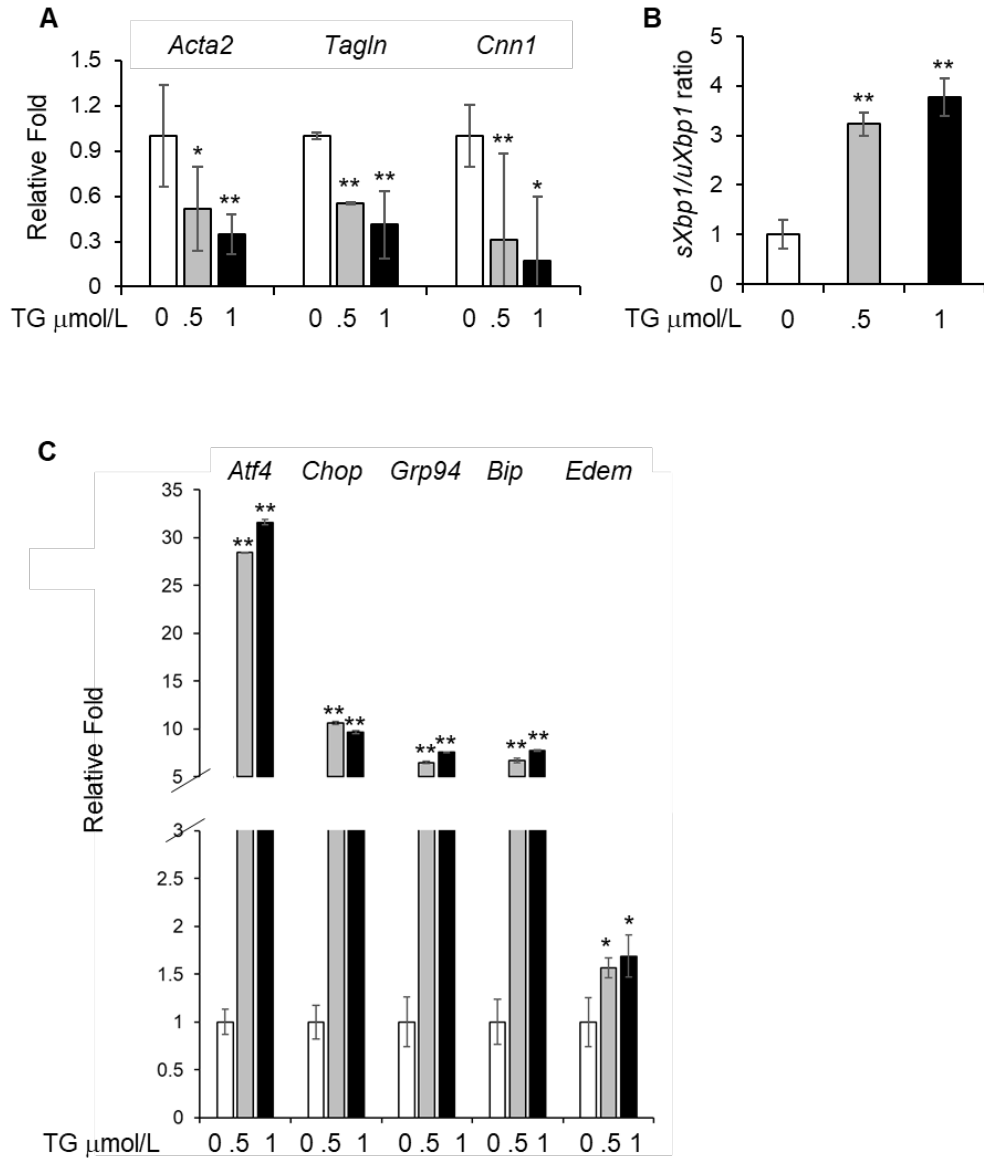


Figure XVI. Thapsigargin induces phenotypic switching in SMCs independent of cholesterol. (A) Thapsigargin (TG) treatment reduces expression of contractile markers in a dose-dependent manner. (B) TG treatment induces *Xbp1* splicing in a dose-dependent manner. (C) TG induces markers of all three arms of UPR. * or ** - indicates $p < 0.05$ or $p < 0.01$ respectively, versus no thapsigargin treatment (DMSO) using unpaired two-tailed Student's t-tests. Each result displayed here is representative of at least three independent biological replicates.

