Novel treatment of small and large artery calcific disease via epigenetic activation of autophagy initiation genes

christian Lino Cardenas (clinocardenas@mgh.harvard.edu)
Massachusetts General Hospital https://orcid.org/0000-0001-5491-4375

Wanlin Jiang
Massachusetts General Hospital, Boston https://orcid.org/0000-0001-9631-2540

Francois Cherbonneau
Massachusetts General Hospital

ZarbaJan Shahrooz
Massachusetts general hospital

Christopher Nicholson
Massachusetts General Hospital

Elizabeth Chou
Massachusetts General Hospital

Rebecca Li
Massachusetts General Hospital, Boston

Sophie Boerboom
Massachusetts General Hospital

Katrina Ostrom
Massachusetts general hospital

Fumito Ichinose
Massachusetts General Hospital/Harvard Medical School https://orcid.org/0000-0003-4535-1632

Donald Bloch
Massachusetts General Hospital

Sagar Nigwekar
Massachusetts general hospital

Patrick Ellinor
The Broad Institute of MIT and Harvard https://orcid.org/0000-0002-2067-0533

Patricia Musolino
Massachusetts General Hospital

Mark Lindsay
Massachusetts General Hospital

Clint Miller
Letter

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Abstract

Vascular calcification is a hallmark of atherosclerotic disease and serves as a strong predictor and risk factor for cardiovascular events. Growing evidence points towards macroautophagy (herein referred to simply as autophagy), a cellular self-degradation process, as being protective during early atherosclerosis. However, autophagy may become dysregulated with advanced atherosclerosis. Vascular smooth muscle cells (VSMC) contribute to early and advanced atherosclerosis by regulating vascular remodeling and calcification. The precise effects of autophagy on VSMC-mediated calcification are unknown. Here, we combined multi-omic profiling and high-resolution structural imaging to pinpoint an epigenetic mechanism by which impaired autophagy drives VSMC calcification. Our studies revealed dysfunctional autophagy initiation to be responsible for altered autophagy flux during VSMC calcification. In vivo pharmacologic and genetic activation of autophagy using chromatin modulator GSK3 and Beclin1F21A knock-in mice, respectively, inhibited vascular calcification and improved survival rates in mouse models of spontaneous large (Mgp-/−) and small (Abcc6-/−) artery calcification. Taken together, these data enhance our mechanistic understanding of vascular calcification by specifically implicating impairment in the initiation of the autophagy pathway and provide important insights for a broad range of cardiovascular diseases involving VSMC phenotype switch.

Full Text

The mature VSMC is not terminally differentiated and exhibits remarkable plasticity even during adulthood. Intimal and medial arterial calcification are characterized by the transdifferentiation or phenotypic transition of VSMCs from contractile cells to proliferative, osteogenic cells. During the course of vascular calcification, VSMC phenotypic transition is associated with both a loss of the contractile apparatus and an increase in osteogenic markers such as Runt-related transcription factor 2 (RUNX2) and alkaline phosphatase (ALPL). Runx2 is a master transcriptional regulator that is required for VSMC phenotypic transition and osteogenic activity. VSMCs undergoing phenotypic transition within atherosclerotic lesions migrate into the intima, where they proliferate, produce calcified extracellular matrix, and participate in fibrous cap formation that alters plaque stability. This process contributes to a range of vascular pathologies including vascular calcification, atherosclerosis, calciphylaxis, vascular restenosis, transplant vasculopathy, and hypertension.

Autophagy is an evolutionarily-conserved, tightly regulated process through which eukaryotic cells recycle cellular components and deliver unnecessary or potentially dangerous cellular materials in double-membrane vesicles for degradation via fusion with lysosomal compartments. Autophagy consists of multiple steps including autophagosome initiation and nucleation, elongation, lysosomal fusion to create autolysosomes, and degradation. Specifically, autophagy initiation/nucleation is characterized by the formation of the autophagy-initiation complex (or ULK1 complex) and this early phase of autophagy is dependent on multiple factors including ULK1, ULK2, ATG101, ATG12, ATG13, PIK3R3, ATG5, ATG7, ATG14, ATG16L1/L2, FIP200, BECN1, VPS34, and VPS13A. Autophagic flux is a term used to describe the
rate of autophagic degradation and impaired autophagic flux can be determine with multiple techniques including assessment of autophagic vacuole accumulation, disrupted autophagosome membrane integrity, and/or lack of lysosomal fusion on transmission electron microscopy (TEM), levels of autophagic protein LC3II and lack of co-localization with lysosomal proteins (ie, LAMP1), accumulation of autophagic substrate SQSTM1 (also known as p62), and changes in the fluorescent signal of mRFP-GFP-LC3 reporter (as detailed below). Autophagy is essential for maintaining normal vascular cell homeostasis and function\textsuperscript{16,17} and is considered protective during early atherosclerosis but can become dysregulated with advanced atherosclerosis\textsuperscript{18,19}. We therefore aimed to ascertain the changes that occur in autophagy flux and the expression of autophagy-related genes in the context of VSMC osteogenic switch and calcification as well as in human and mouse models of vascular diseases of calcification.

