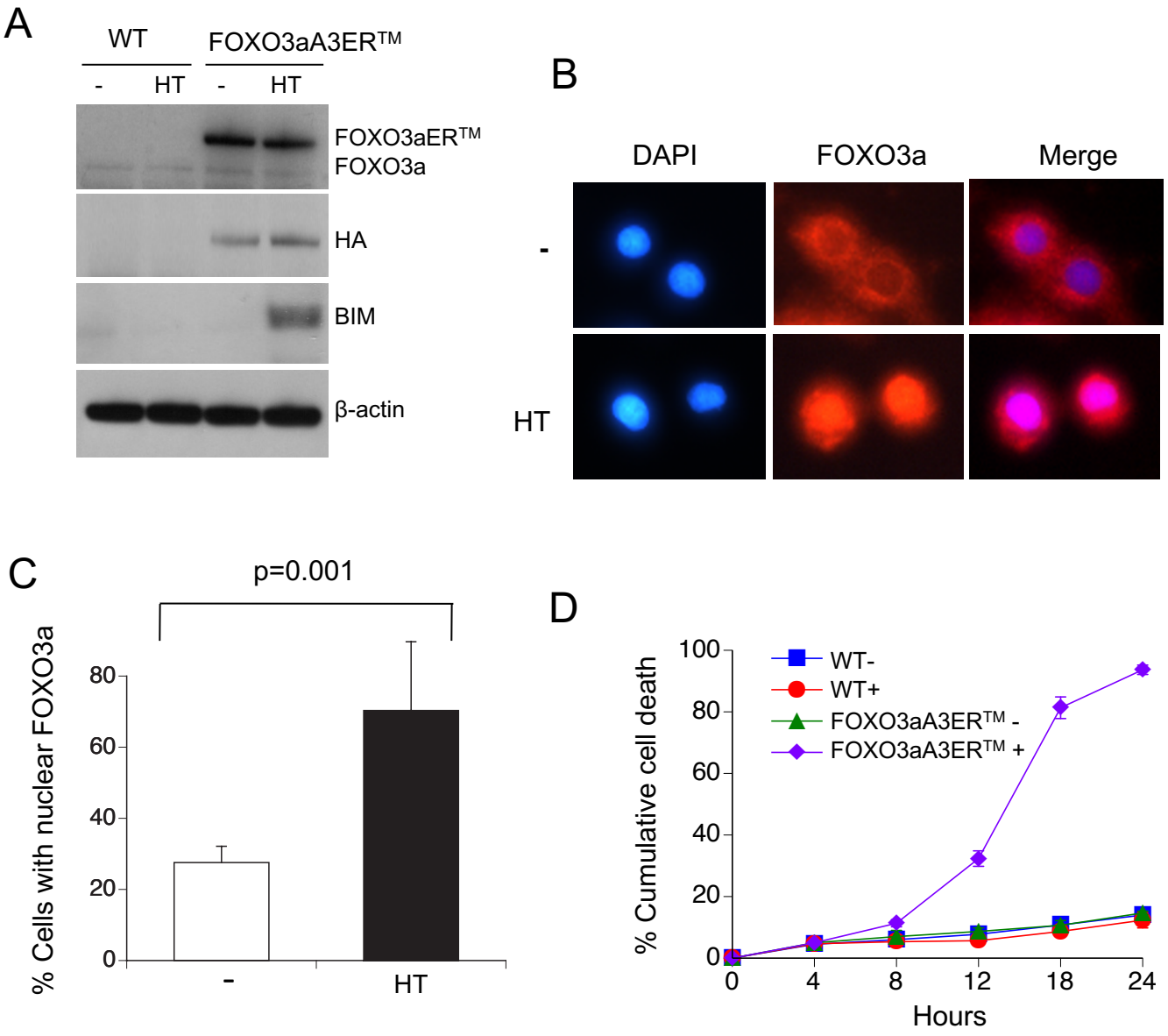
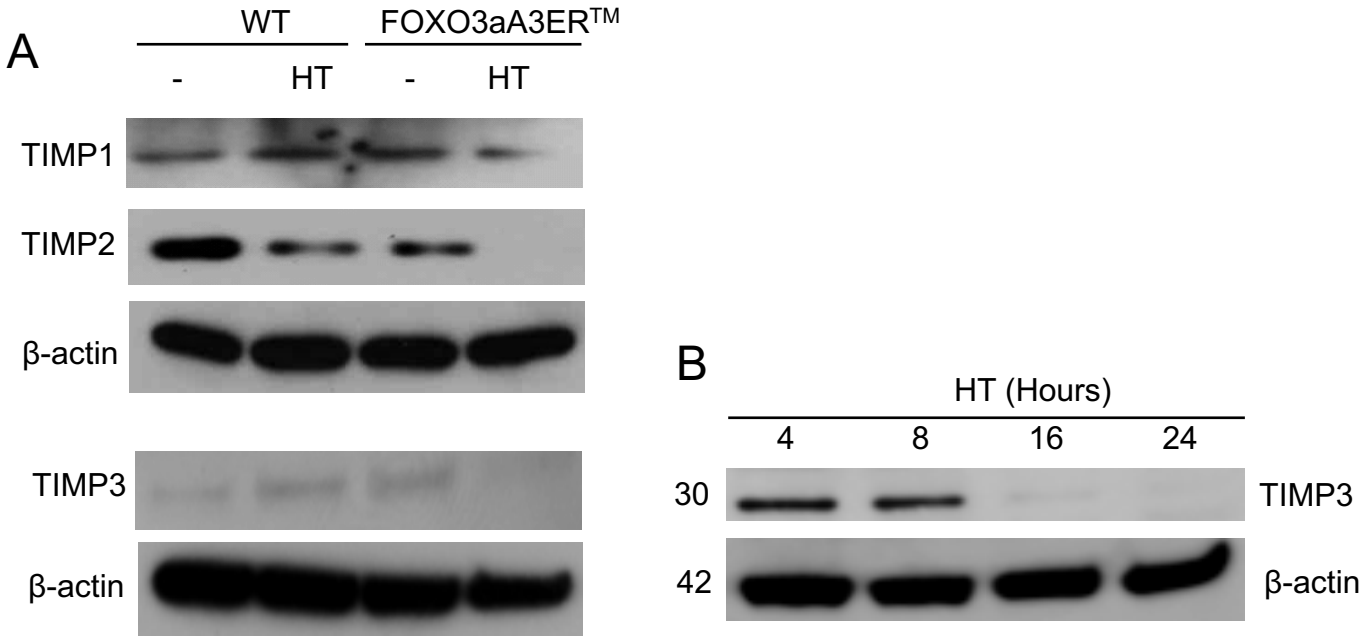
# Supplemental Figures and Tables

