Caffeine prevents restenosis and inhibits vascular smooth muscle cell proliferation through the induction of autophagy

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Abstract:

Caffeine is among the most highly consumed substances worldwide, and it has been associated with decreased cardiovascular risk. Caffeine inhibits the proliferation of vascular smooth muscle cells (VSMCs); however, little is known about the mechanism(s). Here, we demonstrated that caffeine decreased VSMC proliferation and induced autophagy in an *in vivo* vascular injury model of restenosis. Further, we studied the effects of caffeine in primary human and mouse aortic VSMCs and immortalized mouse aortic VSMCs. Caffeine decreased cell proliferation, and induced autophagy flux via inhibition of mTOR signaling in these cells. Genetic deletion of the key autophagic gene, *ATG5*, and its adaptor protein, *SQSTM1*/*p62*, showed the anti-proliferative effect by caffeine was dependent upon autophagy. Interestingly, caffeine also decreased Wnt-signaling and the expression of two Wnt target genes, *AXIN2* and *Cyclin D1*. This effect was mediated by autophagic degradation of a key member of the Wnt signaling cascade, DVL2, by caffeine to decrease Wnt signaling and cell proliferation. *SQSTM1*/*p62*, MAP1LC3B-II and Dvl2 were also shown to interact with each other, and the overexpression of Dvl2 counteracted the inhibition of cell proliferation by caffeine. Taken together, our *in vivo* and *in vitro* findings have demonstrated that induction of autophagy by caffeine significantly reduced vascular restenosis. Caffeine reduced VSMC proliferation by inhibiting Wnt signaling via stimulation of autophagy. Our findings suggest that caffeine and other autophagy-inducing drugs may represent novel cardiovascular therapeutic tools to protect against restenosis after angioplasty and/or stent placement.

Keywords: Caffeine, Vascular Smooth Muscle Cells, Autophagy, Wnt Signaling, Restenosis.

Introduction

Cardiovascular diseases remain the leading cause of mortality worldwide despite significant progress in vascular treatments and interventions during recent decades. Currently, percutaneous coronary intervention (PCI) is the established therapeutic strategy for the treatment of patients with coronary and peripheral artery disease, and acute myocardial infarction[1,2]. However, PCI induces hyperproliferation of vascular smooth muscle cells (VSMCs)[3,4] that can lead to restenosis and the need for re-intervention. Although the introduction of drug-eluting stents has reduced restenosis rates significantly compared to balloon angioplasty[5], restenosis still remains a major concern in clinical practice since 1-year recurrence rates of restenosis are as high as 15%[6, 7]. Therefore, new treatments are needed to reduce rates of restenosis following vascular interventions.

Caffeine (1,3,7-trimethylxanthine) is one of the most frequently ingested natural drugs in the world[8] due to the widespread consumption of coffee and tea. Both beverages are often contraindicated for CVD patients since they can increase blood pressure and heart rate. Several recent studies have shown an inverse relationship between long-term coffee consumption and CVD risk[9, 10] However, it is currently not known whether caffeine or other components of coffee may be playing the major role in these observations [11]. Several studies also have suggested that caffeine may have beneficial pharmacological effects on vascular healing after mechanical interventions. First, caffeine facilitates the recruitment of bone marrow endothelial progenitor cells and their nitric oxide production[12,11]to promote vascular repair after mechanical injury. Second, caffeine inhibits the proliferation of VSMCs and decreases restenosis[13,14]. However, the mechanisms involved in these processes are not known.

Intracellular and/or extracellular stimuli such as reactive oxygen species, oxidized low-density lipoproteins and oxysterols can activated autophagy, decrease proliferation, and protect VSMCs from cell death[15]. Rapamycin-based drugs which activate MTOR-dependent autophagy and trehalose which stimulates MTOR-independent, prevent cell proliferation and promote a contractile phenotype in VSMCs[16-18]. Since we previously observed induction of autophagy by caffeine in the liver[19], we examined whether caffeine could reduce proliferation of VSMCs and protect against neointimal hyperplasia by inducing autophagy. In this study, we found that the induction of autophagy by caffeine reduced VSMC proliferation and neointima formation after vascular injury *in vivo*. We also showed that caffeine inhibited VSMC proliferation by decreasing Wnt signaling via Dvl2 degradation by autophagy. These novel findings demonstrating caffeine's effects on autophagy and cell proliferation in VSMCs suggest that caffeine and other autophagy-inducing drugs may be potential therapies for preventing or treating restenosis after angioplasty and/or stent placement.

Results

Caffeine inhibits neointima formation in a mouse model of restenosis.

Although VSMCs play a pivotal role in the pathogenesis of many proliferative vascular diseases, they rarely proliferate in mature blood vessels, as they mainly perform a contractile function. However, in response to injury, VSMCs can undergo a phenotypic change characterized by decreased contractile marker expression, increased proliferation, and extracellular matrix synthesis [20]. To investigate the role of caffeine on autophagy and VSMC proliferation *in vivo*, we employed a mouse model of mechanical vascular injury-induced restenosis. After de-endothelialization, the mice were treated with and without caffeine for two weeks. The caffeine-treated mice showed significantly less neointima and plaque formation than control mice that did not receive caffeine (Figure 1A) when assessed by Elastica-van Gieson staining. However, there

was no difference observed in media formation in the control and treatment groups. Immunofluorescent staining of the lesion areas showed that the effect of caffeine was mostly due to inhibition of VSMC proliferation (Figure 1B) rather than accumulation of macrophages (Figure 1C). Double staining of Map1lc3b-ii/smooth muscle actin (SMA) showed that the inhibitory effects of caffeine on VSMC proliferation were associated with enhanced autophagy as evidenced by increased Map1lc3b-ii co-staining with α-SMA (Figure 1D). Additionally, the caffeine-mediated decrease in VSMC cell proliferation was associated with decreased collagen production (Figure 1E).

Caffeine caused phenotypic switching and decreased aortic smooth muscle cell proliferation

To examine the direct effects of caffeine on differentiation and proliferation of aortic smooth muscle cells (AoSMCs), we examined its effects in cell culture. Caffeine induced AoSMCs to differentiate from the synthetic phenotype to the contractile phenotype, which undergoes less proliferation by increasing contractile markers such as Acta2, Calponin and SM22a, and decreasing Col1a1 protein expression (Figures 2A-E). We next investigated the direct effect of caffeine on cell proliferation of AoSMCs in cell culture. Interestingly, caffeine decreased AoSMC proliferation in primary human, mouse, and immortalized mouse cell lines as measured by crystal violet cell proliferation assay (Figures 2F-H). We also confirmed this finding using the WST-8 and BrdU cell proliferation assay (Supplementary Figure 1A, B). Furthermore, we observed that caffeine significantly decreased the protein expression of two molecular markers of cell proliferation, proliferating cell nuclear antigen (Pcna) and chromatin assembly factor 1, subunit A (splice variants P60 and P150) (Figures 2I-K). We further observed that the inhibition of cell

proliferation by caffeine did not cause apoptosis since there was no change in caspase-3 cleavage, a hallmark of apoptotic cell death (Supplementary Figure 1C).

Caffeine increased autophagic flux and inhibited MTOR signaling in aortic smooth muscle cells

We next examined the effect of caffeine on autophagy in AoSMCs, and observed that it induced autophagy in primary human and mouse AoSMCs, and immortalized AoSMCs (Figure 3A and Supplementary Figure 2A, B). Caffeine also increased levels of the key autophagy protein, Map1lc3b-ii, and reduced Sqstm1/p62 levels in a dose-dependent manner suggesting that it stimulated autophagy flux (Figure 3B). We examined whether caffeine increased autophagy flux by using bafilomycin A1, a lysosomal acidification inhibitor, to block autophagy and measuring the change in Map1lc3b-ii protein levels. We observed a significant increase in Map1lc3b-ii levels in cells treated with caffeine and bafilomycin A1 (Figure 3C) demonstrating that caffeine increased autophagy flux. Caffeine also increased the number of immunofluorescent Map1lc3b-specific puncta in AoSMCs (Figure 3D, E) confirming that it increased autophagy.

Gene expression analyses of key autophagy genes showed that caffeine significantly increased Map1lc3b-ii, *Atg5, Atg7*, and *Beclin1* mRNA expression. However, there was no change in *Sqstm1/p62* mRNA expression (Figure 3F). Caffeine also decreased phosphorylation of mtor and its downstream target p70S6K (Figures 3G). These pro-autophagic cell signaling changes were observed in both primary human and mouse AoSMCs as well as in immortalized AoSMCs, and suggested that the induction of autophagy by caffeine was mediated by the inhibition of MTOR signaling (Supplementary Figure 3A,B and Figure 3G).

Caffeine inhibited aortic smooth muscle cell proliferation in an autophagy-dependent manner

We then examined whether caffeine's anti-proliferative action was dependent upon its induction of autophagy by knocking down the key autophagy gene, *Atg5*, in AoSMCs. Indeed, caffeine's anti-proliferative effect was significantly reduced with decreased Atg5 expression, showing that an increase in autophagy was critical for caffeine's anti-proliferative activity (Figures 3H-I). In combination with our other *in vivo* and *in vitro* results, these findings were consistent with the notion that caffeine inhibited VSMC proliferation and neointima formation after vascular injury by increasing autophagy in VSMCs.

***Caffeine inhibited Wnt signaling by degrading its mediator protein,* Dvl2**

Autophagy has been reported to inhibit Wnt signaling[21], and Wnt signaling can stimulate VSMC proliferation[22]. Accordingly, we examined the effects of caffeine on the expression of Wnt signaling target genes and found that it significantly reduced *Axin2* and *Cyclin D1* mRNA expression levels (Supplementary Figure 4A, B) in primary and immortalized AoSMCs (Figure 4A). We also found that caffeine decreased Dvl2 protein levels in primary AoSMCs (Supplementary Figure 4C, D) as well as in immortalized AoSMCs (Figure 4B). Interestingly, caffeine decreased Dvl2 protein expression in a dose- and time-dependent manner that was associated with increased autophagy in these cells (Figures 4C and D). We also observed parallel trends between Sqstm1/p62 and Dvl2 protein levels that occurred in a dose- and time-dependent manner (Figures 4C and D). Moreover, inhibition of autophagy by bafilomycin A1 significantly increased the accumulation of Dvl2 in caffeine-treated cells (Figure 4F), suggesting that the degradation of Dvl2 was autophagy-mediated. Thus, we hypothesized caffeine inhibited Wnt signaling by this mechanism since Dvl2 can stabilize and protect β -catenin (another key mediator of Wnt signaling) from degradation[23, 24]. In this connection, we found that caffeine decreased β-catenin expression in a dose- and time-dependent manner (Supplementary Figures 4F and G).