To gain insights into the molecular mechanisms of VSMC calcification, we first performed RNA-seq analysis in VSMCs cultured in normal conditions and in media that promotes calcification (osteogenic media). Differential expression analyses revealed 3,657 upregulated and 3,738 downregulated genes under osteogenic conditions (Fig. 1a). Pathway analyses of differentially expressed genes revealed enrichment of signaling pathways related to autophagy, including p53 and mTOR signaling, which are involved in the initiation of autophagy signaling (Fig. 1b). To complement these transcriptomic analyses showing perturbations in autophagy-related signaling pathways, we investigated the status of autophagic flux in either normal or calcified VSMCs by performing ultrastructural analysis with transmission electron microscopy (TEM). In calcified VSMCs, TEM showed an increase in autophagic vacuoles and lysosomes (dense bodies) when compared to normal VSMCs (Fig. 1c). Based on the RNAseq pathway analysis and TEM results, we sought to determine the specific components of the autophagy pathway that are impaired in VSMC calcification. Data from RNA-seq analysis (Fig. 1d) and subsequent qPCR validation (Extended Data Fig. 1a) showed that \textit{ULK1} was the most down-regulated of the autophagy-related genes. Importantly, ULK1 is required for the formation of the activation complex of autophagy suggesting that a defect in autophagy initiation may be a hallmark of calcified VSMCs. Other genes involved in autophagy initiation were also down-regulated in calcified VSMCs as shown in Fig. 1d. In further evaluation, immunoblot analyses showed increased lipidation of LC3 and accumulation of SQSTM1 (a substrate of autophagosomes), confirming a disruption of autophagic flux in calcified VSMCs (Fig. 1e). This disruption in autophagic flux was associated with both VSMC calcification (Alizarin Red stain) and phenotypic switch revealed by decreased contractile protein expression (calponin, CNN1; SM22α) and increased RUNX2 (Fig. 1e). Immunofluorescence analysis revealed a lack of co-localization between autophagosome (LC3B) and lysosome (LAMP-1) markers, further highlighting a disruption of autolysosome formation in calcified VSMCs (Fig. 1f). Furthermore, in immunofluorescence analyses, ULK1 protein expression was reduced and exhibited abnormal cytoplasmic localization compared to healthy VSMCs (Extended Data Fig. 1b), suggesting a defect in the initiation step of the autophagy pathway and the capacity of ULK1 to appropriately interact with other members of the autophagy initiation complex. Moreover, TEM analysis of autophagy bodies demonstrated a lack of membrane integrity in phagosome vacuoles in calcified VSMCs compared to healthy VSMCs (Fig. 1g).
Taken together, these \textit{in vitro} results demonstrate that VSMC calcification is associated with a defect in the initiation of autophagy and the formation of autophagosomes.

Because ULK1 is a critical component of the complex that initiates autophagosome formation, we sought to determine the effects of inhibiting ULK1 activity on VSMC calcification. Incubation of VSMCs in osteogenic media with SBI0206965, a specific ULK1 inhibitor,\textsuperscript{20} increased calcification of VSMCs in a dose-dependent manner (Fig. 1h). In contrast, activation of autophagy with rapamycin prevented VSMC calcification. Furthermore, in \textit{ex vivo} analysis of wild-type mouse aortas treated with or without SBI0206965 under osteogenic conditions, ULK1 inhibition dramatically exacerbated aortic calcification as evidenced by increased Osteosense signal, Alizarin red staining, MMP activity, and Runx2 expression (Fig. 1i). Overall, our results show that VSMC calcification is characterized by the increased expression but lack of co-localization between autophagosome (LC3B) and lysosome (LAMP-1) markers indicating an impairment of autophagy flux. Furthermore, these results strongly implicate a defect in the initiation of autophagy underlying VSMC-mediated calcification and that inhibition of the autophagy ULK1-initiation complex exacerbates osteogenic VSMC phenotype switching.

To support these \textit{in vitro} and \textit{ex vivo} findings, we next determined whether the expression of autophagy initiation genes associated with vascular disease in humans. To investigate the regulation of autophagy genes in atherosclerosis, we queried differential gene expression in a transcriptomic dataset derived from normal and ischemic human coronary artery tissues. While \textit{ULK1} was not differentially regulated, we identified 5 autophagy initiation genes of those listed above (\textit{ATG12, ATG13, ATG16L1, BECN1,} and \textit{VPS13A}) that were downregulated in ischemic, compared to normal, coronary arteries and 2 autophagy initiation genes (\textit{ATG7} and \textit{ATG16L2}) that were upregulated (Fig. 2a and \textit{Supplemental Table 1}). We next investigated genetic associations of autophagy initiation genes with vascular disease by performing summary-based Mendelian randomization (SMR) analysis of thoracic aortic calcification (TAC) genome-wide association study (GWAS) loci using expression quantitative trait loci loci (eQTL) from atherosclerotic aortic root and mammary artery tissues in the Stockholm-Tartu Atherosclerosis Reverse Networks Engineering Task (STARNET) dataset\textsuperscript{21}. This pleiotropic association of the GWAS and eQTL signals identified \textit{ULK2} and \textit{VPS13A} as the most likely autophagy effector genes whose expression is associated with TAC risk (\textit{Supplemental Table 2}). To complement these results, we leveraged genome-wide epigenomic profiles in coronary artery tissues and smooth muscle cells to determine enhancer-gene activity-by-contact (ABC) scores for disease associated variants.\textsuperscript{22} This analysis identified \textit{ATG16L2} as a the most likely effector gene of peripheral artery disease (PAD) associated regulatory variants from the Million Veteran Program\textsuperscript{23} (Fig. 2b and \textit{Supplemental Table 3}). Taken together, these integrative analyses provide additional human genetic evidence for abnormal expression of autophagy-related genes involved in autophagosome initiation (e.g. \textit{ATG12/13, ATG16L1, BECN1} and \textit{ULK2}). We observed a strong association both in coronary disease and aortic calcific disease with SNPs that are linked to expression levels of autophagy-initiation genes.