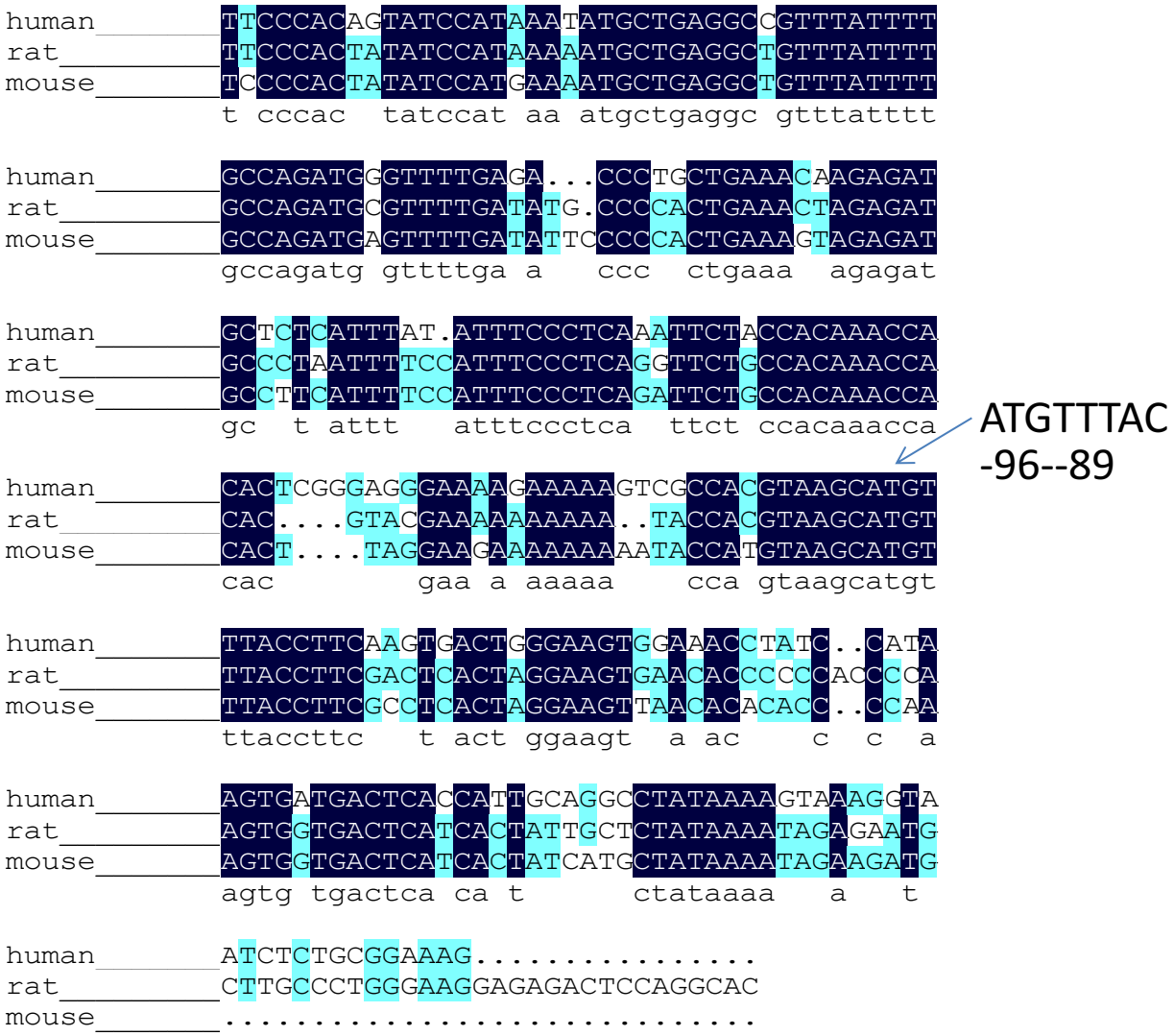



**Supplemental Figure I**
**HT activates FOXO3a and leads to VSMC apoptosis**
**(A)** Western blot of wild type control (WT) or FOXO3aA3ER™ VSMCs for FOXO3a, HA or the FOXO3a transcriptional target BIM after administration of hydroxytamoxifen (HT) vs. the ethanol carrier control (-). **(B)** Immunocytochemistry for FOXO3a (red) or DAPI (blue) with overlay (pink) in FOXO3aA3ER™ VSMCs treated with 1 μM HT or carrier control (-) for 4h. **(C)** Quantification of % cells expressing nuclear FOXO3a in **(B)**. **(D)** Digital videomicroscopic analysis of % cumulative cell death in WT or FOXO3aA3ER™ VSMCs after 1 μM HT (+) or carrier control (-) up to 24h. n=3, data are Means±SD.



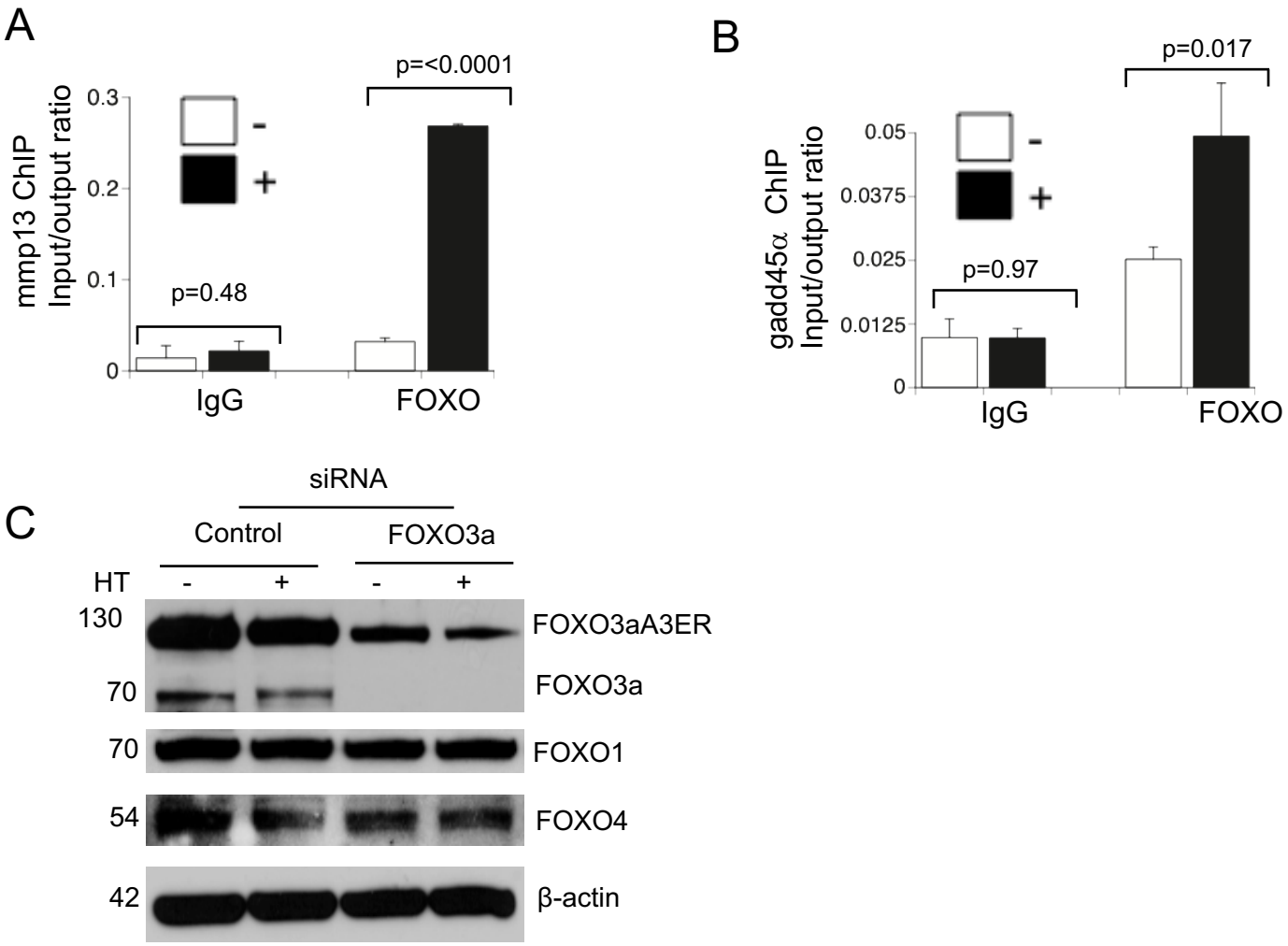

**Supplemental Figure II**

**(A)** Western blot of lysates from wild type (WT) control or FOXO3aA3ER™ VSMCs treated with the ethanol carrier control (-) or HT at 24h. **(B) )** Western blot of lysates from FOXO3aA3ER™ VSMCs treated with HT from 4-24h. Representative of n=3.



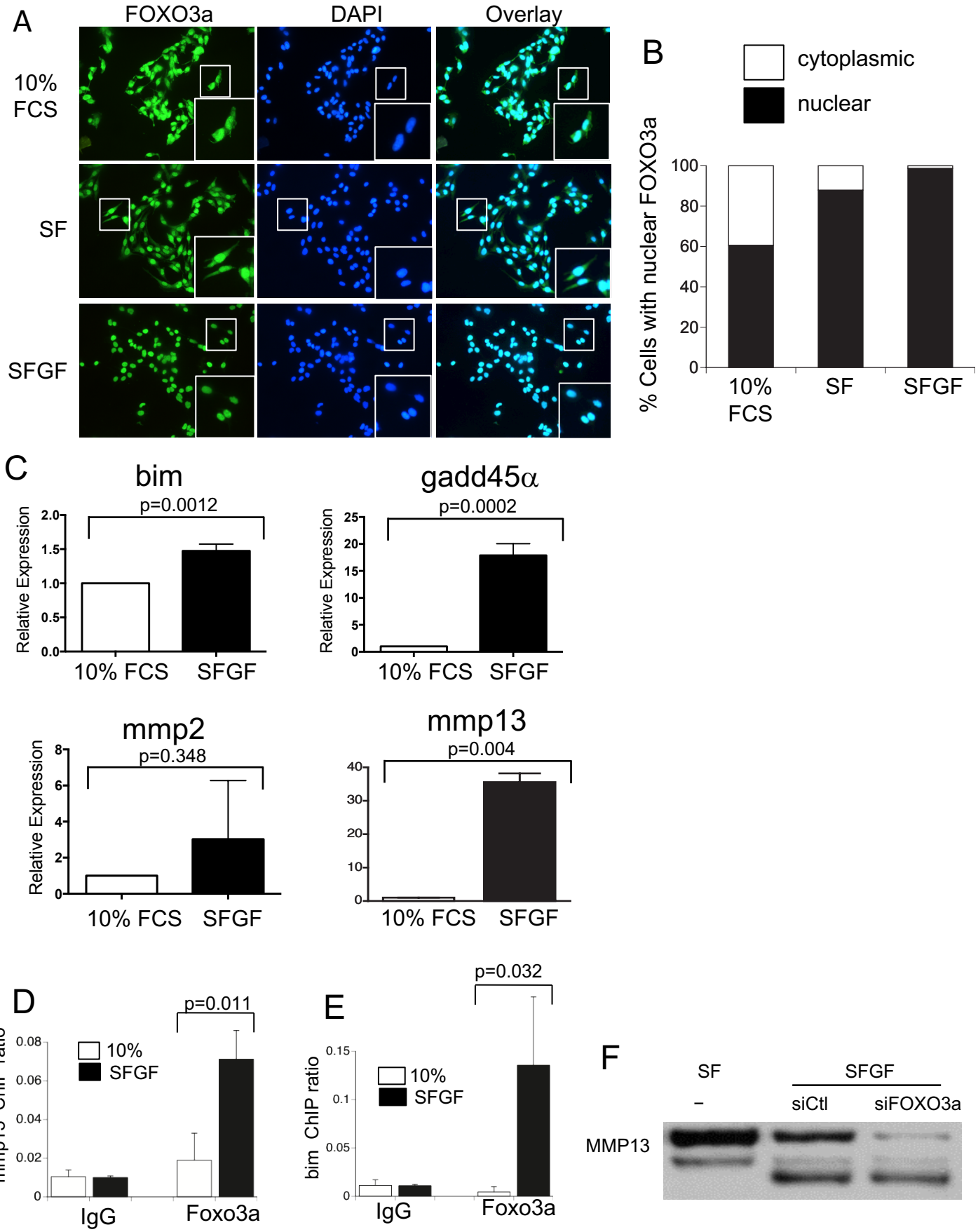

**Supplemental Figure III**

MMP13 promoter proximal site (-96 bp upstream of start codon) contains a complete FOXO3a DNA binding motif ATGTTTAC. Residues shaded dark blue are conserved between species.

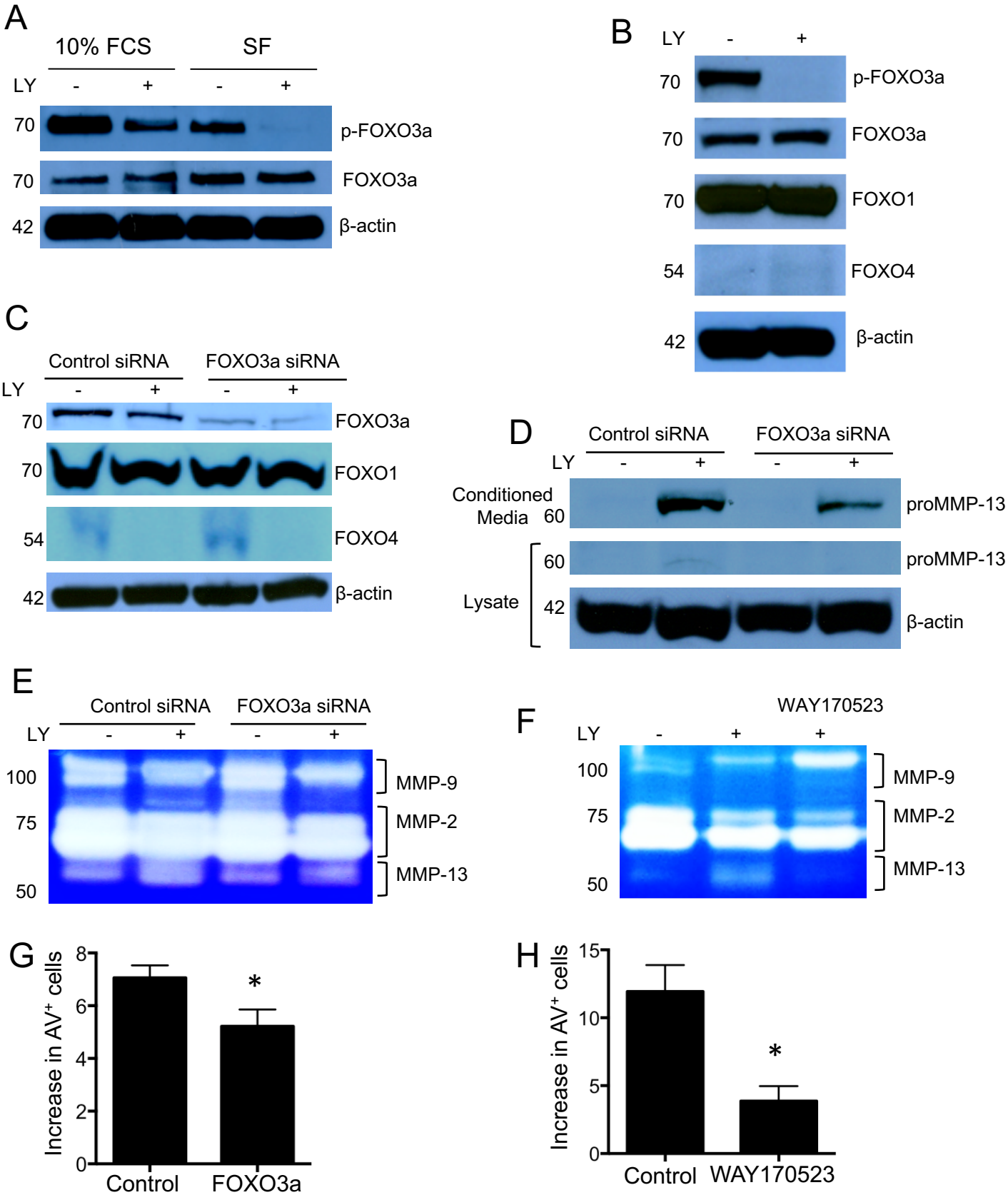



**Supplemental Figure IV**

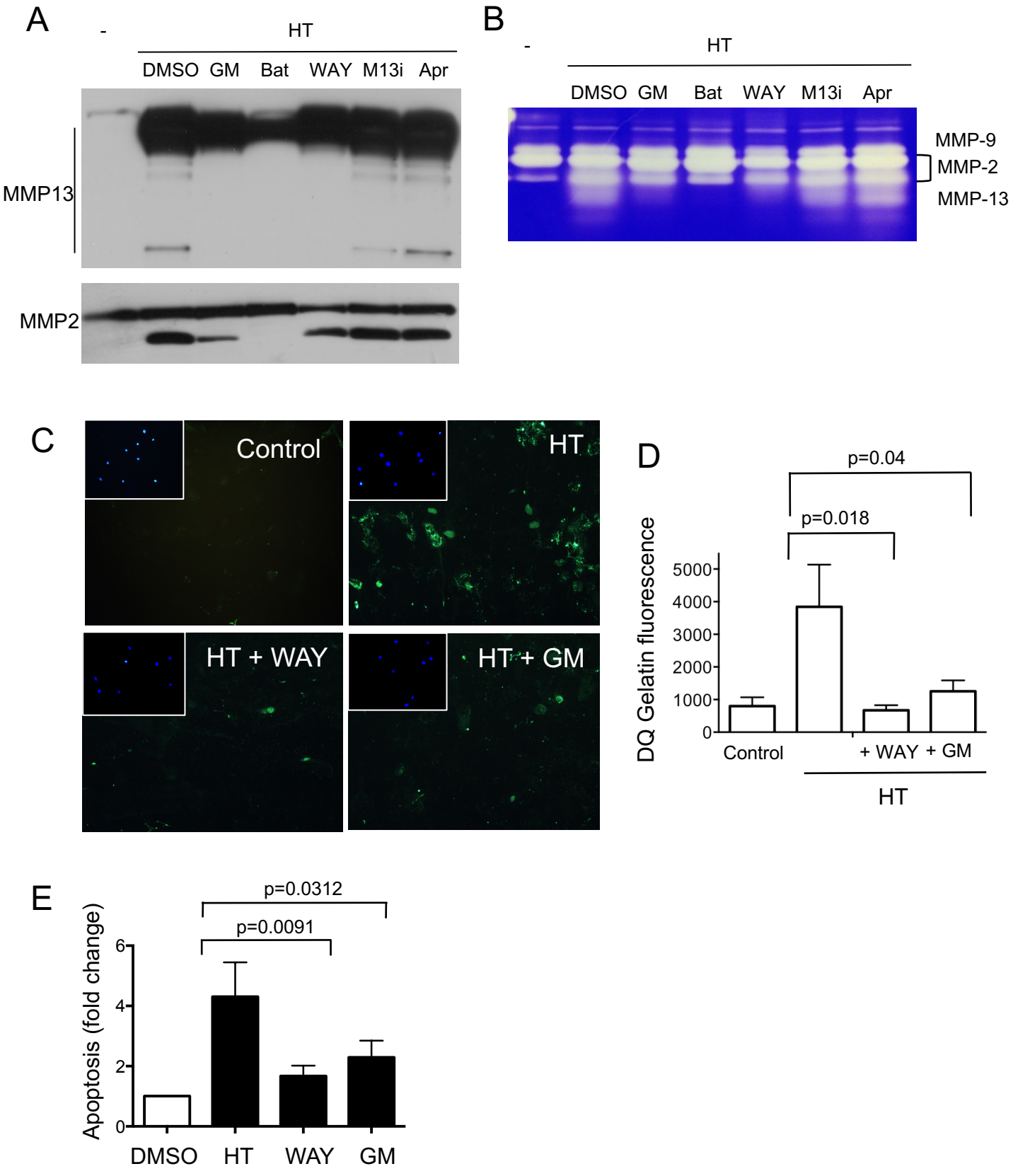
**(A-B)** Chromatin IP (ChIP) for binding of FOXO3a to the mmp13 **(A)** or gadd45α **(B)** promoter in FOXO3aA3ER™ VSMCs treated with carrier control (-) or HT (+) for 24h and immunoprecipitated with an IgG control or MMP13 antibody. **(C)** Western blot for FOXO species in lysates of FOXO3aA3ER™ VSMCs after transfection with either control siRNA (siCtl) or siRNA to human FOXO3a ± 24h treatment with HT.



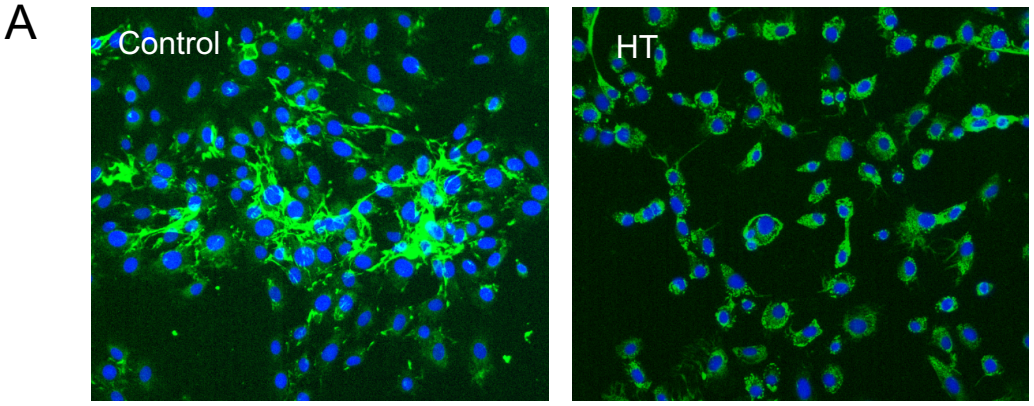

**Supplemental Figure V. Nutrient withdrawal induces Foxo3a transcriptional targets**
**(A-B).** FOXO3a localization in human VSMCs in 10% FCS, or serum-free (SF) or serum-free/ glucose free media (SFGF) for 24h. Insets show high power view of outlined areas. **(C)** qPCR for bim, gadd45α, mmp2 and mmp13 in control VSMCs in 10% FCS, or 24h after transfer to SFGF media. **(D)** Chromatin IP (ChIP) for FOXO3a binding to the mmp13 **(D)** or bim **(E)** promoter in FOXO3aA3ER$^{TM}$ VSMCs in 10% FCS or SFGF medium, immunoprecipitated with an IgG control or MMP13 antibody. **(F)** Western blot of conditioned medium from human VSMCs transfected with siRNA to control sequences (siCtl) or FOXO3a, transferred to SF or SFGF medium for 24h. Data are means±SD. n=3.

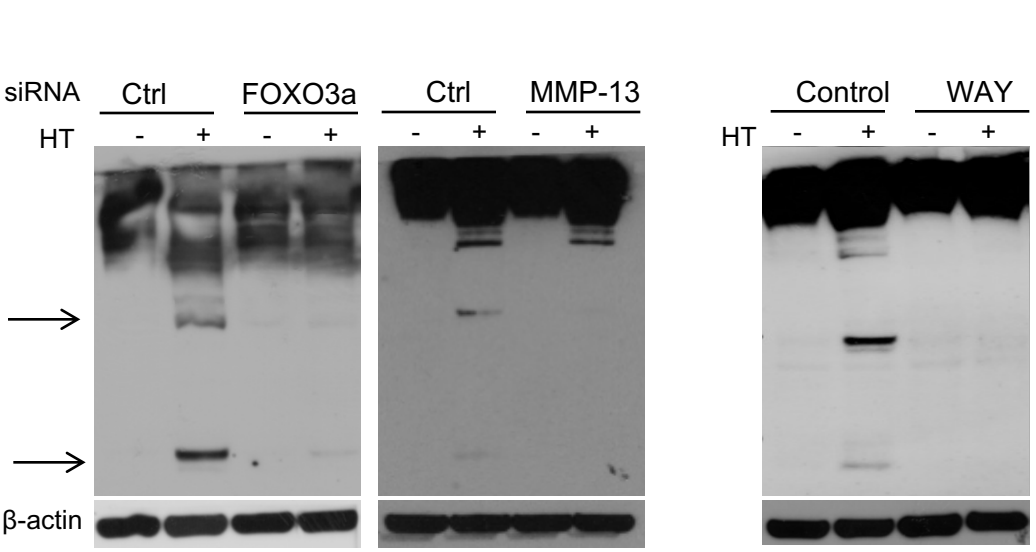


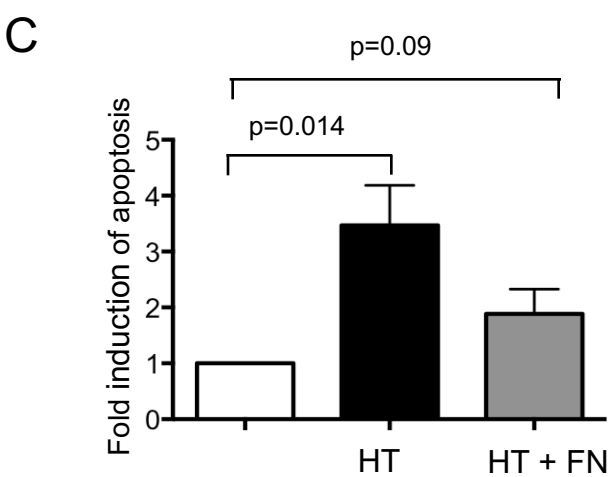

**Supplementary Figure VI. PI-3K inhibition activates FOXO3 and induces MMP13 in human VSMCs** **(A)** Western blot of cell lysates from human VSMCs treated with 10%FCS or 0%FCS medium (serum-free – SF) ± LY294002 (LY) for 24 hours. **(B-C)** Western blot for FOXO species ± LY294002 (LY) for 24h **(B) or** after prior treatment with control siRNA or siRNA to FOXO3a for 48h ± LY294002 (LY) for 24h **(C)**. **(D-E)** Western blot for MMP13 **(D)** or zymogram **(E)** of conditioned media of human VSMCs ± LY294002 (LY) for 24 hours or additionally after prior treatment with control siRNA or siRNA to FOXO3a. **(F)** Zymogram of human VSMCs ± LY294002 (LY) or after treatment with WAY170523. **(G-H)** Increase in Annexin V-positive cells after treatment of human VSMCs with LY after prior treatment with control siRNA or siRNA to FOXO3a **(G)**, or after treatment with WAY170523 **(H).** Data are means ± SEM. Representative of n=3. *p<0.05 vs. Control.

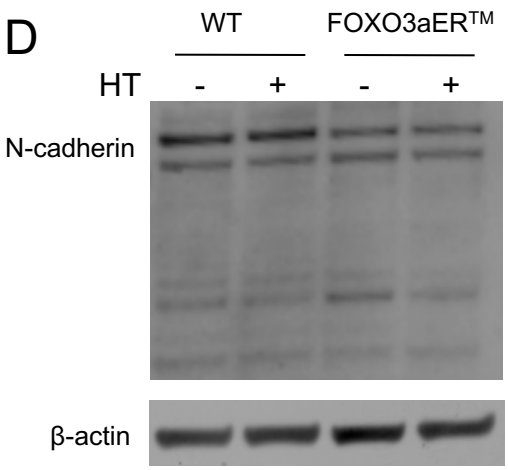



**Supplemental Figure VII. MMP13 promotes apoptosis induced by FOXO3a**
**(A)** Western blot for MMP13 or MMP2 in conditioned media of FOXO3aA3ER$^{TM}$ VSMCs after 24h treatment with HT or control (-), with pre-incubation with 10mM GM6001 (GM), 10μM Batamistat (Bat), 10μM WAY170523 (WAY), 1μM M13i or 2mg/ml Aprotinin, or DMSO control. **(B)** Zymogram of conditioned media from VSMCs treated in **(A)**. **(C-D)** DQ-gelatin fluorescence of FOXO3aA3ER$^{TM}$ VSMCs treated with HT ± WAY or GM for 16h. Insets show DAPI of same field as DQ-gelatin. **(E)** Apoptosis of FOXO3aA3ER$^{TM}$ VSMCs after HT ± WAY170523 or GM6001 treatment for 24h. Data are means ± SD.



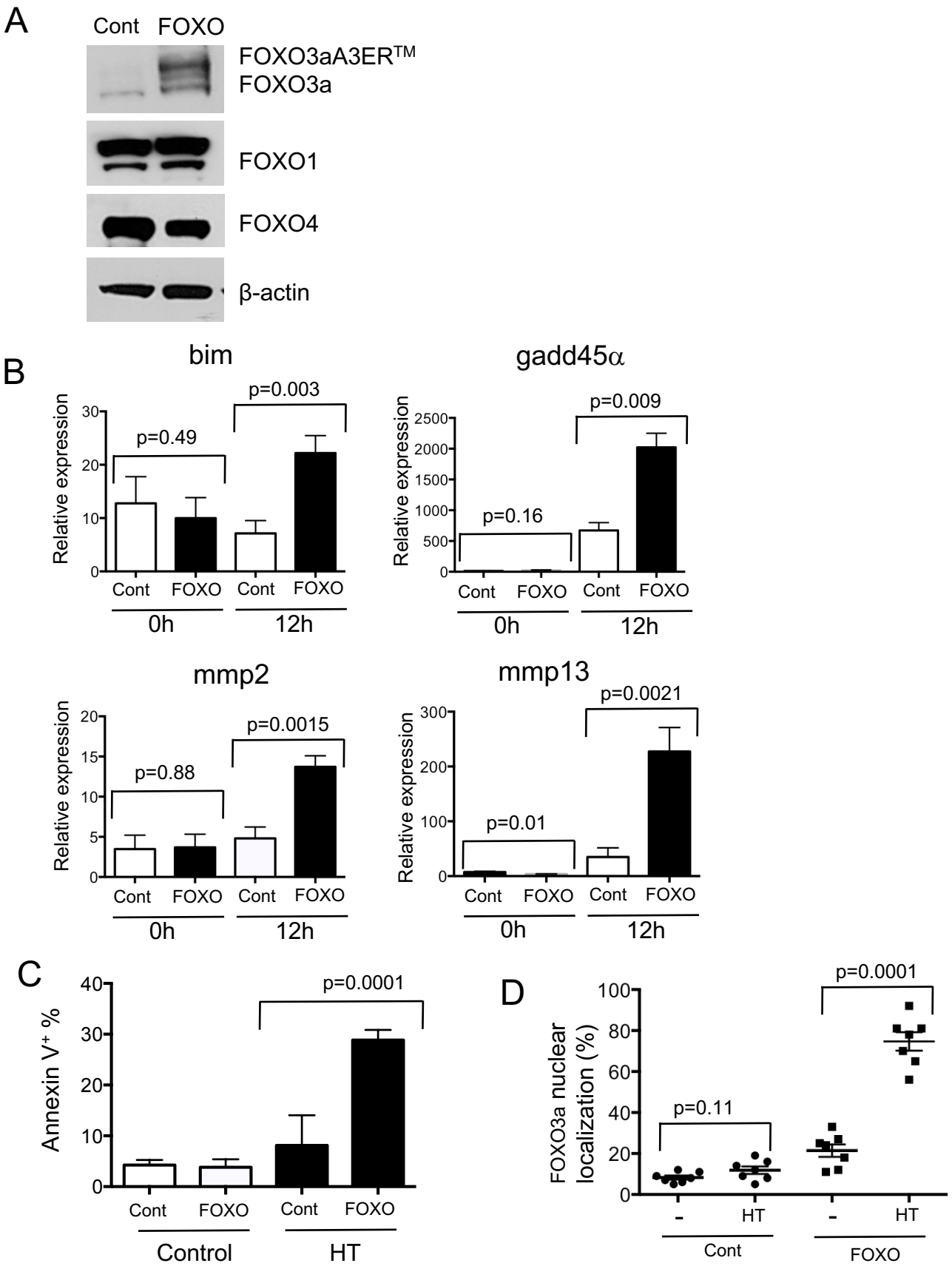



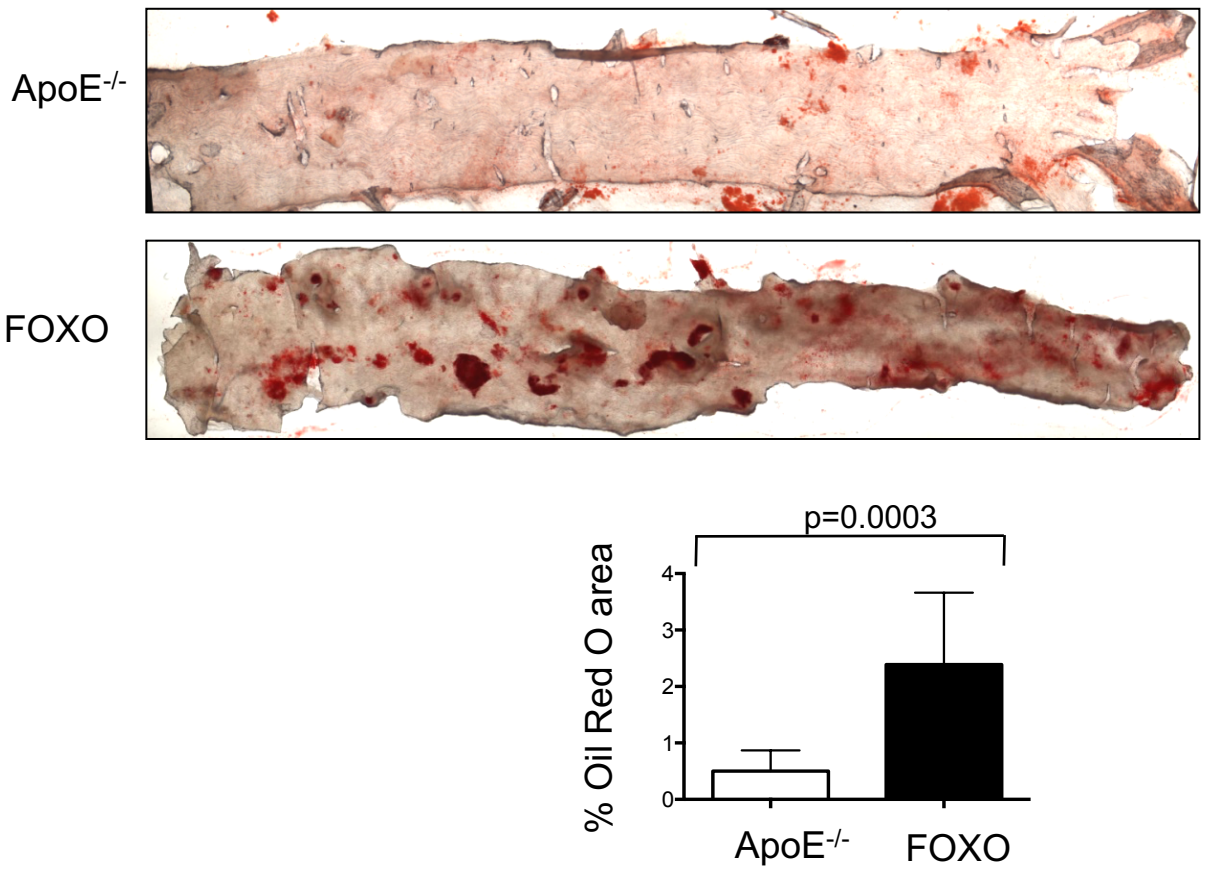

**Supplemental Figure VIII. FOXO3a-induced MMP13-mediated cleavage of fibronectin promotes VSMC apoptosis**

**(A).** Immunocytochemistry for fibronectin in cultures of FOXO3aA3ER$^{TM}$ cells ± HT for 24h. **(B)** Western blot for fibronectin (arrows) in FOXO3aA3ER$^{TM}$ cells ± HT for 24h, preincubated with siRNA to control sequences (Ctrl), FOXO3a or MMP13, or treated with vehicle control or WAY170523. **(C)** Apoptosis of FOXO3aA3ER$^{TM}$ VSMCs 24h after HT ± fibronectin (FN) coating of the culture plate. **(D)** Western blot for N-cadherin in wild type control (WT) or FOXO3aA3ER$^{TM}$ cells ± HT for 24h. Data are Means ± SD, n=3.

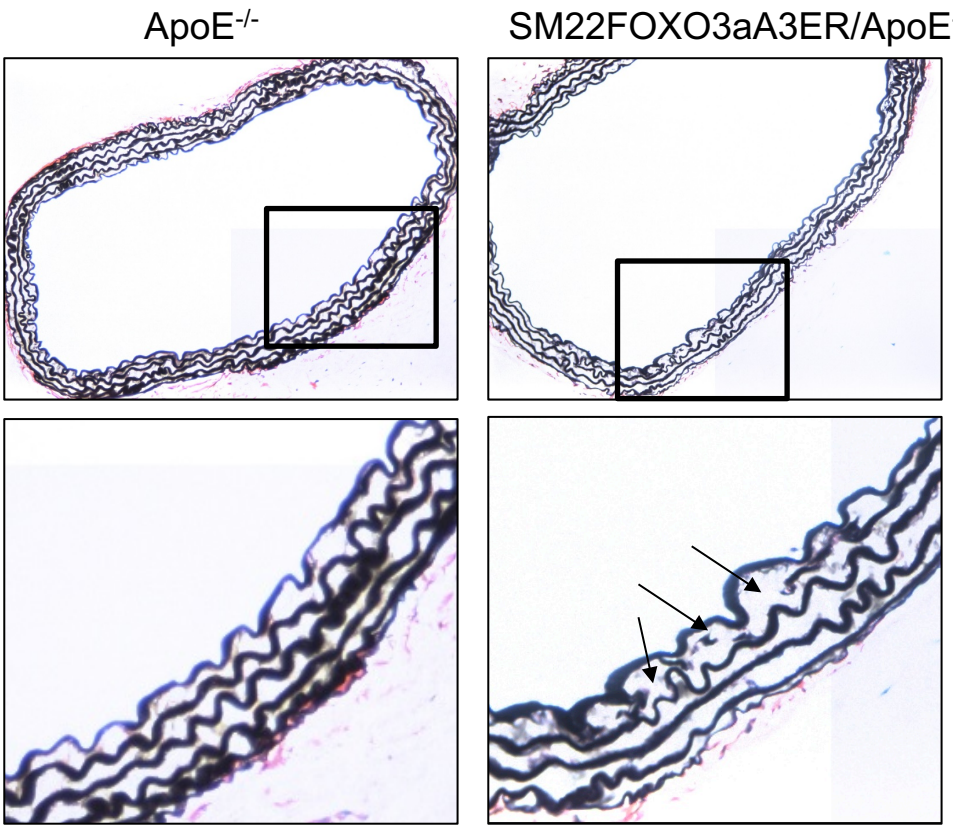



**Supplemental Figure IX. SM22aFOXO3aA3ER™ mouse VSMCs induce Foxo3a targets and apoptosis after HT**

**(A)** Western blot for FOXO species in VSMCs derived from littermate control (Cont) or SM22aFOXO3aA3ER™ (FOXO) mice 24h after treatment with HT**. (B)** Relative expression by qPCR for bim, gadd45α, mmp2 and mmp13 in VSMCs derived from mice in **(A)** at time 0 or 12hr after 1μM HT. **(C)** Apoptosis (Annexin V+) % determined by flow cytometry of VSMCs derived from mice in **(A)** treated with ethanol carrier (Control) or 1μM HT for 24h. **(D)** Quantification of nuclear FOXO3a in littermate control (Cont) or SM22aFOXO3aA3ER™ (FOXO) mice 24h after treatment with HT. Data are means±SD. N=3 mice in **(A-C)** or n=7 for **(D)**.




**Supplemental Figure X**
Oil Red O staining of descending aorta of of Control ApoE$^{-/-}$ (ApoE$^{-/-}$)(n=13) or SM22αFOXO3aA3ER$^{TM}$/ ApoE$^{-/-}$ (FOXO)(n=14) mice after 14 weeks of fat feeding. Data are means±SD.




**Supplemental Figure XI**
Verhoeff Van Gieson stain for elastin in littermate control and SM22αFOXO3aA3ER$^{TM}$/ApoE$^{-/-}$ mice, showing elastin breaks (arrows).

## Up-regulated genes

| Gene name | Encoded protein | Fold change | Potential function |
|---|---|---|---|
| MMP13 | Matrix metallopeptidase 13 | 371.37 | ECM degradation |
| BMP2 | Bone morphogenetic protein 2 | 9.80 | MMP activator |
| Cited2 | Cbp/p300-interacting transactivator 2 | 8.96 | apoptosis |
| CXCL10 | Interferon gamma-induced protein 10 | 7.88 | Chemoattractant |
| Bcl2l11 | Bcl-2-like protein 11, BIM | 6.24 | apoptosis |
| Bcl2 | B-cell lymphoma 2 | 6.24 | apoptosis |
| Adamts5 | A disintegrin and metalloproteinase with thrombospondin motifs, 5 | 5.93 | major aggrecanase in mouse cartilage |
| Hmga2 | The high mobility group A 2 | 5.39 | necrosis |
| Gadd45 | Growth Arrest and DNA Damage | 4.29 | DNA repair |
| Pim1 | Proto-oncogene protein kinase | 3.67 | apoptosis |
| Bcl6 | B-cell lymphoma 6 protein | 3.41 | apoptosis |
| CDKNIB | p27 | 2.85 | growth arrest |
| Adam17 | ADAM metallopeptidase domain 17 | 2.00 | Sheddase |
| Pink1 | PTEN-induced putative kinase 1 | 2.00 | apoptosis |

## Down-regulated genes

| Gene name | Encoded protein | Fold change | Potential function |
|---|---|---|---|
| TIMP3 | tissue inhibitor of metalloproteinase 3 | -27.16 | MMP inhibitor |
| WISP2 | WNT1-inducible-signaling pathway protein 2 | -7.10 | Akt activator and apoptosis inhibition |
| Itga11 | Integrin alpha-11 | -5.54 | Cell attachment to ECM |
| Cdh13 | T-cadherin | -5.18 | Cell adhesion |
| Tagln | SM22α | -4.00 | SMC contractile marker |
| Cnn1 | Calponin 1 | -4.02 | SMC contractile marker |
| WISP1 | WNT1-inducible-signaling pathway protein 1 | -3.42 | Akt activator and apoptosis inhibition |
| Itga7 | Integrin alpha-7 | -2.96 | Cell attachment to ECM |
| Ccnd1 | G1/S-specific cyclin-D1 | -2.72 | Cell cycle progression |
| smtn | Smoothelin | -2.12 | SMC contractile marker |
| Itga5 | Integrin alpha-5 | -2.00 | Cell attachment to ECM |
| Myh10 | SMC myosin heavy chain isoform | -2.00 | SMC contractile marker |
| des | Desmin | -2.00 | SMC contractile marker |

**Supplemental Table I.**
Most upregulated and down-regulated genes in FOXO3aA3ER$^{TM}$ vs. control VSMCs 4 hours after HT.

| Name | Promoter sequence | Comments |
|---|---|---|
| pMMP13-luc | -1600 - +1 | |
| pMMP13M-luc | -1600 - +1 mutant | Site directed mutagenesis of above |
| pMMP2-luc | -1545 - +1 | |
| pMMP3-luc | -1551 - +39 | |
| pMMP9-luc | -1775 - +1 | |
| pFHRE-luc | -743 - -648 | |
| pRL-CMV | 7-803 | |

**Supplemental Table II. MMP or Foxo response element reporter plasmids**
Sequence numbers are relative to the transcriptional start site.

| | ApoE$^{-/-}$ | FOXO | p value |
|---|---|---|---|
| **Cholesterol (mmol/L)** | | | |
| 0 | 7.68±1.99 | 6.94±1.16 | 0.25 |
| 7 | 10.77±1.86 | 10.68±1.25 | 0.73 |
| 14 | 13.30±2.33 | 13.33±1.78 | 0.81 |
| **Triglyceride (mmol/L)** | | | |
| 0 | 1.17±0.26 | 1.22±0.36 | 0.62 |
| 7 | 0.73±0.15 | 0.78±0.14 | 0.40 |
| 14 | 1.28±0.36 | 1.13±0.28 | 0.24 |
| **LDL (mmol/L)** | | | |
| 0 | 6.60±1.91 | 5.78±1.11 | 0.19 |
| 7 | 10.16±1.85 | 10.02±1.18 | 0.65 |
| 14 | 12.38±2.15 | 12.54±1.66 | 0.92 |
| **HDL (mmol/L)** | | | |
| 0 | 0.55±0.20 | 0.61±0.17 | 0.48 |
| 7 | 0.28±0.07 | 0.31±0.04 | 0.099 |
| 14 | 0.34±0.10 | 0.29±0.05 | 0.10 |

**Supplemental Table III**
Serum lipids in ApoE$^{-/-}$ littermate control (ApoE$^{-/-}$)(n=13) or SM22αFOXO3aA3ER$^{TM}$/ApoE$^{-/-}$ (FOXO)(n=14) mice at 0, 7 and 14w of high fat diet and HT administration. Data are means±SD.

| | Systolic (mmHg) | Diastolic (mmHg) | Mean (mmHg) | HR (beats/min) |
|---|---|---|---|---|
| **ApoE$^{-/-}$** | 107.51±11 | 58.32±13 | 68.78±13 | 659.77±33 |
| **FOXO** | 113.89±10 | 56.32±12 | 68.44±12 | 626.44±48 |
| **p value** | 0.151 | 0.694 | 0.946 | 0.056 |

**Supplemental Table IV**
Blood pressure and heart rate of ApoE$^{-/-}$ littermate control (ApoE$^{-/-}$)(n=13) or SM22αFOXO3aA3ER$^{TM}$/ApoE$^{-/-}$ (FOXO) mice (n=14) after 14 weeks high fat diet and HT administration. Data are means±SD.

| Cytokine (pg/mL) | ApoE$^{-/-}$ | FOXO | p value |
|---|---|---|---|
| CXCL-9 | 80.60±39.97 | 109.37±86.39 | 0.47 |
| IFN-γ | 1.00±0.42 | 1.43±1.82 | 0.57 |
| IL-10 | 47.49±17.15 | 43.91±6.53 | 0.62 |
| IL-1β | 1.26±0.59 | 1.03±0.32 | 0.37 |
| IL-2 | 2.29±1.01 | 2.35±1.09 | 0.88 |
| IL-5 | 25.04±9.57 | 23.68±8.83 | 0.79 |
| IL-6 | 73.42±45.54 | 69.96±40.71 | 0.89 |
| TNF-α | 22.47±8.31 | 22.26±4.53 | 0.96 |

**Supplemental Table V**
Serum cytokines in ApoE$^{-/-}$ littermate control (ApoE$^{-/-}$)(n=6) or SM22αFOXO3aA3ER$^{TM}$/ApoE$^{-/-}$ (FOXO) mice (n=7) after 14 weeks high fat diet and HT administration. Data are means±SD.

| | Control (ApoE$^{-/-}$) | SM22α-FOXO3aER$^{TM}$/ApoE$^{-/-}$ | p Value |
|---|---|---|---|
| **Aortic root** | | | |
| Plaque area ($\mu m^2$) | 307808 ± 33937 | 490227 ± 41346 | **0.0024** |
| Necrotic core area ($\mu m^2$) | 67723 ± 12840 | 203535 ± 36488 | **0.0022** |
| Core/Plaque ratio | 0.219±0.02 | 0.402±0.06 | **0.014** |
| Cap area ($\mu m^2$) | 72111 ± 11377 | 50207 ± 10319 | 0.1653 |
| Cap cell count | 181.3 ± 26.94 | 152.1 ± 39.66 | 0.5536 |
| Cap/Core ratio | 1.479 ± 0.40 | 0.265 ± 0.026 | **0.0044** |
| Cap/Plaque ratio | 0.225±0.02 | 0.0986±0.02 | **0.0001** |
| TUNEL (% cells) | 6.154 ± 1.480 | 19.08 ± 1.943 | **< 0.0001** |
| BrdU (% cells) | 3.45 ± 1.58 | 4.24 ±2.61 | 0.191 |

**Supplemental Table VI**
Morphometric and immunohistochemical quantification of aortic root atherosclerotic plaques in Control ApoE$^{-/-}$ and SM22a-FOXO3aER$^{TM}$/ApoE$^{-/-}$ mice at 22w after fat feeding for 14w and Tamoxifen administration. Data are means±SD (n=11-14).

| | Control + vehicle | Control + WAY | p value vs. vehicle control | SM22α-FOXO3aER$^{TM}$ + vehicle | SM22α-FOXO3aER$^{TM}$ + WAY | P value vs. vehicle control |
|---|---|---|---|---|---|---|
| Lumen area ($\mu m^2$) | 34710±7543 | 22440±2856 | 0.079 | 24383±6310 | 34943±11564 | 0.223 |
| Intimal area ($\mu m^2$) | 17222±4726 | 8376±3731 | 0.083 | 37301±9442 | 9886±4552 | **0.011** |
| Medial area ($\mu m^2$) | 40291±6299 | 49636±4850 | 0.130 | 40926±4437 | 62958±16227 | 0.120 |
| Intimal Cell count | 127±31 | 106±39 | 0.346 | 331±90 | 130±59 | **0.043** |
| Medial Cell count | 164±23 | 147±12 | 0.260 | 196±26 | 153±27 | 0.138 |
| Intimal Cellularity (cells/$mm^2$) | 11765±1807 | 14681±1700 | 0.131 | 10511±703 | 13006±423 | **0.005** |
| Medial Cellularity (cells/$mm^2$) | 4617±613 | 3016±197 | 0.018 | 4818±325 | 2833±418 | **0.003** |
| Cell proliferation (No of cells) | 197.7±63.9 | 166.8±81.3 | 0.38 | 611.4±144.1 | 115.8±89.0 | **0.027** |
| Apoptosis (No of cells) | 1.1±0.5 | 0.0±0.0 | **0.020** | 2.4±0.5 | 0.6±0.6 | **0.024** |

**Supplemental Table VII**
Morphometry of left carotid artery at 28 days post ligation of FOXO3aA3ER$^{TM}$ or littermate control mice, treated with HT throughout ± WAY170523 or vehicle control from 0-7 days. Cell proliferation and apoptosis represent number of cells positive for BrdU or TUNEL in both intima and media. Data are means±SEM, n=6-11.