Inhibition of caffeine-stimulated autophagy by bafilomycin A1 also increased the accumulation of β-catenin generated by caffeine (Supplementary Figure 4H).

To further demonstrate a critical role of autophagy on Dvl2 expression, we performed *Atg5* siRNA knockdown in immortalized mouse AoSMCs, and found that Atg5 siRNA knockdown blocked caffeine-induced Dvl2 degradation (Figure 4F). ATG5 siRNA also inhibited caffeine-induced autophagy by causing decreased Map1lc3b-ii and increased Sqstm1/p62 expression (Figure 4F). Furthermore, *Atg5* knockdown significantly the increased mRNA expression levels of the Wnt target genes *Axin2* and *Cyclin D1* in immortalized AoSMCs (Figure 4G). Significantly, there were no changes in *Dvl2* or β-*catenin* (*Ctnnb1*) mRNA levels in immortalized mouse AoSMCs treated with caffeine (Supplementary Figure 4H), which further suggested that caffeine decreased *Dvl2* and β-*catenin* protein levels post-transcriptionally. Taken together, these results strongly suggested that caffeine inhibited Wnt signaling and its downstream target gene expression via autophagy-dependent degradation of Dvl2.

Caffeine's anti-proliferative action in aortic smooth muscle cells is Dvl2-dependent

To confirm the role of Dvl2 protein expression level in the caffeine-mediated inhibition of AoSMC proliferation, we performed gain-of-function as well as loss-of-function experiments in immortalized mouse AoSMCs by overexpressing wild-type Dvl2 in the presence of caffeine or by knocking down Dvl2 expression in the basal state. Overexpression of wild-type Dvl2 blocked the caffeine-mediated inhibition of cell proliferation as measured by both Pcna protein expression and crystal violet cell proliferation assays (Figures 5A-C). Interestingly, Dvl2 knockdown mimicked caffeine's effects on Pcna protein expression (Figure 5A), cell proliferation, and the mRNA expression of *Axin2* and *Cyclin D1* (Supplementary Figure 4I). In contrast, overexpression of

DVL2 completely rescued the caffeine-mediated inhibition of *Axin2* and *Cyclin D1* mRNA expression (Supplementary Figure 4I). Taken together, our data confirmed the key role of caffeine induced Dvl2 degradation in decreasing the effects of Wnt signaling on AoSMC proliferation.

Caffeine stimulation of Dvl2 degradation by autophagy mediated by direct Sqstm1/p62, Maplc3b-ii, and Dvl2 interaction

Previously, it was shown that Dvl2 and Sqstm1/p62 were able to interact with each other [21]. We thus performed co-immunoprecipitation assays to examine the potential interaction of endogenous Dvl2 and Sqstm1/p62. Interestingly, caffeine increased the interaction between Dvl2 and Sqstm1/p62 as well as the autophagosomal protein, Map1lc3b-ii (Figure 5D). Furthermore, knockdown of Sqstm1/p62 prevented the degradation of Dvl2 by caffeine (Figure 5E) and caffeine-mediated inhibition of cell proliferation (Figure 5F). Moreover, Sqstm1/p62 knockdown increased expression of the Wnt target genes *Axin2* and *Cyclin D1* (Supplementary Figure 4J) in a manner similar to Atg5 knockdown. These data showed that caffeine's inhibition of Wnt signaling depended upon the interaction of Dvl2 with Sqstm1/p62 and Map1lc3b-ii to promote autophagic degradation of Dvl2. Additionally, we also looked if the degradation of Dvl2 was autophagy-mediated. To confirm this, we treated cells with MG132 (proteasomal inhibitor). Cells treated with caffeine modestly increased ubiquitinated proteins while inducing Dvl2 degradation. On the other hand, MG132 robustly increased ubiquitinated proteins but did not decrease Dvl2 degradation. These findings suggested that caffeine-mediated degradation of Dvl2 was dependent on autophagy (Supplementary Figure 5).

Discussion:

VSMC proliferation and migration are involved in plaque formation and stability, and occur during early atherogenesis and vascular remodeling after mechanical injury[25]. VSMC proliferation

has been associated with intimal thickening, inflammation, foam cell formation, pathological angiogenesis, and calcification[26-29]. It previously was suggested that caffeine may inhibit the cell proliferation of VSMCs[13,14]. In order to determine whether caffeine could decrease AoSMC restenosis *in vivo*, we employed an *in vivo* mouse model of mechanical de-endothelialization, which mimics the restenosis process. In this *in vivo* model, we observed that caffeine reduced smooth muscle cell proliferation and induced autophagy. Furthermore, we observed that caffeine decreased cell proliferation and increased the autophagic flux in both human and mouse primary VSMCs and immortalized mouse VSMCs as evident by the increased MAP1LC3B-II and decreased SQSTM1/p62 levels, increased LC3B-II levels after bafilomycin treatment, and increased LC3B puncta by immunostaining. We also found that inhibition of cell proliferation by caffeine was critically dependent upon autophagy since ATG5 siRNA abrogated the inhibition of cell proliferation. We observed a decrease in the phosphorylation of MTOR and its downstream target, p70S6K in the VSMCs. This decrease in MTOR activity and stimulation of autophagy was similar to our previous findings for caffeine in the liver[19].

Wnt signaling plays an important role in VSMC proliferation[22, 30-32]. Dvl2 and its downstream target, β-catenin, are the critical mediator proteins of canonical and non-canonical Wnt signaling[28, 29]. Dvl2 is degraded via both the proteasome and autophagy-lysosome pathways, respectively[33]. Accordingly, we examined whether the Wnt signaling pathway was involved caffeine's induction of autophagy and reduction of cell proliferation. Interestingly, we found that caffeine markedly decreased both Dvl2 and β-catenin levels in VSMCs, and decreased the expression of several target genes regulated by β-catenin. We also found that the autophagic adaptor protein, SQSTM1/p62 and MAP1LC3B-II, interacted with Dvl2, and this complex was degraded by caffeine-induced autophagy. Of note, a previous report also showed that autophagy

reduced Dvl2 expression[21]. In that study, starvation induced the aggregation of Dvl2 and SQSTM1/p62, which then led to the recruitment and degradation of Dvl2 by MAP1LC3B-II - mediated autophagy. Surprisingly, we found that caffeine decreased Wnt signaling by enhancing the interaction of Dvl2 with SQSTM1/p62 and MAP1LC3B-II, to promote the autophagic degradation of Dvl2. Genetic ablation of Dvl2 inhibited VSMC proliferation by a similar amount as caffeine and overexpression of Dvl2 completely abolished caffeine's anti-proliferative effects on immortalized VSMCs further ascertained the critical role of DVL2 on cell proliferation and caffeine's role in Dvl2 degradation.

Taken together, our *in vivo* and *in vitro* findings suggest that caffeine inhibited VSMC proliferation as well as neointima formation after vascular injury by increasing autophagy in VSMCs. Caffeine's action on VSMC proliferation was primarily mediated by inhibition of Wnt signaling due to autophagic degradation of Dvl2. We and others previously showed that caffeine stimulated autophagy in the liver and muscle [19, 34, 35]; however, to the best of our knowledge, this is the first report showing a role for caffeine-induced autophagy in the cell proliferation of VSMCs. Further studies are needed to determine whether caffeine may cause similar effects on autophagy and cell proliferation in other tissues. Our findings on the mechanism (s) underlying caffeine-mediated inhibition of VSMC proliferation also may have clinical implications since there is a need for therapies to prevent VSMC hyper-proliferation. So far, there have not been any published reports on the potential association between caffeine, coffee, or tea consumption and the risk for restenosis. Our results suggest that such studies would be useful in determining whether there are potential protective effects of caffeine on restenosis after stent placement or angioplasty in certain patient populations. If so, caffeine and other autophagy-inducing drugs may be useful as

preventive and/or therapeutic treatments for VSMC hyper-proliferation after vascular injury and help decrease the risk for restenosis.

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Materials and Methods

Drugs and Reagents: Caffeine (1,3,7-Trimethylxanthine) was from Sigma-Aldrich. Antibodies against: LC3B, #2775; SQSTM1/p62, #5114; MTOR (7C10), #2983; phosphoMTOR (Ser2448) (D9C2), #5536; Dvl2 (30D2), #3224; Cleaved Caspase-3 (Asp175) (5A1E), #9664; Phospho-p70S6Kinase (Thr389), #9234; p70S6kinase (49D7), #2708; Atg5 (D5F5U), #12994; Non-phosphoβ-Catenin (Active) (Ser33/37/Thr41) (D13A1), #8814; GAPDH (D16H11), #5174; α-

Smooth Muscle Actin Antibody, (#14968) were from Cell Signaling Technology. Collagen I antibody, #ab34710 was from Abcam, while PCNA, #sc-7907 and β-Actin, #sc-130300 were from Santa Cruz Biotechnology. Culture media and Pen/Strep were from Gibco. Lipofectamine P3000 was from Invitrogen, while DharmaFECT™ 2 transfection reagent was from Dharmacon. 3XFlag DVL2 (WT) (#21074) was from Addgene. Anti-SMA (Smooth Muscle Actin, M0851 clone 1A4) from DAKO, Anti-Mac2(CL8942AP) from Cedarlane and LC3BII (ab48394) were from Abcam.

***In vivo* Model of Restenosis**

Animal model of restenosis. Male, 10 week-old *ApoE*$^{-/-}$ mice (C57BL/6J background) from Jax maintained on 12-hour dark/light cycle and fed an atherogenic high-fat diet (21% fat, 0.15% cholesterol; D12079B, Research Diets, NJ) for 1 week before and 2 weeks after injury were randomized into 2 groups (n=8), one receiving sterile water, and the other receiving 0.05% w/v caffeine in sterile water 2 weeks before and 2 weeks following injury. For endothelial denudation[36],[37], mice were anesthetized (100 mg/kg ketamine hydrochloride/10 mg/kg xylazine i.p.), and the left common carotid artery was de-endothelialized by the insertion of a 0.14 mm guide wire through a transverse arteriotomy of the external carotid artery. After 2 weeks, the mice were euthanized and perfused in-situ with 4% paraformaldehyde. The injured and un-injured control carotid arteries were isolated, fixed in 10% formalin, dehydrated, and embedded in paraffin. Serial 5μm transverse sections were collected within a distance of 0 to 50μm from the bifurcation, each 10th section was stained using Elastica-van Gieson, and areas of lumen, neointima (between lumen and internal elastic lamina), and media (between internal and external elastic laminae) were measured by planimetry using Diskus Software (Hilgers). Neointimal macrophages, vascular smooth muscle cells, autophagy, and fibrosis were visualized by immunofluorescence staining for MAC-2, SMA and LC3BII, respectively, followed by

fluorescein isothiocyanate (FITC)-conjugated or Cy3-conjugated secondary antibody staining (Jackson ImmunoResearch) as described. Animal studies were approved by the Biomedical Sciences Institute Singapore Institutional Animal Care Committee (Protocol #161165).