The association of differential autophagy initiation gene expression in vascular calcification was further underscored by \textit{in situ} hybridization analysis of human aortic sections with atherocalcific disease as well
as dermal biopsies from patients with calciphylaxis. Aortic calcification represents large vessel disease whereas calciphylaxis is a disease characterized by rapidly progressive arteriolar calcification in fat and skin tissue\textsuperscript{24}. Interestingly, both types of calcification exhibit similar underlying molecular mechanisms. We observed that, both in aortic calcification (Fig. 2c, upper panel) and in calciphylaxis (Fig. 2c, lower panel and Extended Data Fig. 2a), the presence of arterial calcification was associated with reduced ULK1 mRNA levels compared to healthy, non-calcified control tissues. To determine if activation of autophagy improves vascular calcification and prevents VSMC phenotype switch \textit{in vitro}, we used two activators of autophagy, GSK343 (an inhibitor of Enhancer of zeste homolog 2, EZH2)\textsuperscript{25,26} and rapamycin (an mTOR inhibitor). Treatment of VSMCs under osteogenic conditions with either GSK343 or rapamycin inhibited calcification by >80%, as determined by Alizarin Red staining and the cresolphthalein method for calcium quantification (Fig. 2d and Extended Data Fig. 3a) and preserved a normal contractile phenotype of VSMCs grown in osteogenic conditions (Fig. 2e and Extended Data Fig. 3b). Furthermore, activation of autophagy with GSK343 or rapamycin, as evidenced by increased LAMP1 and increased lipidation of LC3 (indicating increased autophagy flux) (Fig. 2f), was associated with reduced RUNX2 protein levels (a master activator of calcification). Treatment of VSMCs under osteogenic conditions with GSK343 rescued the mRNA (Fig. 2g) and protein (Fig. 2h) expression of autophagy-initiation genes, most notably a dose-dependent increase in ULK1 with GSK343 treatment. Given that GSK343 alters chromatin structure via inhibition of EZH2 catalytic activity (histone trimethylation modifications), we sought to determine if the inhibition of calcification observed with GSK343 occurs via altering chromatin accessibility at key autophagy-related and calcification genes using ATAC-seq (\textit{assay for transposase accessible chromatin combined with high-throughput DNA sequencing})\textsuperscript{27}. Compared to normal VSMCs, calcified VSMCs exhibited a ~3% rearrangement of chromatin organization with specifically a loss of chromatin accessibility at proximal promoter and 5' UTR regions, which was restored to more normal levels when treated with GSK343 (Fig. 2i and Extended Data Fig. 4a). In assessing the promoter regions of specific autophagy-initiation genes as well as contractile and calcification genes, promoter accessibility was significantly reduced at the $\text{ULK1}$, $\text{ATG13A}$, and $\text{BECN1}$ (autophagy-initiation) and $\text{CNN1}$ (contractile protein) promoters and increased at the $\text{RUNX2}$ (calcification) promoter in calcified VSMCs compared to normal VSMCs. Interestingly, treatment of VSMCs in osteogenic media with GSK343 augmented chromatin accessibility in the promoter regions of ULK1 and ATG13A (Fig. 2j). Altogether, these data point towards an important epigenetic regulation of autophagy-initiation genes with downstream effects on mRNA expression in VSMC calcification and identifies GSK343 as a novel inhibitor of VSMC calcification.

We next sought to determine whether the restoration of autophagy-initiation gene expression with GSK343 treatment improves the observed defect in autophagosome and autolysosome formation in calcified VSMCs. In TEM analysis, VSMCs in osteogenic media treated with GSK343 demonstrated a greater number of autophagosomes with intact membranes associated with increased numbers of autolysosomes compared to DMSO-treated cells (Fig. 3a). VSMCs in osteogenic media treated with GSK343 exhibited a preponderance of intact autophagosome structures characterized by the sequestration of SQSTM1 (a specific autophagosome substrate) inside the autophagic vacuole...
compared to DMSO-treated VSMCs (Fig. 3b). To corroborate the therapeutic potential of GSK343 in vascular calcification, we assessed *ex vivo* human tissue samples from calcified abdominal aortas (and from healthy controls) and dermal biopsies from end-stage kidney disease (ESKD) patients afflicted with calciphylaxis (and control patients with ESKD but not calciphylaxis). Immunofluorescence analysis of fixed tissues demonstrated reduced protein expression of ULK1 and the contractile protein CNN1 in calcified aortae (Fig. 3c, upper panel) and arterioles of calciphylaxis patients (Fig. 3c, lower panel) compared to their respective control samples. Using fresh tissue samples, aortas and dermal biopsies from individual patients were sectioned and incubated in osteogenic conditions for 14 days in the presence or absence of GSK343 followed by fixation and histologic analysis. Treatment of *ex vivo* human aortic samples (Fig. 3d) and calciphylaxis samples (Fig. 3e) with GSK343 inhibited calcification induced by osteogenic media, and this reduction in calcification was associated with increased ULK1 and CNN1 protein levels by immunofluorescence. Taken together, our results point to GSK343 as a potent inhibitor of VSMC calcification through epigenetic regulation of autophagy-initiation genes (Fig. 3f).