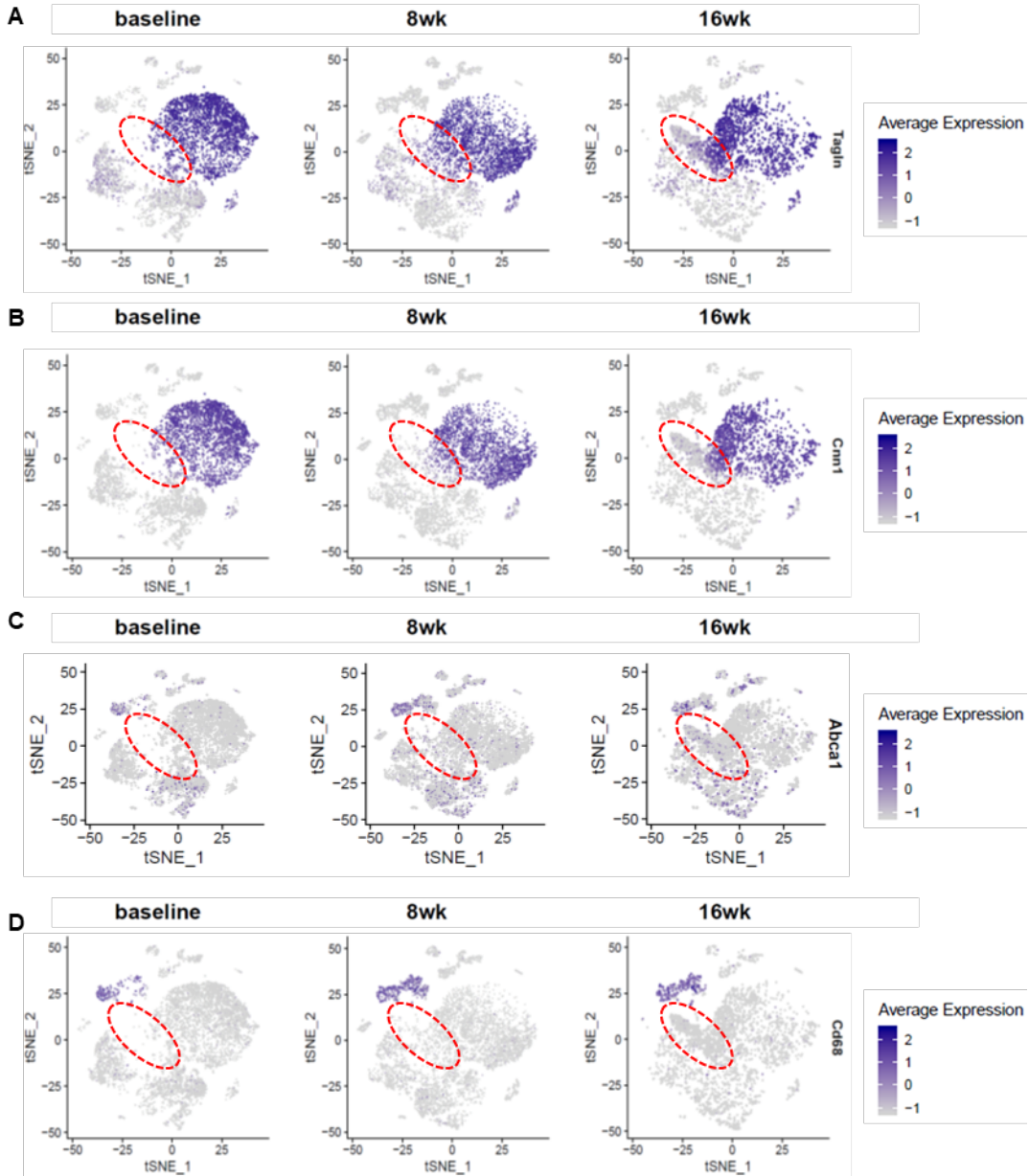


Figure XVII. Transcriptomic profiling of mouse aortic root atherosclerotic plaque shows decreased contractile markers and increased *Abca1* in modulated SMCs. (A-D) t-SNE visualization of cell clusters at the three time points overlaid with expression of various genes show decreased expression the contractile markers *Tagln* (A) and *Cnn1* (B), increased expression of the cholesterol metabolism gene *Abca1* (C), but no appreciable increase in the expression of the macrophage marker *Cd68* (D) in the modulated SMC cluster that appeared over time on high fat diet. The scale on the right of each gene indicates level of expression. The disease-specific modulated SMC cluster, as identified by Wirka *et al*, is highlighted by the dotted red circle. Analysis was performed on published scRNA-Seq data (GSE131780) from Wirka *et al*, where n=3 mice were used at each time point.