Cell culture, maintenance and in vitro treatment

Primary Mouse Aortic Smooth muscle cell Culture: Mouse primary AoSMCs were isolated from thoracic aortas of 6-9 weeks old male C57BL/6J mice. The intimal layer of the aorta was scraped off and the aorta was cut into smaller pieces and pressed down flat using 18-mm circular cover slips for 7-10 days in complete growth medium (1:1 DMEM/F12, 2 mM L-glutamine, 20% FBS, 1x penicillin/streptomycin). AoSMCs were used after the first passage.

Primary Human AoSMC Culture: Primary human AoSMCs were procured from Lonza (CC-2571; Clonetics™ AoSMCs) and cultured using SmGM-2 BulletKits (CC-3181 and CC-4149) containing SmBM Basal Medium and SingleQuots™ Kits (growth factors, cytokines, and supplements) as per manufacturer's description. The first 2 passages of AoSMCs were used in the experiments.

Immortalized AoSMC Culture: Mouse primary vascular AoSMCs, that were isolated by collagenase-elastase digestion and immortalized by transducing SV40 large T antigen, were procured from ATCC (MOVAS ATCC® CRL-2797™). Cells were cultured in DMEM, 10% fetal bovine serum, 1 x Pen/Strep and were passaged every 3-4 days.

In vitro Treatments: Cells were seeded in 6x24 well plates as required. Cells at ~50% confluency were serum starved overnight for synchronization and treated with caffeine (2 mM) for 48 h unless mentioned otherwise. For co-immunoprecipitation assays, cells were treated with caffeine (2 mM) overnight. For autophagic flux analyses, caffeine-treated cells were incubated with bafilomycin A1 (50 nM) for 5 h and cell lysate (Protein) was prepared using 1x Lammeli buffer.

Crystal Violet Cell Proliferation Assay: The assay was performed in 12 or 24 well plates in duplicate. AoSMCs were seeded and synchronized overnight by serum starvation followed by caffeine treatment for 48 h. After treatment, the plates were washed with PBS and fixed in 90% ethanol for 30 min. Plates were then washed with PBS and incubated with crystal violet stain (0.1%) for 30 min. Plates were washed with water, air dried and scanned. For quantification, 10% acetic acid was added to each well to dissolve the crystal violet stain, 100 □l aliquots from each well in triplicate were transferred to 96 well plates and absorbance was quantified at 592 nM using a Tecan spectrophotometer.

WST-8 Cell Proliferation Assay: AoSMCs were seeded in 96 well plates at 10^4 cells/well. Following overnight incubation, caffeine treatment was performed for 48 h and the WST-8 cell proliferation assay (Cayman chemicals #10010199*)* was carried out as per the manufacturer's protocol. Absorbance was measured at 450 nM using a Tecan spectrophotometer and the data was calculated as percentage change in cell proliferation.

BrdU Cell Proliferation ELISA Kit: AoSMCs were seeded in 96 well plates at 10^4 cells/well. Following overnight incubation, caffeine treatment was performed for 48 h and the BrdU cell proliferation assay (Cayman chemicals #ab126556) was carried out as per the manufacturer's protocol. Absorbance was measured at 450 nM using a Tecan spectrophotometer and the data was calculated as percentage change in cell proliferation.

Immunofluorescence and Confocal Microscopy: LC3B Antibody (Cell Signaling Technology®, #2775) was used (1:200 dilution) to analyze endogenous LC3B puncta formation in immortalized AoSMCs. Cells were cultured in chambered slides and a standard protocol was used (immunofluorescence protocol for suspension cells, Cell Signaling Technology®) to fix, permeabilize and immunostain the cells. Cells were mounted in VECTASHIELD Antifade

Mounting Medium (H-1000; Vector Laboratories, USA), visualized using the LSM710 Carl Zeiss confocal microscope (Carl Zeiss Microscopy GmbH, Germany) and images were captured using ZEN software (Black edition, Leica) at 40x magnification. Relative LC3B puncta/fluorescence per cell was calculated using ImageJ software (NIH).

Genetic Ablation of ATG5, SQSTM1, and DVL2 In Vitro: Silencer® Select siRNAs (Thermo Fisher Scientific) were used to silence *ATG5* and *SQSTM1*, and SMARTpool (ON-TARGETplus siRNA, GE Dharmacon) siRNA was used to silence *DVL2* in immortalized AoSMCs. When immortalized AoSMCs reached 70-80% confluency, they were transfected with Dharmacon™ DharmaFECT™ 2 transfection reagent as per manufacturer's protocols. 24 h after siRNA transfection, the medium was changed and cells were treated with caffeine for 48 h.

Plasmid Transfection and Confocal Microscopy: Immortalized AoSMCs were grown on chambered slides until 80% confluency. 3XFlag DVL2 (WT) (Addgene plasmid #21074; a gift from Jeff Wrana[38]) plasmid was transfected using lipofectamine 3000 (Invitrogen) as described in manufacturer's protocol. After 24 h of transfection, cells were treated with caffeine for 48 h.

Co-immunoprecipitation Assay: DVL2 antibody (30D2: #3224; Cell Signaling Technology) was used to immunoprecipitate (IP) endogenous DVL2 from immortalized AoSMCs. SQSTM1 antibody (sc-28359; Santa Cruz) and LC3B (2775; CST) was used to detect the IP of SQSTM1 and LC3B. IP was performed using Immunoprecipitation Starter Pack (GE Healthcare Life Sciences) as per the manufacturer's protocol. Immortalized AoSMCs were treated with caffeine (2 mM) overnight to perform Co-IP as long term treatment decreased DVL2 protein levels substantially. Detected SQSTM1 and LC3B in IP samples was normalized with IP-DVL2 levels and relative densitometric values were determined.

Western Blot Analysis: Cells were lysed using CelLytic™ M Cell Lysis Reagent (Sigma, C2978). Protein samples were prepared in 2x Laemmli Sample Buffer (BioRad #1610737), separated on SDS-PAGE and immunoblotted using the standard protocol described elsewhere[39]. Densitometric analysis was performed using ImageJ software (NIH, Bethesda, MD, USA).

RNA Isolation and Real-Time PCR: Total RNA was isolated using InviTrap Spin Universal RNA Mini Kit (Stratec, Germany) and RT-qPCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen, 204141) in Rotor-Gene® Q (Qiagen) according to the manufacturer's instructions. KiCqStart™ SYBR Green optimized primers from Sigma-Aldrich (KSPQ12012) were used to measure mRNA expression of genes and *POLR2A* was used as an internal control.

Quantitative and Statistical Analyses: Results are expressed as Mean±SD. Statistical significance was defined as $P<0.05$ and was assessed by either student's *t* -test or one-way ANOVA followed by Tukey's *post-hoc* test as required, using GraphPad Prism version 7.0 (GraphPad Software, La Jolla California USA, www.graphpad.com).

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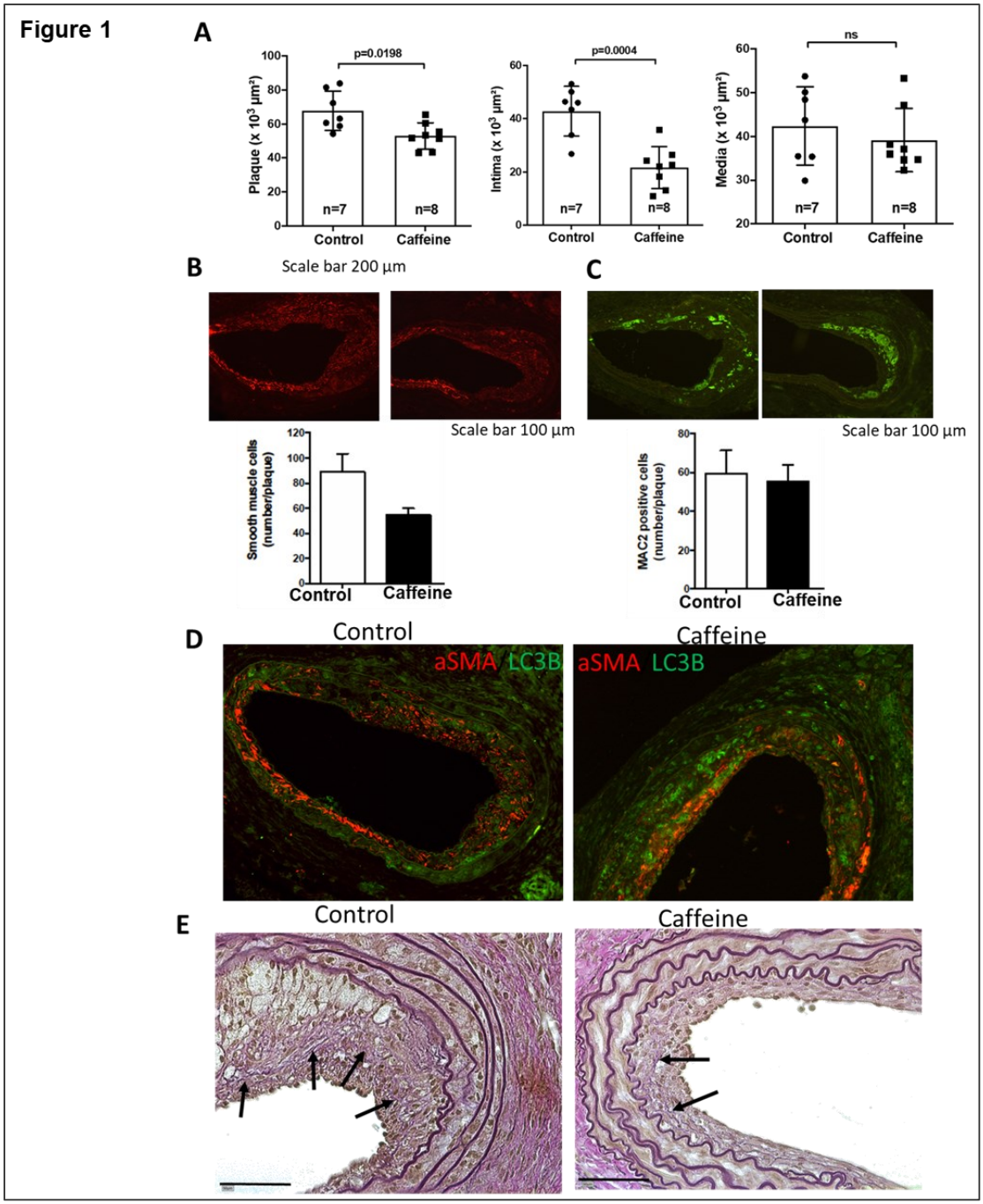
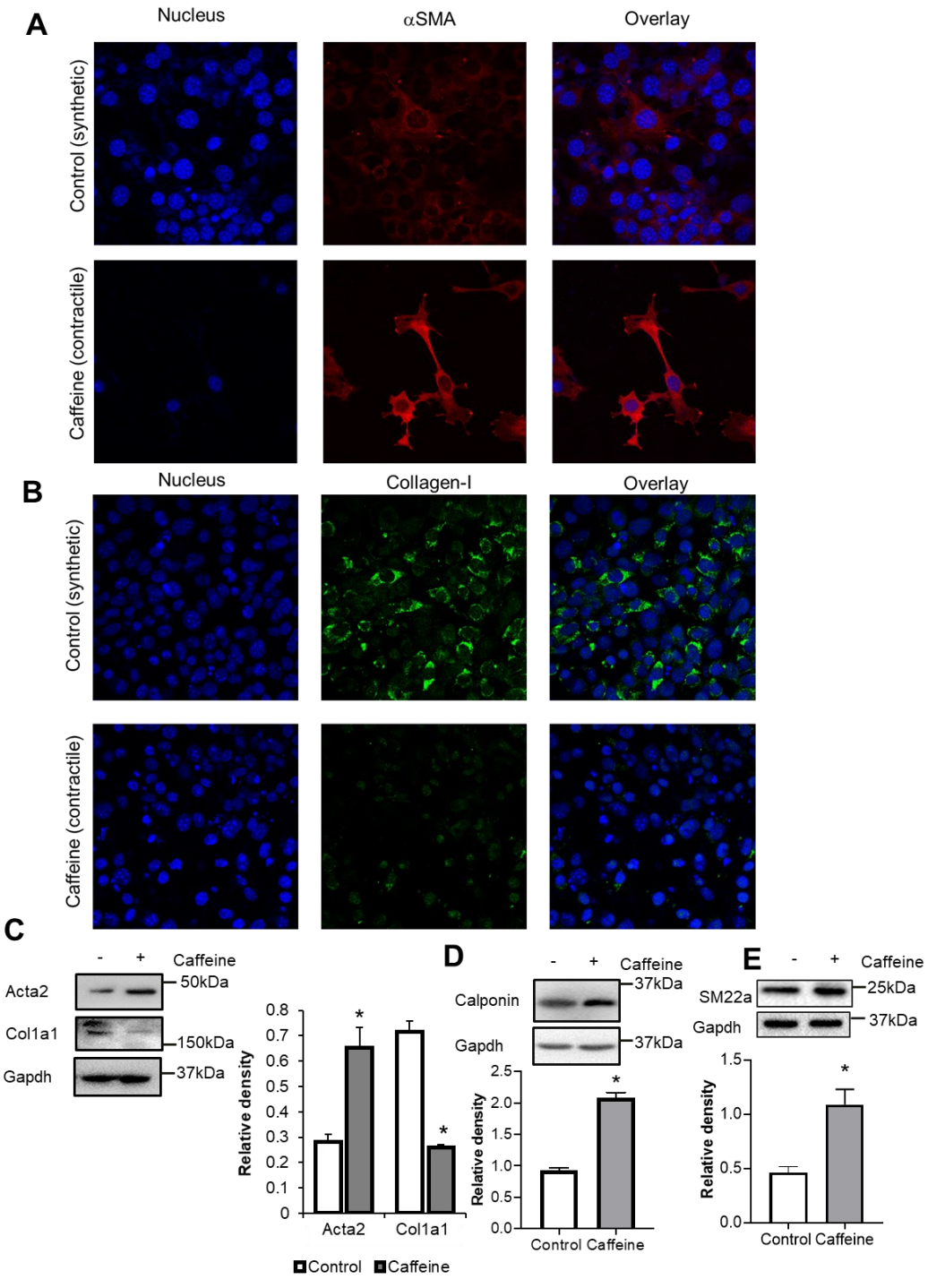


Figure 1. Caffeine inhibits neointima formation in a mouse model of restenosis.

(A) Mice treated with caffeine showed an improved healing and reduced neointima formation and plaque formation than the untreated mice in a mouse model of vascular injury-induced restenosis. Representative images of Elastica-van Gieson showing reduced plaque and unmodified media are presented (scale bar 200 µm). The reduction

in plaque size is due to inhibition of VSMC proliferation **(B)** as seen in SMA staining (red, scale bar 100 μm) and not macrophages **(C)**, as seen in Mac2 staining (green, scale bar 100 μm). Double staining of LC3BII/smooth muscle actin (SMA) showed that the inhibitory effects of caffeine on VSMC proliferation were associated with increased autophagy as increased LC3B co-stained with αSMA **(D)** and decreased collagen production (red fibers, scale bar 50 μm) **(E)**. Results are expressed as mean ± SD. The statistical significance of differences (*P < 0.05) was assessed by a one-way or two-way ANOVA wherever applicable, followed by Tukey's multiple-comparisons test, N=7-8 animals/group.

Figure 2: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αSMA
Overlay
Control (synthetic)
Caffeine (contractile)
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Nucleus
Collagen-I
Overlay
Control (synthetic)
Caffeine (contractile)
C
- + Caffeine
Acta2
50kDa
Col1a1
150kDa
Gapdh
37kDa
Relative density
Acta2
Col1a1
Control Caffeine
D
- + Caffeine
Calponin
37kDa
Gapdh
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Relative density
Control Caffeine
E
- + Caffeine
SM22a
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37kDa
Relative density
Control Caffeine

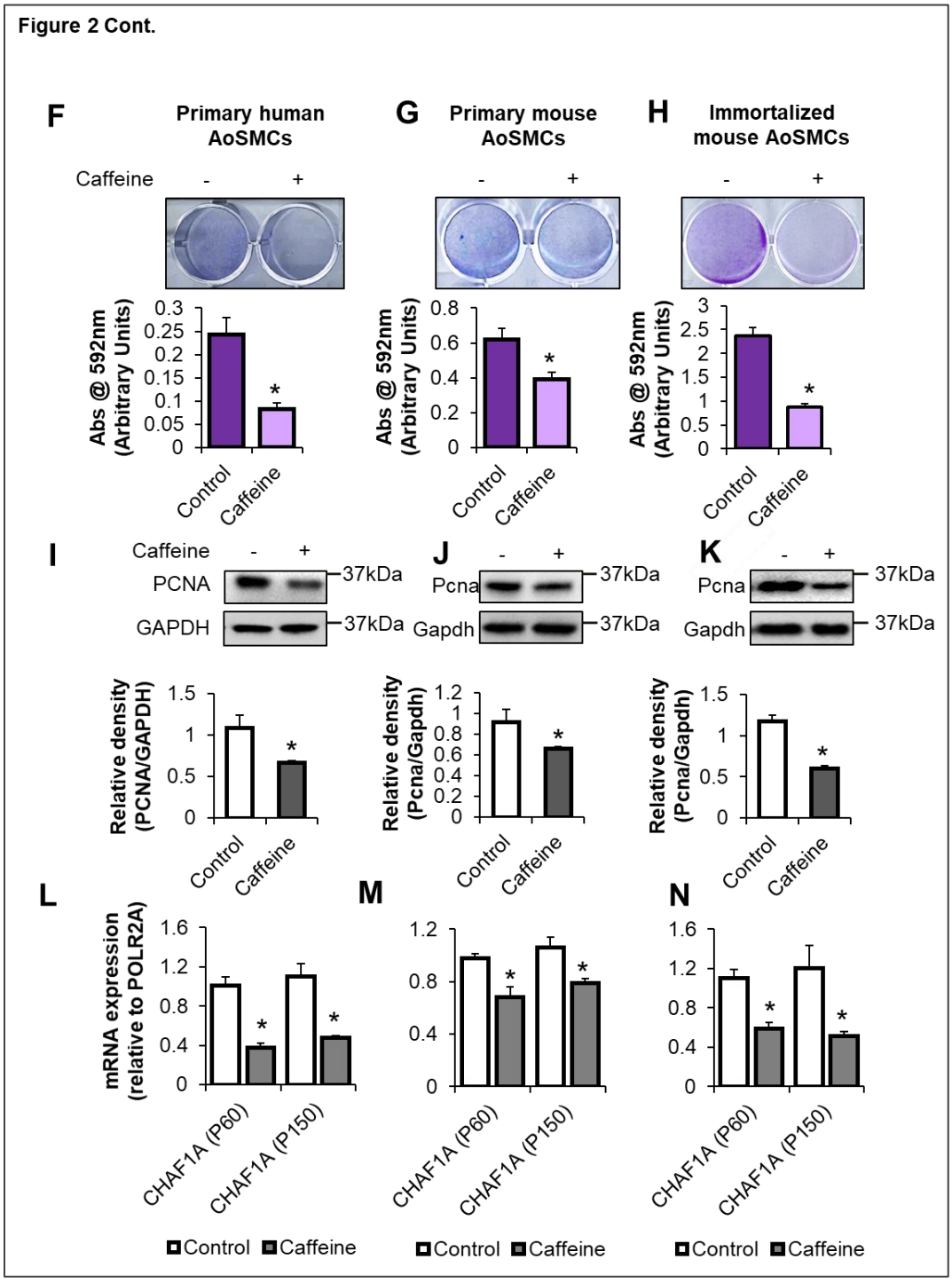


Figure 2. Caffeine stimulates phenotypic switching and inhibits the proliferation of primary and immortalized aortic smooth muscle cells.

Immunofluorescence analysis of AoSMCs phenotypic markers **(A)** alpha smooth muscle actin and **(B)** collagen type I respectively. Images were captured at 40x magnification and representative pictures were shown. Western blot analysis of AoSMCs phenotypic markers. **(C)** alpha smooth muscle actin and collagen type I and **(D)** Calponin in immortalized mouse AoSMCs treated with or without caffeine (2 mM) for 48 h. **(E)** SM22A in immortalized mouse AoSMCs treated with or without caffeine (2 mM) for 48 h. Quantitative analysis of AoSMCs phenotypic markers alpha smooth muscle actin (Acta2), collagen type I (Col1a1 and Calponin and SM22a was done and plotted as bar graphs. Crystal violet staining was performed on **(F)** primary human AoSMCs, **(G)** primary mouse AoSMCs, and **(H)** immortalized mouse AoSMCs, and absorbance was quantified as arbitrary units. Shown are representative images of wells containing cells treated ± caffeine (2 mM) for 48 h. Western blot analysis to determine PCNA was performed on **(I)** primary human AoSMCs, **(J)** primary mouse AoSMCs, and **(K)** immortalized mouse AoSMCs, treated ± caffeine (2 mM) for 48 h. RT-qPCR analyses of proliferation marker *CHAF1A* (splice variants P60 and P150) in **(L)** primary human AoSMCs, **(M)** primary mouse AoSMCs, and **(N)** immortalized mouse AoSMCs treated ± caffeine (2 mM) for 48 h. Results are expressed as mean ± SD. The statistical significance of differences (*$P < 0.05$) was assessed by a one-way or two-way ANOVA wherever applicable, followed by Tukey's multiple-comparisons test, N=3.

Figure 4:

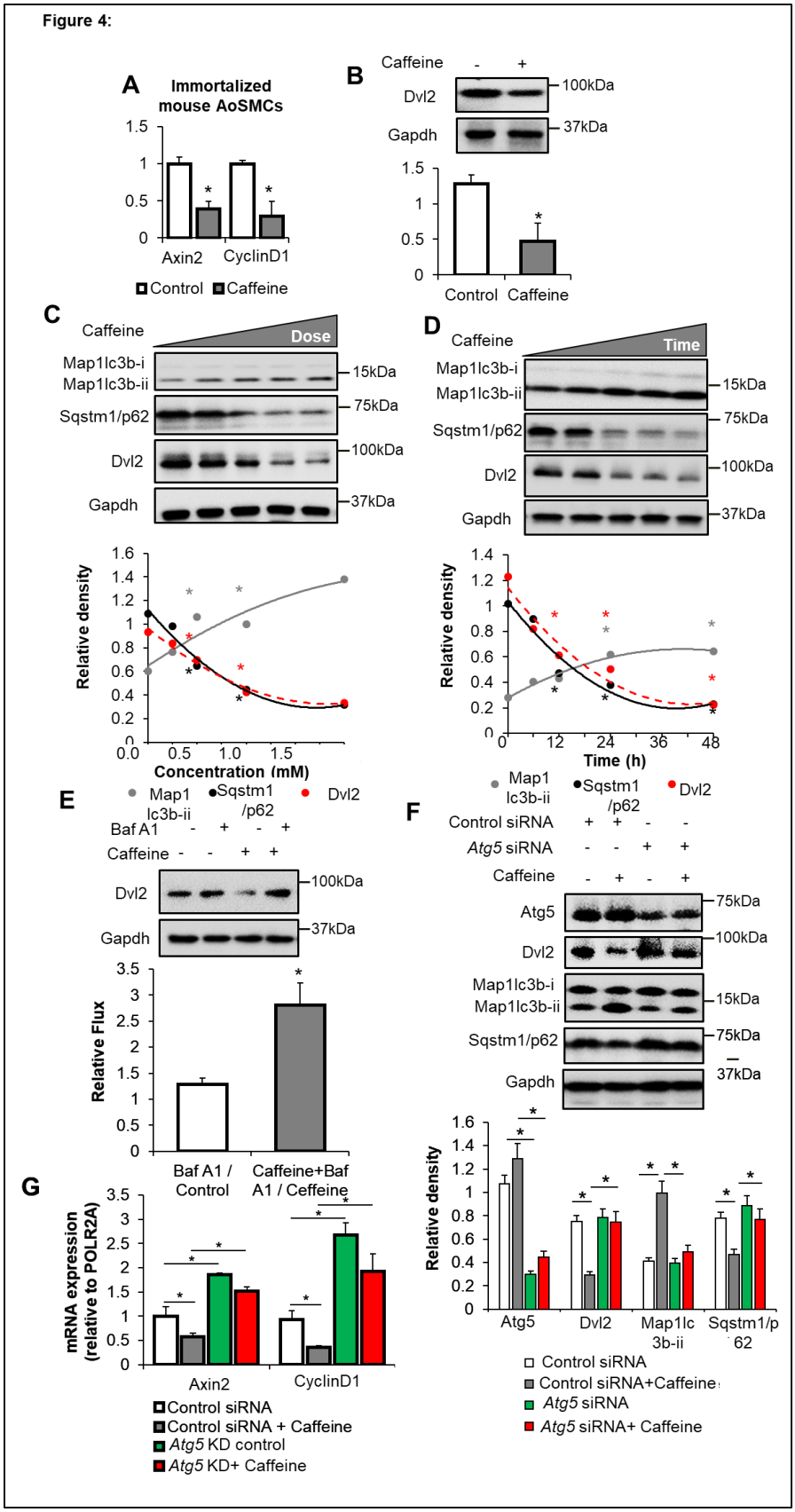

A
Immortalized mouse AoSMCs
Axin2
CyclinD1
Control
Caffeine
B
Caffeine
Dvl2
Gapdh
100kDa
37kDa
Control
Caffeine
C
Caffeine
Dose
Map1lc3b-i
Map1lc3b-ii
Sqstm1/p62
Dvl2
Gapdh
15kDa
75kDa
100kDa
37kDa
Relative density
Concentration (mM)
D
Caffeine
Time
Time (h)
E
Map1 lc3b-ii
Sqstm1 /p62
Dvl2
Baf A1
Caffeine
Relative Flux
Baf A1 / Control
Caffeine+Baf A1 / Ceffeine
F
Control siRNA
Atg5 siRNA
Caffeine
Atg5
Control siRNA+Caffeine
Atg5 siRNA
Atg5 siRNA+ Caffeine
G
mRNA expression (relative to POLR2A)
Axin2
CyclinD1
Control siRNA
Control siRNA + Caffeine
Atg5 KD control
Atg5 KD+ Caffeine

Figure 3. Caffeine induces autophagy by inhibiting MTOR signaling. Knockdown of ATG5 abolished the inhibition of aortic smooth muscle cell proliferation by caffeine.

Immunoblot analyses of the key autophagic marker proteins Microtubule-associated protein 1A/1B-light chain 3B (Maplc3b-ii) and Sqstm1/p62 were performed in **(A)** immortalized mouse AoSMCs treated ± caffeine (2 mM) for 48 h. Representative immunoblots and quantitative analyses of LC3B-II and SQSTM1 are shown. Relative densities are plotted as bar graphs. **(B)** Dose-dependent effects of caffeine on LC3B-II and Sqstm1/p62 in immortalized mouse AoSMCs treated with serial dilutions of caffeine (0.0, 0.25, 0.50, 1.0 and 2.0 mM) for 48 h. Immunoblots were performed and quantified. **(C)** Autophagic flux analyses by immunoblot of immortalized mouse AoSMCs treated ± caffeine (2 mM for 48 h) ± Bafilomycin A1 (50 nM for 5 h). Quantitative analyses of autophagy flux was calculated by determining the ratio of blot intensities of Maplc3b-ii in bafilomycin A1 (lysosomal inhibitor)-treated cells to those that were not treated with bafilomycin A1. Results are plotted as bar graphs showing autophagic flux. **(D)** Immunofluorescence analyses of endogenous Maplc3b for autophagic puncta formation in cells treated ± caffeine (2 mM for 48 h). Images were taken at 40x magnification and representative images are shown. **(E)** Quantitative analysis of Maplc3b-ii fluorescence normalized to nuclear stain (Hoechst 33258, Sigma). Results shown are the number of Maplc3b-ii positive puncta per cell. **(F)** RT-qPCR analyses in immortalized mouse AoSMCs treated with caffeine (2 mM; 48h) for key autophagy marker genes (Maplc3b-ii*, Sqstm1, Atg5, Atg7* and *Beclin1*). Immunoblotting to analyze mtor signaling

(phosphorylation of mtor and its downstream target p70S6K) was performed on **(G)** immortalized mouse AoSMCs, treated ± caffeine (2 mM) for 48 h. Crystal violet staining performed on immortalized Atg 5 Knock down mouse AoSMCs treated ± caffeine (2 mM for 48 h) and absorbance was plotted as an arbitrary units. Shown here are representative images of wells **(H)** containing cultured cells treated ± caffeine (2 mM) for 48 h. Crystal violet stain was dissolved in 10% acetic acid and colorimetric measurement was performed (**I**). Results are expressed as mean ± SD. The statistical significance of differences (*P < 0.05) was assessed by a one-way or two-way ANOVA wherever applicable, followed by Tukey's multiple-comparisons test, N=3.

Figure 4:

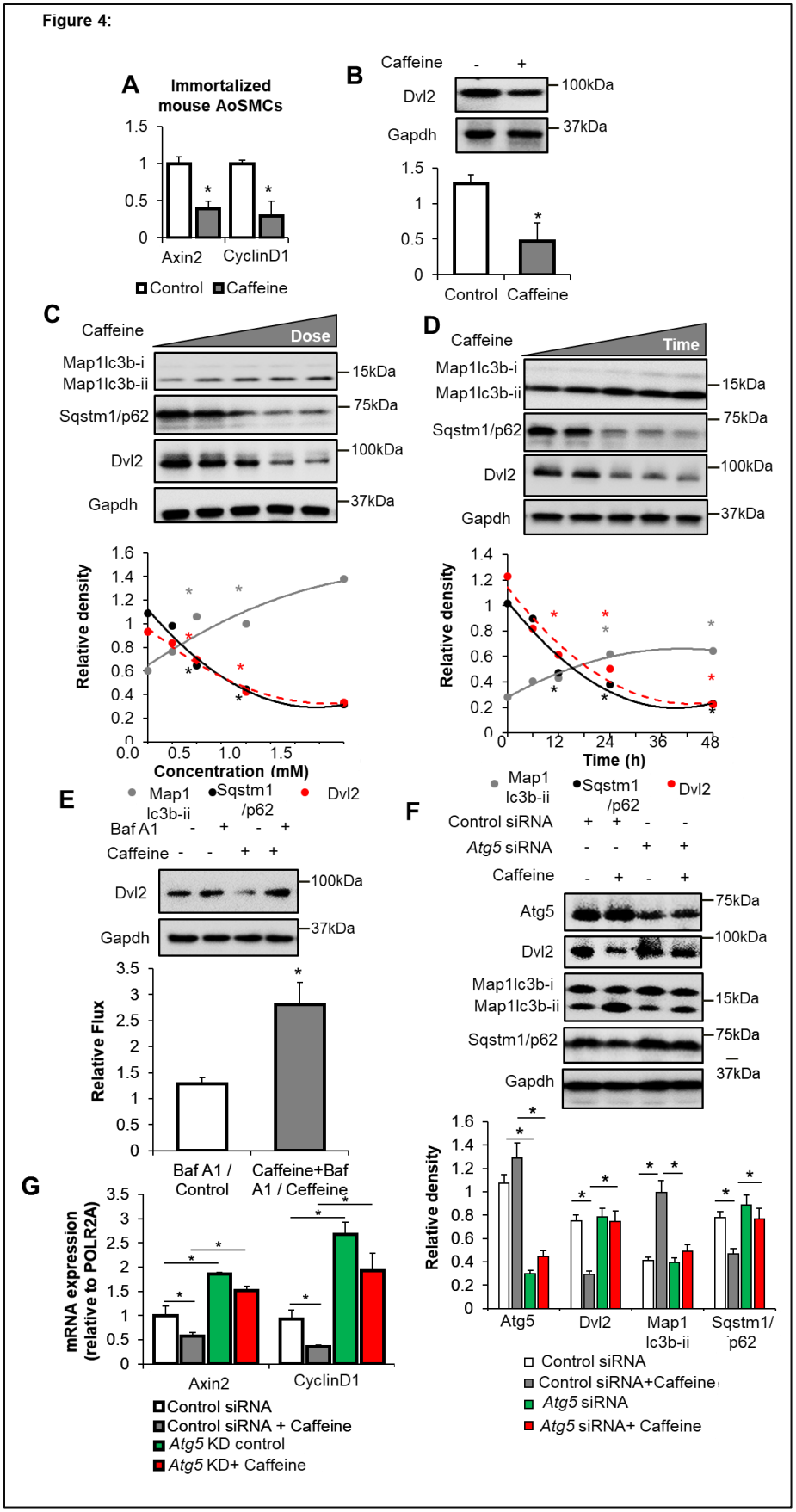
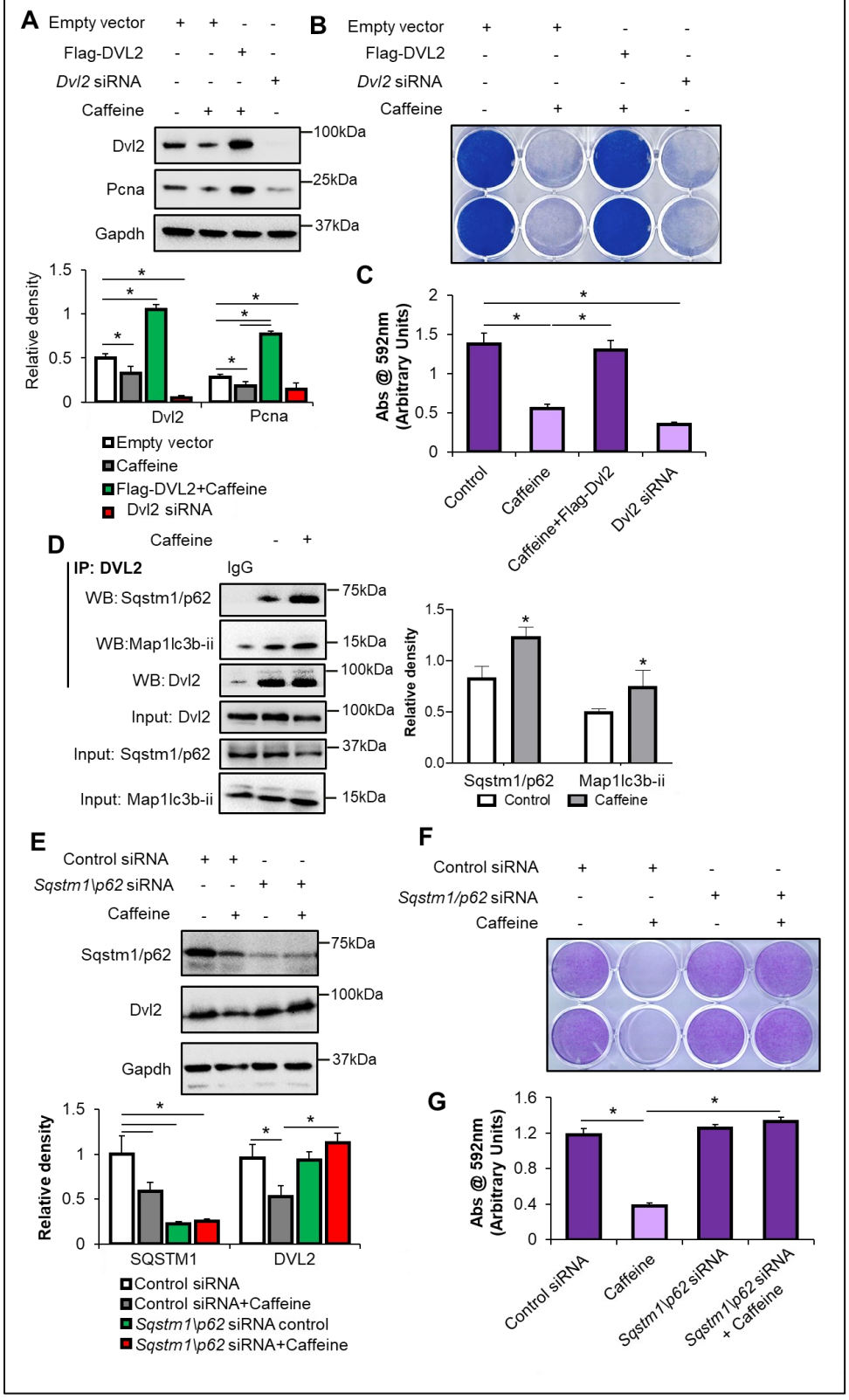

A
Immortalized mouse AoSMCs
Axin2
CyclinD1
Control
Caffeine
B
Caffeine
Dvl2
Gapdh
100kDa
37kDa
C
Caffeine
Dose
Map1lc3b-i
Map1lc3b-ii
Sqstm1/p62
15kDa
75kDa
Relative density
Concentration (mM)
D
Time
Time (h)
E
Baf A1
Relative Flux
Baf A1 / Control
Caffeine+Baf A1 / Ceffeine
F
Control siRNA
Atg5 siRNA
Atg5
Map1 lc3b-ii
Sqstm1 /p62
G
mRNA expression (relative to POLR2A)
Control siRNA
Control siRNA + Caffeine
Atg5 KD control
Atg5 KD+ Caffeine
Control siRNA+Caffeine
Atg5 siRNA
Atg5 siRNA+ Caffeine

Figure 4. Caffeine inhibits Wnt signaling and increases Dvl2 degradation in aortic smooth muscle cells.

RT-qPCR analyses of Wnt signaling target genes *Axin2 and CyclinD1* **(A)** immortalized mouse AoSMCs cells treated ± caffeine (2 mM) for 48 h. Immunoblotting for the key mediator of Wnt signaling, DVL2 was performed on **(B)** immortalized mouse AoSMCs, treated ± caffeine (2 mM) for 48 h. Quantitative analysis of their relative densities are shown. Immunoblot analyses to analyze caffeine dose-response (**C)** (0.0, 0.25, 0.50, 1.0 and 2.0 mM for 48 h) and time-course **(D)** (0, 6, 12, 24 and 48 h at 2 mM) in immortalized mouse AoSMCs. Quantitative analyses of their relative densities are shown. **(E)** Dvl2 degradation by autophagy was assessed using autophagic flux analyses in response to Bafilomycin A1 (50 nM for 5 h) in caffeine (2 mM for 48 h) ± treated immortalized mouse AoSMCs. Quantitative analyses of Dvl2 degradation was calculated by determining the ratio of blot intensities of bafilomycin A1 (lysosomal inhibitor)-treated cells to those that were not treated with bafilomycin A1. Bar graphs of autophagic flux are shown. Data is shown as Mean±SD and **P*<0.05 was considered statistically significant. **(F)** Immunoblotting of the key mediator of Wnt signaling, Dvl2 was performed on immortalized mouse AoSMCs in which Atg5 was knocked down, treated ± caffeine (2mM) for 48 h. Quantitative analyses of the relative densities of Atg5 knockdown, Dvl2 degradation and autophagic markers Maplc3b-ii and Sqstm1/p62 are shown. **(G)** RT-qPCR analyses of target genes of the Wnt signaling pathway, *Axin2 and CyclinD1* in immortalized mouse AoSMCs in which Atg5 was knocked down, treated ± caffeine (2 mM) for 48 h. Results are expressed as mean ± SD. The statistical significance of differences (*P < 0.05) was

assessed by a one-way or two-way ANOVA wherever applicable, followed by Tukey's multiple-comparisons test, N=3.