A mouse model of autophagy flux based on the LC3 double reporter (RFP-GFP-LC3) mouse was used to characterize autophagy in vascular calcification. We sought to understand the effect of pharmacologic agonism (GSK343) or genetic activation (Bcln1-F121A hyperactivating mutant knock-in) of autophagy in two different *in vivo* models, one of diffuse arterial calcification (*Mgp*<sup>−/−</sup>) and a second of dermal arterial calcification (*Abcc6*<sup>−/−</sup>). In Fig. 4b (left panel), compared to wild-type mice, untreated *Mgp*<sup>−/−</sup> mice show an accumulation of LC3 (high RFP-GFP) suggesting impaired autophagy flux. However, *Mgp*<sup>−/−</sup> mice treated with GSK343 exhibited a dose-dependent decrease in LC3 signal suggesting restoration of autophagy flux with appropriate recycling of the autolysosome. Similarly, genetic augmentation of autophagy initiation with Bcln1-F121A knock-in also decreased LC3 signal in *Mgp*<sup>−/−</sup> aortas suggesting restoration of autophagy flux (Fig. 4b, right panel). Due to severe aortic calcification, *Mgp*<sup>−/−</sup> mice typically die by ~5–6 weeks of age from aortic rupture. However, activation of autophagy in *Mgp*<sup>−/−</sup> mice treated with GSK343 (median survival 69 days) or with Bcln1-F121A knock-in (median survival 56 days for heterozygote knock-in and 68 days for homozygous knock-in mice) improved survival (Log-rank p < 0.0001 for each pairwise comparison with control *Mgp*<sup>−/−</sup>, Fig. 4c) compared with control *Mgp*<sup>−/−</sup> mice with a median survival of 39.5 days. Improved survival with increased autophagy was associated with reduced calcium deposition in the aortas of 35-day-old mice as assessed by Alizarin Red staining, improvement in aortic wall integrity as observed by elastin VVG stain, and increased expression of VSMC contractile protein CNN1 by immunofluorescence (Fig. 4d). A similar strategy to augment autophagy initiation was employed with *Abcc6*<sup>−/−</sup> mice, a model of spontaneous dermal and arterial calcification bearing a deficiency in the gene that is mutated in pseudoxanthoma elasticum. In Fig. 4e, compared to wild-type mice, the skin of *Abcc6*<sup>−/−</sup> mice show an accumulation of LC3 (high RFP-GFP) suggesting impaired autophagy flux. However, *Abcc6*<sup>−/−</sup> mice treated with GSK343 exhibited a decrease in LC3 signal suggesting improved autophagy flux with appropriate recycling of the autolysosome. Similarly, genetic augmentation of autophagy initiation with hyperactive Becn1-F121A also decreased LC3 signal in *Abcc6*<sup>−/−</sup> mice suggesting restoration of autophagy flux. Furthermore,
activation of autophagy with GSK343 or with Bcln1-F121A in Abcc6−/− mice reduced dermal calcification as assessed by Alizarin red stain (Fig. 4f, lower panel). Interestingly the subcutaneous vascular anomalies characterized by reduced microvascular density in Abcc6-deficiency also improved in either pharmacologic or genetic activation of autophagy (Fig. 4f, upper panel). Overall, these results provide evidence for impaired autophagy flux in two in vivo models of vascular calcification and highlight the potential therapeutic benefits of autophagy activation with GSK343 in treating vascular calcification.

Our findings point towards impaired autophagy in the pathogenesis of vascular calcification. SNPs in multiple genes critical for autophagy initiation as well as mRNA expression of autophagy initiation genes are tightly linked to calcification-related diseases including ischemic coronary artery disease and peripheral arterial disease. In vitro assays demonstrated that calcification of VSMCs is characterized by decreased accessibility in the promoters of key autophagy initiation genes, which was concordant with decreased expression of autophagy genes associated with osteogenic VSMCs. Furthermore, pharmacologic activation of autophagy with GSK343 prevented the osteogenic switch and calcification of VSMCs by rescuing the expression of autophagy initiation genes. In in vivo studies, impaired autophagy flux was a hallmark of large- and small-vessel calcification in two mouse models, and either pharmacologic or genetic restoration of autophagy flux improved survival and reduced vascular calcification.

The state of chromatin defined by the packaging of DNA with histone and nonhistone proteins into areas of relative accessibility and repression profoundly affects gene expression and regulates cellular identity. Autophagy, in response to environmental cues and stimuli, has been implicated in regulating the differentiation of numerous cell types including endothelial cells and plays an important role in angiogenesis and vascular homeostasis. Our results provide evidence that exposure of VSMCs to calcifying conditions results in reduced autophagy at least in part through large-scale adjustments in chromatin state and that activation of autophagy inhibits the phenotype switch of VSMCs. Prior work identified important roles for epigenetic regulators in vascular calcification including histone deacetylases and methyltransferases. A number of studies have demonstrated that histone methyltransferases play an important role in autophagy. EZH2 is the active subunit of the polycomb repressive complex 2 (PRC2) and functions as a histone methyltransferase that di- and tri-methylates H3 at lys27 (H3K27) to suppress autophagy-related gene transcription. We demonstrated that EZH2 inhibition with GSK343 increases chromatin accessibility at the sites of autophagy initiation genes in VSMCs and that this was associated with an inhibition of osteogenic phenotype switch and calcification.

Impaired autophagy has been implicated in numerous human diseases including atherosclerosis, neurodegenerative diseases, autoimmune diseases, and cancer. To our knowledge, this is the first association of dysregulated autophagy in calciphylaxis and pseudoxanthoma elasticum, two highly morbid conditions that have no targeted therapy. Similar to our findings, autophagy activation is protective against uremic medial calcification as well as atherosclerotic intimal calcification, underscoring an important role for autophagy in vascular homeostasis. Autophagy consists of
multiple steps including autophagosome initiation, elongation, fusion, and degradation. Our results implicate impaired autophagy initiation in vascular calcification. There is no targeted medical treatment to prevent vascular calcification. This proof-of-concept preclinical study suggests that pharmacologic autophagy activators may have a role in the future medical treatment of vascular calcific disorders.

**Methods**

**Human aortic tissue and study design.** Human aortic tissue samples were collected from surgical specimens and through rapid autopsy of individuals within 4 hours of cardiac arrest. Aortic tissue was collected by a primary surgeon and were snap frozen. Skin tissue samples from calciphylaxis and control patients were obtained from Dr. Sagar Nigwekar, principal investigator of the Partners Calciphylaxis Biorepository and Patient Registry (ClinicalTrials.gov ID:NCT03032835). Both aortic and skin samples were collected at the Massachusetts General Hospital. Control aortic tissue was obtained from patients undergoing orthotopic cardiac transplant. IRB permissions do not allow for demographic information from discarded tissue to be collected or stored. All tissue samples were collected in compliance with the Mass General Brigham Institutional Review Board (IRB) requiring written informed consent.