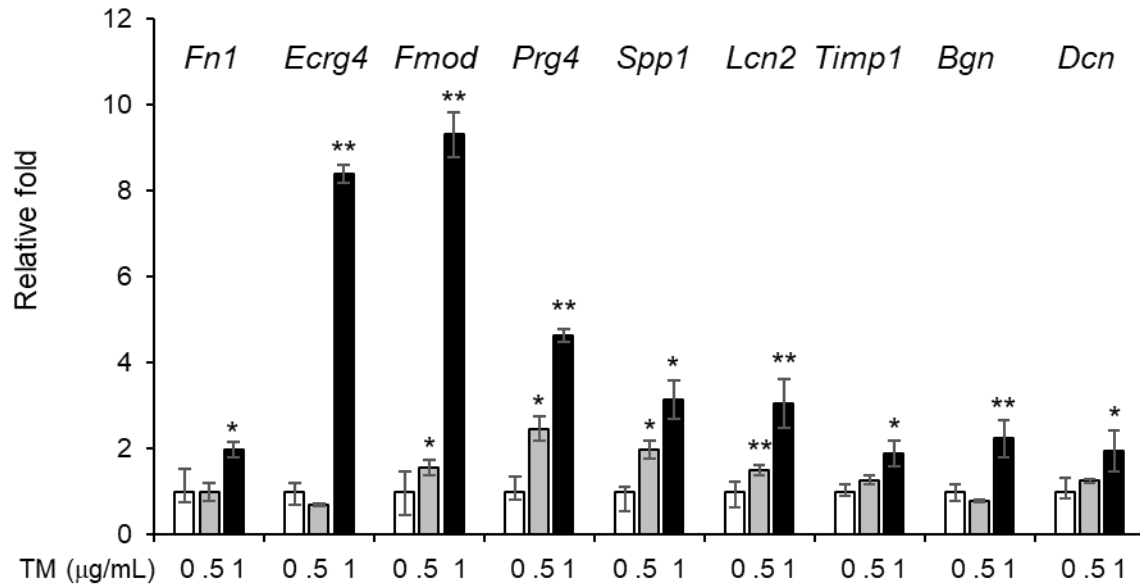


Figure XVIII. Tunicamycin induces expression of fibroblast markers indicative of modulated SMCs. TM treatment increases expression of a subset of fibroblast marker genes identified in modulated SMCs. Each result displayed here is representative of at least three independent biological replicates. * or ** - indicates $p < 0.05$ or $p < 0.01$ respectively, versus no tunicamycin treatment (DMSO) using unpaired two-tailed Student's t-tests.

Major Resources Table

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
<i>Mus musculus</i>	The Jackson Laboratory	C57BL/6J	M/F	https://www.jax.org/strain/000664

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Persistent ID / URL
Klf4	RnD Systems	AF3158	0.1µg/mL	https://www.rndsystems.com/products/mouse-klf4-antibody_af3158
Cd68	Abcam	ab125212	0.2µg/mL	https://www.abcam.com/cd68-antibody-ab125212.html
Lgals3	Abcam	ab76245/EP2775Y	0.1µg/mL	https://www.abcam.com/galectin-3-antibody-ep2775y-ab76245.html
Gapdh	Cell Signaling Technologies	2118/14C10	0.05µg/mL	https://www.cellsignal.com/products/primary-antibodies/gapdh-14c10-rabbit-mab/2118
Ire1α	Abcam	ab37073	1µg/mL	https://www.abcam.com/ire1-antibody-ab37073.html
Phospho-Ire1α (phospho S724)	Abcam	ab48187	1µg/mL	https://www.abcam.com/ire1-phospho-s724-antibody-ab48187.html
Atf6	ThermoFisher Scientific	NBP14025601	2µg/mL	https://www.fishersci.com/shop/products/mouse-anti-atf6-clone-70b1413-1-novus-biologicals/nbp14025601#?keyword=70B1413.1
eIF2α	Cell Signaling Technologies	5342 S/D7D3	0.05µg/mL	https://www.cellsignal.com/products/primary-antibodies/eif2a-d7d3-xp-rabbit-mab/5342
Phospho-eIF2α	Cell Signaling Technologies	3398 S/D9G8	0.1µg/mL	https://www.cellsignal.com/products/primary-antibodies/phospho-eif2a-ser51-d9g8-xp-rabbit-mab/3398