Figure 5: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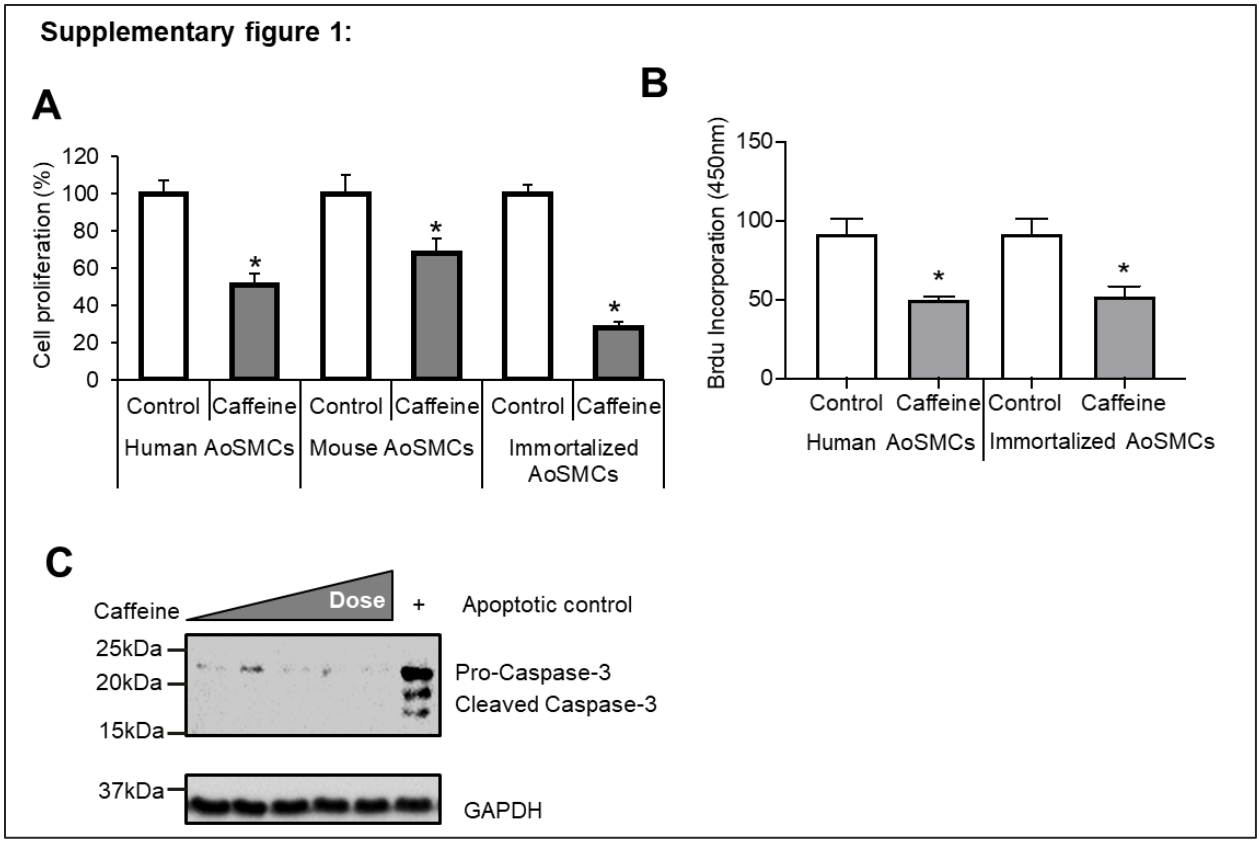
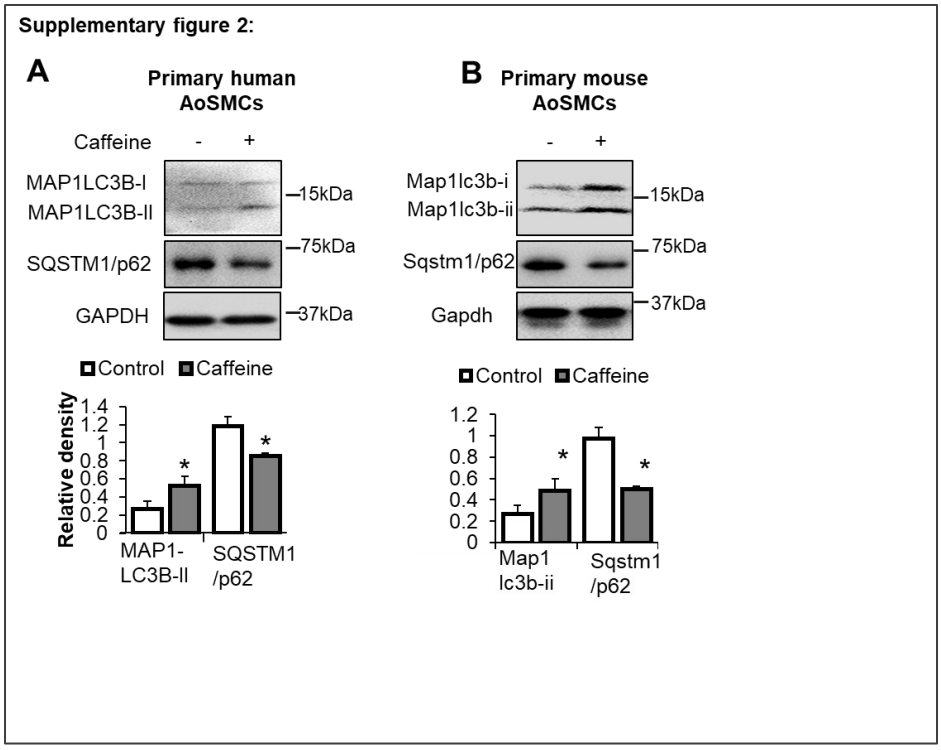
Pcna
Gapdh
100kDa
25kDa
37kDa
Relative density
Dvl2
Pcna
Empty vector
Caffeine
Flag-DVL2+Caffeine
Dvl2 siRNA
B
Empty vector + + - -
Flag-DVL2 - - + -
Dvl2 siRNA - - - +
Caffeine - + + -
C
Abs @ 592nm (Arbitrary Units)
Control
Caffeine
Caffeine+Flag-Dvl2
Dvl2 siRNA
D
Caffeine - +
IP: DVL2
IgG
WB: Sqstm1/p62
WB:Map1lc3b-ii
WB: Dvl2
Input: Dvl2
Input: Sqstm1/p62
Input: Map1lc3b-ii
75kDa
15kDa
100kDa
100kDa
37kDa
15kDa
Relative density
Sqstm1/p62
Map1lc3b-ii
Control
Caffeine
E
Control siRNA + + - -
Sqstm1\p62 siRNA - - + +
Caffeine - + - +
Sqstm1/p62
Dvl2
Gapdh
75kDa
100kDa
37kDa
Relative density
SQSTM1
DVL2
Control siRNA
Control siRNA+Caffeine
Sqstm1\p62 siRNA control
Sqstm1\p62 siRNA+Caffeine
F
Control siRNA + + - -
Sqstm1/p62 siRNA - - + +
Caffeine - + - +
G
Abs @ 592nm (Arbitrary Units)
Control siRNA
Caffeine
Sqstm1\p62 siRNA
Sqstm1\p62 siRNA + Caffeine

Figure 5. Dvl2 is required for inhibition of aortic smooth muscle cell proliferation by caffeine also it increases Sqstm1/p62 and Map1lc3b-ii -mediated interaction and degradation of Dvl2 and inhibited Wnt signaling

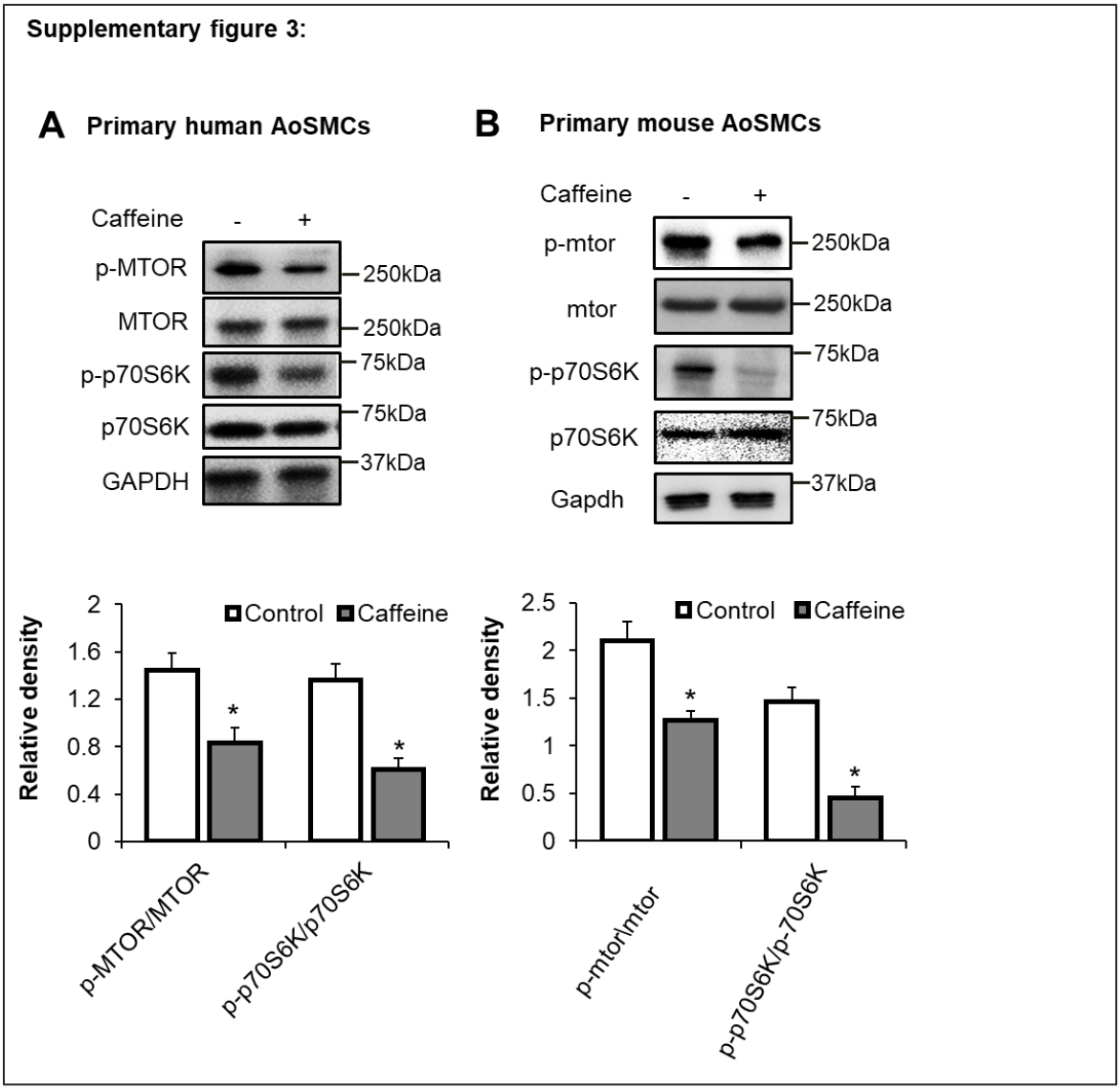
(A) Western Blot analysis of immortalized mouse AoSMC proliferation during Dvl2 overexpression with caffeine treatment (2 mM) for 48 h or during Dvl2 knockdown. Quantitative analysis of Dvl2 and proliferation marker Pcna were done and their relative densities shown. **(B)** Crystal violet staining of immortalized mouse AoSMC's with either overexpression or siRNA knockdown of Dvl2 and caffeine (2 mM) treatment for 48 h. **(C)** Crystal violet stain was dissolved in 10% acetic acid and colorimetric measurements were performed at 592 nm. Absorbance is shown as arbitrary units. Dvl2, Sqstm1/p62 and Map1lc3b-ii protein interaction was analyzed by (**D)** Co-immunoprecipitation/Immunoblot analyses in immortalized mouse AoSMCs, treated ± caffeine (2mM) for 16 h. Relative density of Sqstm1\p62 and Map1lc3b-ii was normalized to that of IP-Dvl2 and result is shown. **(E)** Western blotting of Dvl2 degradation was performed on Sqstm1/p62 knocked down immortalized mouse AoSMCs, treated ± caffeine (2 mM) for 48 h. Quantitative analysis of SQSTM1 knockdown and Dvl2 degradation were done and their relative densities were plotted. Crystal violet staining assays performed on Sqstm1/p62 knocked down immortalized mouse AoSMCs treated ± caffeine (2 mM for 48 h) (**F**) and absorbance was plotted as an arbitrary units (**G**) Crystal violet stain was dissolved in 10% acetic acid. Results are expressed as mean ± SD. The statistical significance of differences (*P < 0.05) was assessed by a one-way or two-way ANOVA wherever applicable, followed by Tukey's multiple-comparisons test, N=3.



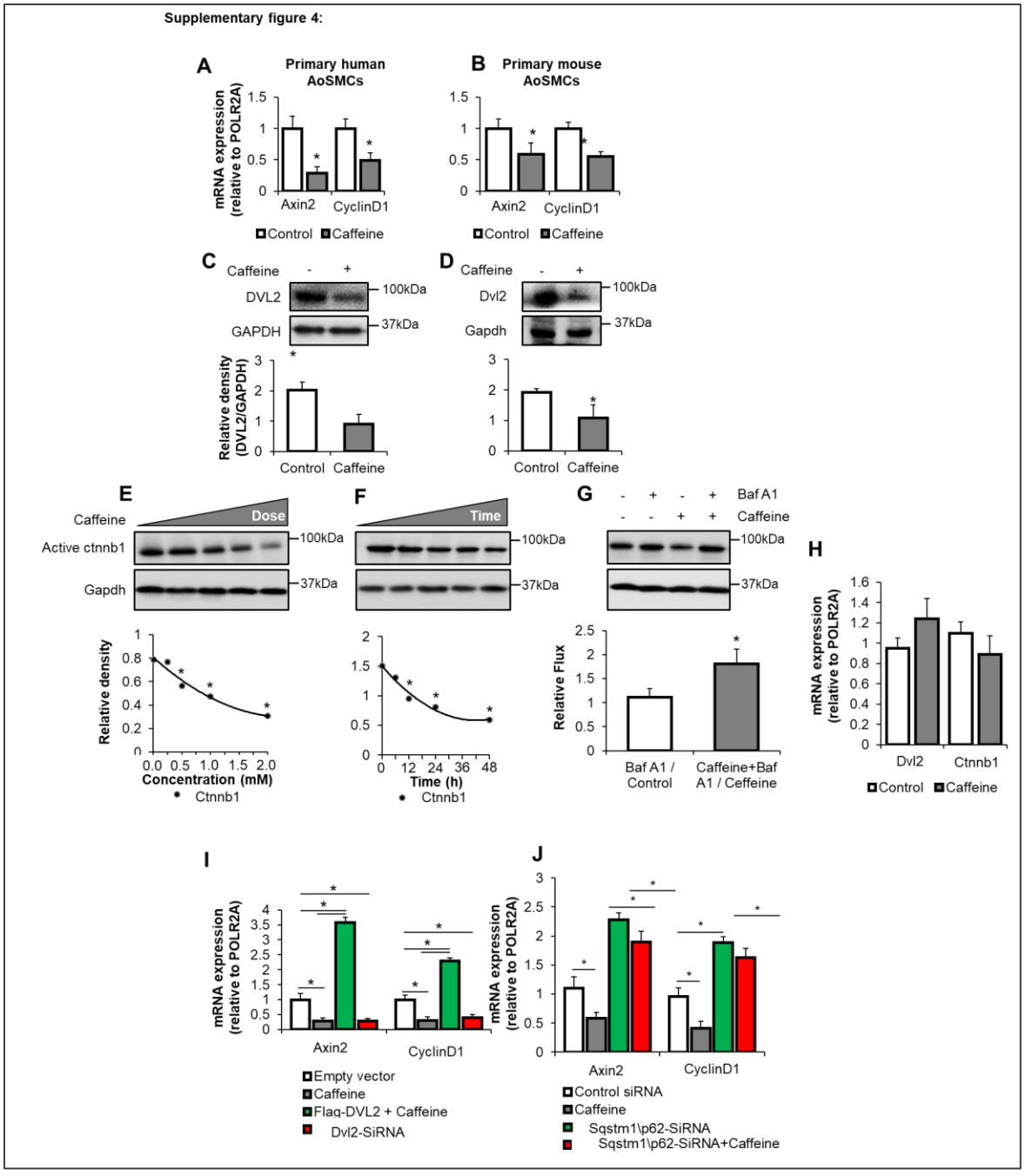
Supplementary Figure 1. Caffeine decreased AoSMCs proliferation without inducing apoptosis. (A) WST-8 cell proliferation analysis of primary human, mouse AoSMCs and immortalized mouse AoSMCs treated with caffeine (2 mM) for 48 h. **(B)** Western blot analysis of immortalized mouse AoSMCs treated with caffeine (serial dilution form 0-2 mM for 48 h) and Palmitic acid (1 mM for 8 h, as apoptotic control) for apoptosis analysis. The statistical significance of differences (*P < 0.05) was assessed by a one-way or two-way ANOVA wherever applicable, followed by Tukey's multiple-comparisons test, N=3.



Supplementary Figure 2. Caffeine induces autophagy in primary human and mouse aortic smooth muscle cells. Immunoblot analyses of the key autophagic marker proteins Microtubule-associated protein 1A/1B-light chain 3B (MAP1LC3B-II) and SQSTM1/p62 were performed in **(A)** primary human AoSMCs, **(B)** primary mouse AoSMCs. The statistical significance of differences (*P < 0.05) was assessed by a one-way or two-way ANOVA wherever applicable, followed by Tukey's multiple-comparisons test, N=3.



Supplementary Figure 3. Caffeine inhibits MTOR signaling in primary human and mouse smooth muscle cells. Immunoblotting to analyze MTOR signaling (phosphorylation of MTOR and its downstream target p70S6K) was performed on **(A)** primary human AoSMCs, **(B)** primary mouse AoSMCs. The statistical significance of differences (*P < 0.05) was assessed by a one-way or two-way ANOVA wherever applicable, followed by Tukey's multiple-comparisons test, N=3.



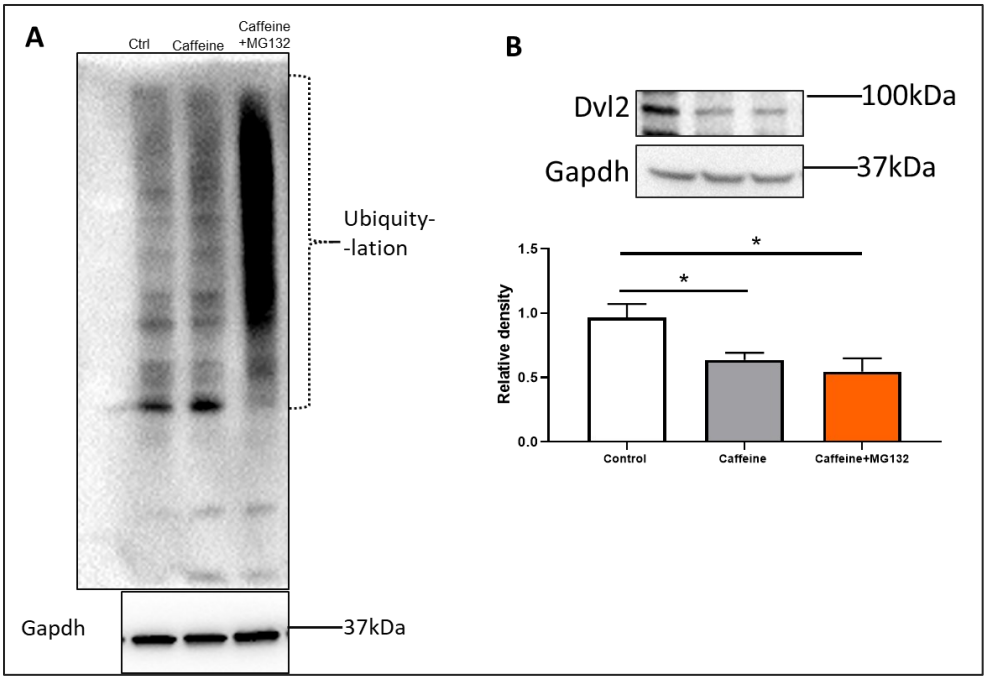
Supplementary Figure 4. Caffeine inhibits Wnt signaling and decreases Dvl2 degradation. Caffeine degraded β-catenin in a dose- and time-dependent manner via autophagy70S6K

RT-qPCR analyses of Wnt signaling target genes *Axin2* and *CyclinD1* in **(A)** primary human AoSMCs, **(B)** primary mouse AoSMCs. Immunoblotting for the key mediator of

Wnt signaling, Dvl2 was performed on **(C)** primary human AoSMCs, **(D)** primary mouse AoSMCs, treated±caffeine (2 mM) for 48 h. Quantitative analysis of their relative densities are shown. Immunoblot analyses to analyze caffeine dose-response.

(E and F) Western blot analysis of immortalized mouse AoSMCs treated with caffeine (serial dilution from 0-2 mM for Dose response; and 2 mM for different time points for Time course).Quantitative analysis of active *β*-catenin was done, normalized with Gapdh and plotted as bar graphs. **(G)** Autophagic flux analysis by western blot in caffeine (2 mM for 48 h) and/or Bafilomycin A1 (50 nM for 5 h) treated immortalized mouse AoSMCs. Quantitative analysis of active *β* -catenin for autophagic degradation was done by taking ratio of bafilomycin A1 (lysosomal inhibitor) treated cells compare to untreated and caffeine treated samples respectively. **(H)** RT-qPCR analysis of immortalized mouse AoSMCs treated with or without caffeine (2 mM) for 48 h, for Dvl2 and *b*-catenin (Ctnnb1) gene expression. Results were plotted as bar graphs.

(I) RT-qPCR analyses of Wnt target genes *Axin2* and *Cyclin D1* in immortalized mouse AoSMC's with either overexpression or siRNA knockdown of DVL2 and caffeine (2 mM) **(J)** RT-qPCR analysis of Wnt signaling target genes *Axin2* and *Cyclin D1* in Sqstm1/p62 knocked down immortalized mouse AoSMCs, treated ± caffeine (2 mM) for 48 h treatment for 48 h. The statistical significance of differences (*P < 0.05) was assessed by a one-way or two-way ANOVA wherever applicable, followed by Tukey's multiple-comparisons test, N=3.



Supplementary Figure 5. Caffeine promotes DVL2 degradation via autophagy.

Immortalized mouse AoSMCs treated with caffeine (2 mM) for 48 h and MG132 (a proteasomal inhibitor) along with caffeine. Ubiquitynylation was detected using immunoblotting, **(A)** Ubiquitinated proteins **(B)** Relative density of Dvl2, normalized with Gapdh.

The statistical significance of differences (*P < 0.05) was assessed by a one-way or two-way ANOVA wherever applicable, followed by Tukey's multiple-comparisons test, N=3.