**Human coronary artery tissue procurement.** Ischemic human coronary artery tissue biospecimens were obtained at Stanford University from diseased heart transplant donors consenting for research studies. Hearts were arrested in cardioplegic solution and transported on ice prior to dissecting proximal coronary artery segments from main branches of left anterior descending, circumflex or right coronary arteries. Epicardial and perivascular adipose was trimmed on ice, rinsed in cold phosphate buffered saline, and rapidly frozen in liquid nitrogen, and stored at -80°C. Normal human coronary artery tissue biospecimens were also obtained at Stanford University from non-diseased donor hearts rejected for orthotopic heart transplantation processed following the same protocol as hearts for transplant. Tissues were de-identified and clinical and histopathology information was used to classify ischemic and non-ischemic arteries. All normal arteries originated from hearts with left ventricular ejection fraction (LVEF) greater than 50%. Frozen tissues were transferred to the University of Virginia through a material transfer agreement and Institutional Review Board approved protocols.

**Coronary artery RNA extraction, QC, library construction and sequencing.** Total RNA was extracted from frozen coronary artery segments using the Qiagen miRNeasy Mini RNA Extraction kit (catalog #217004). Approximately 50 mg of frozen tissue was pulverized using a mortar and pestle under liquid nitrogen. Tissue powder was then further homogenized in Qiazol lysis buffer using stainless steel beads in a Bullet Blender (Next Advance) homogenizer, followed by column-based purification. RNA concentration was determined using Qubit 3.0 and RNA quality was determined using Agilent 4200 TapeStation. Samples with RNA Integrity Number (RIN) greater than 5.5 and Illumina DV200 values greater than 75 were including for library construction. Total RNA libraries were constructed using the Illumina TruSeq Stranded Total RNA Gold kit (catalog #20020599) and barcoded using Illumina TruSeq RNA unique dual indexes (catalog # 20022371). After re-evaluating library quality using TapeStation, individually barcoded libraries were sent to Novogene for next generation sequencing. After passing additional QC, libraries
were multiplexed and subjected to paired end 150 bp read sequencing on an Illumina NovaSeq S4 Flowcell to a median depth of 100 million total reads (>30 G) per library.

**Coronary artery RNA-seq processing and analysis.** The raw passed filter sequencing reads obtained from Novogene were demultiplexed using the bcl2fastq script. The quality of the reads was assessed using FASTQC and the adapter sequences were trimmed using trimgalore. Trimmed reads were aligned to the hg38 human reference genome using STAR v2.7.3a according to the GATK Best Practices for RNA-seq. To increase mapping efficiency and sensitivity, novel splice junctions discovered in a first alignment pass with high stringency, were used as annotation in a second pass to permit lower stringency alignment and therefore increase sensitivity. PCR duplicates were marked using Picard and WASP was used to filter reads prone to mapping bias. Total read counts and RPKM were calculated with RNA-SeQC v1.1.8 using default parameters and additional flags “-n 1000 -noDoC -strictMode” and GENCODE v30 reference annotation. The transcript and isoform expression levels were estimated using the RSEM package.

**Coronary artery differential gene expression analysis.** Differential expression analysis was performed on a subset of normal coronary artery (n=24) and ischemic coronary artery (n=36) raw read counts using DEseq2 (v3.1) after correcting for age, sex, RIN Score, ethnicity and hidden confounding variables (surrogate variables). Surrogate variables in the RNA-seq data were estimated in sva v3.2.0. These variables were then adjusted in DEseq2 along with other covariates. Differentially expressed genes were called at FDR <0.05 significance cut-off, which resulted in more than 2300 upregulated and 1400 downregulated protein coding genes. P-values were adjusted for multiple testing using the Benjamini & Hochberg method.

**Aortic smooth muscle cell lines.** Primary human aortic vascular smooth muscle cells (VSMC) were purchased from Cell Applications Inc. (354K-05a), California, USA. To preserve cell identity all experiments were carried out at passages 1-5. Vascular smooth muscle cell identity (contractile phenotype) was assessed by immunofluorescence staining of contractile markers including SM22α, Calponin-1, and MYH11.

**In vitro model of VSMC calcification.** VSMCs at 40% confluence were treated with DMEM supplemented with 10% fetal bovine serum, 10 mM β-glycerophosphate disodium, 50 μg/mL L-ascorbic acid, and 10 nM dexamethasone, as previously described for 12 days. To detect calcification, cells were fixed in 10% formalin and incubated with Alizarin Red at 2% (pH 4.1-4.3) for 15-30 min followed by 3x washing step with distilled water. For GSK343 treatment in vitro, VSMCs were supplemented with osteogenic media containing DMSO (0.05%) or GSK343 (15μM) for 12 days. Fresh media containing DMSO or GSK343 was applied every 2 days.

**Immunoblotting.** Protein lysates were extracted using RIPA buffer (ThermoFisher, CA, USA) and supplemented with 1× of protease inhibitor cocktail (Roche) according to the manufacturer’s instruction. 30 μg of total extracts were mixed with denaturing buffer (1× Laemmli loading buffer with 10% of β-mercaptoethanol) and analyzed by SDS–PAGE/western blot. Separated proteins were transferred onto a
nitrocellulose membrane using the iBlot transfer system (Novex, ThermoFisher, USA). In general, primary antibodies were used at concentration of 1:100 and secondary at concentration of 1:10000.

**RNA isolation and RNA-seq analysis.** Total RNA was extracted using a miRNeasy kit (Qiagen, Cat. No. ID: 217084) following the manufacturer’s protocol. For RNA-seq, we used the BGISEQ platform, on average generating about 4.28G bases per sample. The average alignment ratio with the reference genome was 97.01%, and the average mapping of the gene set was 74.05%. A total of 17029 genes were detected. We used HISAT to align the clean reads to the reference genome and Bowtie2 to align the clean reads to the reference genes. For qPCR, 100 ng of total RNA was used as the starting template for cDNA synthesis. The cDNA was prepared by reverse transcription (RT), and gene expression was analyzed by quantitative PCR (qPCR) on a SYBR green system (Applied Biosystems). Expression results were analyzed by the DDCT method, and GAPDH (encoding glyceraldehyde-3-phosphate dehydrogenase) was used as a housekeeping gene. Fold changes were calculated as the average relative to control cells growing in normal media.

**ATAC-seq.** 1 million cells were plated under normal or osteogenic media containing DMSO or GSK343 for 12 days as described above. The ATAC-seq analysis was outsourced and performed by Active Motif company ([https://www.activemotif.com/blog-atac-seq](https://www.activemotif.com/blog-atac-seq)) using the following protocol, as previously described41. Briefly, to prepare nuclei, 1 million cells were spun at 500 × g for 5 minutes, followed by a wash using 1000 μL of cold 1x PBS Then, cells were lysed using cold lysis buffer (10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 3 mM MgCl2 and 0.1% IGEPAL CA-630) followed by 10 min of centrifugation at 500x. The transposition reaction was carried out for 30 minutes at 37 °C. For PCR, library fragments were amplified using 1x NEBnext PCR master mix and 1.25 μM of custom Nextera PCR primers.

**FISH and immunofluorescence microscopy.** Stellaris® FISH Probes recognizing human ULK1 (SMF-1082-5) and labeled with Quasar® 570-labeled oligos (Biosearch Technologies, Inc., Petaluma, CA) were hybridized to tissue samples, following the manufacturer's instructions available online at [www.biosearchtech.com/stellarisprotocols](http://www.biosearchtech.com/stellarisprotocols). Two-dimensional and white light images were analyzed using ImageJ software.

**TEM analyses.** Cultured aortic vascular smooth muscle cells under normal or osteogenic conditions were fixed in dishes with 2.0% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4, Electron Microscopy Sciences, Hatfield, PA) at least 2hr at room temperature on a gentle rotator, then fresh fixative was added, and specimens were allowed to infiltrate overnight at 4°C. Fixed cells were rinsed several times (in dishes) in 0.1M sodium cacodylate buffer, then scraped and suspensions transferred into 15ml centrifuge tubes. Specimens were centrifuged (4500rpm for 15min @ 4°C), supernatant removed, and resulting pellets post-fixed in 1.0% osmium tetroxide in cacodylate buffer for 1hr at room temperature. The pellets were rinsed several times in cacodylate buffer, then embedded in warm 2% agarose. After agarose hardened, small blocks containing pelleted material were collected and dehydrated through a graded series of ethanol to 100%, followed by brief dehydration in 100% propylene oxide. Specimens were pre-infiltrated 2hr in a 2:1 mix of propylene oxide and Eponate resin (Ted Pella, Redding, CA), then transferred into a 1:1
mix of propylene oxide and Eponate resin for overnight infiltration at room temperature on a gentle rotator. The following day, samples were allowed to infiltrate several hours in fresh 100% Eponate resin, transferred into flat molds with 100% Eponate resin and molds placed in a 60°C oven for resin polymerization (24-48hrs). Thin (70nm) sections were cut using a Leica EM UC7 ultramicrotome, collected onto formvar-coated grids, stained with 2% uranyl acetate and Reynold's lead citrate and examined in a JEOL JEM 1011 transmission electron microscope at 80 kV. Images were collected using an AMT digital imaging system with proprietary image capture software (Advanced Microscopy Techniques, Danvers, MA).

**Animals.** This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Housing and all procedures involving mice described in this study were specifically approved by the Institutional Animal Care and Use Committees of Massachusetts General Hospital (Subcommittee on Research Animal Care, protocol #2008N000169). Mgp+/− mice were generated by G. Karsenty and colleagues. The model of small vessel dermal calcification (Abcc6+/− mice) was kindly provided by Dr. Jouni J. Uitto and Ida J. Jacobs from the Thomas Jefferson University, Philadelphia, USA. The animal model of hyperactivated autophagy (B6.129(Cg)-Becn1tm1.1Hecc/J) was kindly provided by Dr. Beth Levine from UT Southwestern Medical Center, USA. The animal model of autophagy maturation reporter (C57BL/6-Tg (CAG-RFP/EGFP/Map1lc3b)1Hill/J, Stock No: 027139) was purchased from Jackson Laboratory. Animals were maintained on a standard diet. Survival studies were performed and the Kaplan-Meier statistic with log-rank testing was used to compare survival of mice.

**Histology.** Removed tissues were then fixed in formalin (10%) for 24 hours before transfer to 70% ethanol followed by paraffinization and sectioning (7 μM). Slides were produced for tissue staining or stained with standard stains including H&E, collagen deposition (Trichrome Stain Kit, MilliporeSigma), elastin (Verhoeff-Van Gieson, Thermo Fisher Scientific), or F-actin (ActinGreen 488 ReadyProbes, Thermo Fisher Scientific) for quantitative analysis. Elastin integrity score was rated by blinded observers and graded on an arbitrary scale of 5 (indicating high-quality elastic fiber) to 1 (indicating severe elastin fragmentation).

**Summary-based Mendelian Randomization analysis.** Gene-level prioritization of calcification GWAS variants was performed using summary-based Mendelian Randomization (SMR)(Zhu:2016). GWAS summary statistics were obtained from the Cohorts for Heart and Aging Research in Genome Epidemiology (CHARGE) consortium on thoracic aortic calcification (TAC) from 8422 European ancestry individuals (Malhotra:2019). These data were integrated with cis-eQTL summary data from the Stockholm-Tartu Atherosclerosis Reverse Network Engineering Task (STARNET) containing atherosclerotic aortic root (AOR) and mammary artery (MAM) tissues derived from ~600 European individuals (Franzén:2016). We used SMR to test the pleiotropic association of top TAC associated SNPs with gene expression in the STARNET eQTL datasets, as done previously (Franceschini:2018,Liu:2018). We used the post-hoc heterogeneity in dependent instruments (HEIDI) test (Zhu:2016) to exclude loci with evidence of linkage or heterogeneity in the genetic instruments (P¬HEIDI < 0.01) rather than pleiotropy.
using the 1000G European LD reference panel. We considered only genes with at least 1 cis-eQTL p-value < 5E-5 for colocalization.

**Activity-by-Contact (ABC) enhancer-gene analysis.** We used peripheral artery disease (PAD) GWAS summary data from the Million Veteran Program (MVP) meta-analysis with variants subset to suggestive associations (P<5E-05) to intersect with genome-wide enhancer-gene predictions calculated using the activity-by-contact (ABC) model. We used both the ENCODE coronary artery tissue and human coronary artery smooth muscle cell based annotations from H3K27ac histone modification ChIP-seq and chromatin accessibility (ATAC-seq) datasets, and we considered significant enhancer-gene predictions with ABC scores > 0.02, as previously described. Interaction windows were defined by HiC contact boundaries averaged across multiple human tissues.

**Statistics.** Statistical analysis for *in vitro* and *in vivo* experiments was performed using Graph Pad Prism 8.0 (GraphPad Software, La Jolla, CA). Data are reported as mean ± SD, unless otherwise indicated. Two group comparisons of continuous variables were performed using the two-tailed Student t test. For more than 2 group comparisons of continuous variables, two-tailed one-way analysis of variance (ANOVA). For all experiments, at least 3 experimental replicates were performed. The Kaplan-Meier statistic with log-rank testing was used to compare survival of groups of mice. A two-tailed p<0.05 was considered to indicate statistical significance.

**References**


Figures
Figure 1

**Impaired autophagy is a determinant of VSMC calcification.** (a) Differential gene expression analysis for calcified versus healthy human VSMCs. Calcified VSMCs were grown in osteogenic media for 3 days. (b) Analysis of canonical pathways impacted by global transcriptomic differential gene expression in calcified VSMCs. (c) Transmission electron microscopy (TEM) analysis of human VSMCs under normal or osteogenic conditions. Note that Bafilomycin A1 (Baf-A1) and Chloroquine (CQ), compounds that alter the acidity of the lysosome, allow the identification and quantification of autophagosomes and lysosomes by inhibiting the maturation of autophagy flux. (d) Heat map of mRNA expression of autophagy-related genes in healthy versus calcified VSMCs. (e) Quantitative western blot analysis of calcification (RUNX2), autophagy (ULK1, ULK2, ATG13, ATG5, LC3, SQSTM1), lysosome (LAMP1) and VSMC contractile (CNN1, SM22α) markers along with Alizarin Red stain for calcium. (f) Quantification of autolysosome (yellow) formation by immunofluorescence staining and co-localization of LC3B (red) and LAMP1 (green). (g) TEM analysis of autophagic vacuolar ultra-structures in healthy and calcified VSMCs. Four main structures were identified: (1) Phagosome vacuoles, (2) Autophagosomes containing undegraded cargo which appear as cellular debris (dense due to ongoing protein breakdown) in normal
cells. In contrast, calcified VSMCs show autophagosomes with thinner membranes and nonspecific protein sequestration (lighter and granulated material). (3) Lysosomes, dark dense bodies indicating a late lysosomal state. Calcified VSMCs show irregular and dilated lysosomal bodies. (4) Autolysosomes, double membrane bounded structures as a result of autophagosome fusion with lysosomes. Calcified VSMCs showed fewer autolysosomes than normal VSMCs. (h) Alizarin red staining of human VSMCs under osteogenic conditions indicates calcification is inhibited in cells treated with rapamycin (autophagy activator) and is increased in cells treated with a ULK1 inhibitor (SBI-0206965; autophagy inhibition). (i) Left panel, ex vivo treatment of mouse aortas with ULK1 inhibitor shows increased calcification (Osteosense-Red) and inflammation as measured by MMP activity (MMPsense-green). Right panel shows increased calcium deposition in aortas treated with ULK1 inhibitor (Alizarin red staining) under osteogenic conditions and increased nuclear Runx2 signal (red) as assessed by immunofluorescence.

Figure 2
Differential expression of autophagy-initiation genes is associated with human coronary artery disease and therapeutic activation of autophagy inhibits vascular calcification. (a) Volcano plot of differentially expressed genes in normal (n=24) versus ischemic (n=36) human coronary artery tissues as detected using DESeq2. Significant differentially expressed genes (DEGs) were considered using FDR adjusted p-value < 0.05, as indicated by the green colored points. Autophagy genes are labeled with human gene symbols. (b) Violin plots of differentially regulated autophagy genes in normal versus ischemic coronary artery tissues. Values represent log_{10} normalized gene expression levels quantified using a collapsed gene model and RNA-SeQC. P-values shown are derived from DESeq2 analysis in normal (n=24) versus ischemic (n=36) human coronary artery samples. (c) Alizarin red staining and in situ hybridization for ULK1 mRNA (green) in fixed human tissue from aortic calcification and calciphylaxis patients were compared with healthy tissue, demonstrating reduced ULK1 mRNA levels in the setting of large and small vessel calcification. (d) Treatment with either GSK343 (10µM) or rapamycin (100nM), activators of autophagy, inhibited the calcification of human VSMCs grown in calcification media for 10 days, as evidenced by Alizarin Red stain and cellular calcium content (mg/dL) by colorimetric cresolphthalein method. (e) Collagen gel contraction assay was performed demonstrating that autophagy activation with either GSK343 or rapamycin restores the contractility of VSMCs that is disrupted when grown in osteogenic media. (f) Immunoblot analysis of RUNX2, LAMP1 and LC3BI/II from total lysates of human VSMCs under normal or osteogenic conditions and treated with GSK343 (10µM) or rapamycin (100nM). Treatment with GSK343 or rapamycin inhibits the increase in RUNX2 induced by osteogenic media. (g) Heatmap of the fold-change in mRNA levels of autophagy-related genes from human VSMCs grown under osteogenic media treated without or with GSK343 at low (5µM) or high (10µM) concentrations compared to VSMCs in normal media. (h) Immunoblot analysis of ULK1, ULK2, ATG13 and LC3BI/II from total lysates of human VSMCs under normal or osteogenic conditions and treated with GSK343 (10µM). (i) Pie chart showing the genomic distribution of the differentially accessible chromatin regions from human healthy VSMCs (top chart), calcified VSMCs (middle chart) and calcified VSMCs treated with GSK343 (lower chart). (j) Overlapping ATAC-seq tracks of the promoter regions of the autophagy-initiation genes (ULK1, ATG13A, BECN1), of the contractile gene (CNN1), of the calcification marker (RUNX2), and of the lysosome marker (LAMP1). ATAC-seq peaks for healthy VSMCs are presented in green and for calcified VSMCs in red. The ATAC-seq data have been normalized to take depth into account and the scale on the y-axis was chosen for optimal visualization of peaks. Also, representative ATAC-seq tracks of the autophagy-initiation genes (ULK1 and ATG13A) from VSMCs under osteogenic conditions treated with DMSO (red peaks) or GSK343 (yellow peaks). Shown are a portion of the promoter regions (1-2Kb).
Figure 3

Therapeutic activation of autophagy by GSK343 inhibits calcification in ex vivo calcification.

(a) Ultrastructural TEM analysis of autophagic vacuoles and membrane integrity from calcified VSMCs treated with DMSO or GSK343 (10µM) shows increased autolysosome numbers and membrane integrity with GSK343 treatment. (b) Immunofluorescence staining of autophagosome formation markers from VSMCs under osteogenic conditions treated with DMSO or GSK343. VSMCs were treated with Baf-A1 at 5nM overnight before fixation. (c) Immunofluorescence staining showing reduced ULK1 (green) and CNN1 (magenta) levels in fresh-fixed tissue of human calcified aortas and dermal biopsies from calciphylaxis compared to healthy controls. (d) Ex vivo calcification assays were performed with fresh surgical biopsies from patients with either aortic calcification (left panel) or (e) calciphylaxis (right panel). Alizarin red staining shows the calcification levels of surgical biopsies in normal or osteogenic media treated for 10 days with DMSO or GSK343 (20µM). Treatment with GSK343 inhibited the ex vivo calcification of aortic and calciphylaxis tissues (middle panels) and was associated with increased ULK1 and CNN1 expression (lower panel). (f) In our summative model of autophagy in VSMC calcification, chromatin rearrangement occurs during vascular calcification affecting chromatin accessibility at key
autophagy initiation genes. Defects in the autophagy initiation machinery impair autophagy flux and exacerbate calcification of VSMCs. GSK343 pharmacologically activates autophagy by relaxing chromatin and restoring expression of autophagy initiation genes (e.g., ULK1, BECN1, ATG13) to favor the formation of autolysosomes which associates with inhibition of VSMC calcification.
Therapeutic and genetic activation of autophagy initiation inhibits vascular calcification in two different mouse models. (a) Schematic representation of the in vivo model of assessing autophagy flux using the LC3 double reporter (RFP-GFP-LC3) mouse. (b) Pharmacologic (GSK343, 60 mg/kg/day) and genetic (Bcn1<sup>F121A</sup> mouse) activation of autophagy initiation in an animal model of aortic calcification (Mgp<sup>-/-</sup> mouse) shows restoration of autophagy flux as assessed by the RFP-GFP-LC3 reporter. (c) Either pharmacologic activation of autophagy with GSK343 treatment or genetic activation of autophagy with the Bcn1<sup>F121A</sup> mouse improves survival in Mgp<sup>-/-</sup> mice Kaplan-Meier survival curves are depicted. Log-rank P< 0.0001 for Mgp<sup>-/-</sup> vs Mgp<sup>-/-</sup>/F121<sup>KI/KI</sup> mice. (d) Representative micrographs of Alizarin red, VVG, and CNN1-stained sections of aortas. (e) Pharmacologic (GSK343, 60 mg/kg/day) and genetic (Bcn1<sup>F121A</sup> mouse) activation of autophagy initiation in the animal model of skin calcification (Abcc6<sup>-/-</sup> mouse) shows restoration of autophagy flux as assessed by the RFP-GFP-LC3 reporter. (f) Representative micrographs (GFP channel) of abdominal skin small arteries (top panel), with a schematic representation in the middle panel showing normal skin microvasculature in Abcc6<sup>-/-</sup> mice with autophagy activation compared to control Abcc6<sup>-/-</sup> mice. Lower panel shows Alizarin red staining of histologic skin sections with reduced calcification in Abcc6<sup>-/-</sup> mice with autophagy activation induced either pharmacologically with GSK343 or genetically (Becn1<sup>F121A</sup>).

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalTable13.xlsx
- Onlineoatimage3.png
- floatimage4.jpeg
- floatimage5.jpeg
- floatimage6.jpeg
- floatimage7.jpeg