Atf4	Cell Signaling Technologies	11815 S/D4 B8	0.1µg/mL	https://www.cellsignal.com/products/primary-antibodies/atf-4-d4b8-rabbit-mab/11815
α-smooth muscle actin (α-SMA)	Millipore Sigma	A5228	0.5µg/mL	https://www.sigmaaldrich.com/catalog/product/sigma/a5228?lang=en&region=US
SM22α/TAGLN/transgelin	Abcam	ab14106	1µg/mL	https://www.abcam.com/taglntransgelin-antibody-ab14106.html
Calponin	Thermo Fisher Scientific	PIMA511620	0.5µg/mL	https://www.fishersci.com/shop/products/anti-calponin-1-clone-calp-thermo-scientific-pierce/pima511620?searchHijack=true&searchTerm=PIMA511620&searchType=RAPID&matchedCatNo=PIMA511620

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
Mouse WT SMC – primary and immortalized	Milewicz Lab, explanted from C57BL/6J mice obtained from The Jackson Laboratory	F, M	

TABLE 1: List of SYBR primers used for quantitative real time PCR (qPCR)

Gene	Forward primer (5' -> 3')	Reverse primer (5' -> 3')
<i>Cd68</i>	CAAGGTCCAGGGAGGTTGTG	CCAAAGGTAAGCTGTCCATAA GGA
<i>Lgals3</i>	AGGAGAGGGAATGATGTTGCC	GGTTTGCCACTCTCAAAGGG
<i>Abca1</i>	GCGGACCTCCTGGGTGTT	CAAGAATCTCCGGGCTTTAGG
<i>Klf4</i>	CTGAACAGCAGGGACTGTCA	GTGTGGGTGGCTGTTCTTTT
Unspliced <i>Xbp1</i> (<i>uXbp1</i>)	CAGCACTCAGACTATGTGCA	GTCCATGGGAAGATGTTCTGG

<i>Spliced Xbp1</i> (<i>sXbp1</i>)	CTGAGTCCGAATCAGGTGCAG	GTCCATGGGAAGATGTTCTGG
<i>Atf4</i>	GGGTTCTGTCTTCCACTCCA	AAGCAGCAGAGTCAGGCTTTC
<i>Chop</i>	CCACCACACCTGAAAGCAGAA	AGGTGAAAGGCAGGGACTCA
<i>Grp94</i>	AAGAATGAAGGAAAAACAGGACA AAA	CAAATGGAGAAGATTCCGCC
<i>BiP</i>	TTCAGCCAATTATCAGCAAACCTCT	TTTTCTGATGTATCCTCTTCAC CAGT
<i>Edem</i>	CTACCTGCGAAGAGGCCG	GTTTCATGAGCTGCCCACTGA
<i>Perk</i>	CCTTGGTTTCATCTAGCCTCA	ATCCAGGGAGGGGATGAT
<i>Serping1</i>	GCCCAATTTCGATGACCATAC	AAGTTGGTGCTTTGGGAACA
<i>Fn1</i>	CGAGGTGACAGAGACCACAA	CTGGAGTCAAGCCAGACACA
<i>Ecr4</i> (1500015O10Rik)	AAGCGTGCCAAACGACAGCTGTG GGAC	TTAATAGTCATCATAGTTGACA CTGGC
<i>Fmod</i>	CAACACCAACCTGGAGAATCTTT	GTGCAGAAGCTGCTGATGGA
<i>Prg4</i>	GAAAATACTTCCCGTCTGCTTGT	ACTCCATGTAGTGCTGACAGT TA
<i>Spp1</i>	TCACCATTTCGGATGAGTCTG	ACTTGTGGCTCTGATGTTCC
<i>Lcn2</i>	TGCCACTCCATCTTTCCTGTT	GGGAGTGCTGGCCAAATAAG
<i>Timp1</i>	CCAGAGCCGTCACCTTTGCTT	AGGAAAAGTAGACAGTGTTCA GGCTT
<i>Bgn</i>	ATTGCCCTACCCAGAACTTGAC	GCAGAGTATGAACCCTTTCCT G
<i>Dcn</i>	TTCCTACTCGGCTGTGAGTC	AAGTTGAATGGCAGAACGC
<i>Tnfrsf11b</i>	TCCCGAGGACCACAATGAAC	TCCTGGGTTGTCCATTCAATG
<i>Tcf21</i>	CATTCACCCAGTCAACCTGA	CCACTTCCTTCAGGTCATTCTC
<i>Col1a1</i>	CCTCAGGGTATTGCTGGACAAC	CAGAAGGACCTTGTTTGCCAG G

<i>Lum</i>	GGATGGCAATCCTCTCACTC	TCATTTGCTACACGTAGACACT CAT
<i>18S rRNA</i>	GTAACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG