The Histone Methyltransferase SETDB2 Modulates Tissue Inhibitors of Metalloproteinase–Matrix Metalloproteinase Activity During Abdominal Aortic Aneurysm Development

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Objective: To determine macrophage-specific alterations in epigenetic enzyme function contributing to the development of abdominal aortic aneurysms (AAAs).

Background: AAA is a life-threatening disease, characterized by pathologic vascular remodeling driven by an imbalance of matrix metalloproteinases and tissue inhibitors of metalloproteinases (TIMPs). Identifying mechanisms regulating macrophage-mediated extracellular matrix degradation is of critical importance to developing novel therapies.

Methods: The role of SET Domain Bifurcated Histone Lysine Methyltransferase 2 (SETDB2) in AAA formation was examined in human aortic tissue samples by single-cell RNA sequencing and in a myeloid-specific SETDB2 deficient murine model induced by challenging mice with a combination of a high-fat diet and angiotensin II.

Results: Single-cell RNA sequencing of human AAA tissues identified SETDB2 was upregulated in aortic monocyte/macrophages and murine AAA models compared with controls. Mechanistically, interferon-β regulates *SETDB2* expression through Janus kinase/signal transducer and activator of transcription signaling, which trimethylates histone 3 lysine 9 on the TIMP1-3 gene promoters thereby suppressing *TIMP1-3* transcription and leading to unregulated matrix metalloproteinase activity. Macrophage-specific knockout of SETDB2 ($Setdb2^{fl/fl}Lyz2^{Cre+}$) protected mice from AAA formation with suppression of vascular inflammation, macrophage infiltration, and elastin fragmentation. Genetic depletion of SETDB2 prevented AAA development due to the removal of the repressive histone 3 lysine 9 trimethylation mark on the *TIMP1-3* gene promoter resulting in increased TIMP expression, decreased protease activity, and preserved aortic architecture. Lastly, inhibition of the Janus kinase/signal transducer and activator of the transcription pathway with an FDA-approved inhibitor, Tofacitinib, limited *SETDB2* expression in aortic macrophages.

Conclusions: These findings identify SETDB2 as a critical regulator of macrophage-mediated protease activity in AAAs and identify SETDB2 as a mechanistic target for the management of AAAs.

Key Words: aneurysm, inflammation, vascular biology

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Abdominal aortic aneurysm (AAA) is a common vascular disease that can progress to aortic rupture, which has a mortality of over 80%.[1] Recent studies have suggested that AAAs have a reported incidence of 1.5 to 2 per 1000 people per year in the United States.[2] Despite advances in the surgical and endovascular management of AAAs, to date, there are no proven pharmacological interventions that slow AAA growth or prevent rupture.[3] Cumulative efforts to understand mechanisms that contribute to AAA dilation have consistently demonstrated inflammation and extracellular matrix (ECM) degradation, 2 processes primarily controlled by aortic macrophages (MΦ), as critical drivers of aneurysmal development.[4–7] ECM degradation of aortic tissue is primarily mediated by the matrix metalloproteinase (MMP) family of proteolytic enzymes. Under inflammatory conditions, infiltrating MΦ secrete pro-MMPs; where cleavage of the pro-MMP subunit activates the MMPs, causing ECM degradation. MMP activity may be naturally suppressed by tissue inhibitors of MMPs [tissue inhibitors of metalloproteinase (TIMPs)], which comprise a family of 4 protease inhibitors: TIMP 1, TIMP 2, TIMP 3, and TIMP-4.[8] An improper balance between MMPs and TIMPs shifts the equilibrium towards matrix degradation during AAA development. Studies have shown that genetic and pharmacological approaches to mitigate MMP proteolytic activity can protect against AAA development in several murine models.[9–11] Despite the critical importance of MMPs in AAA development and expansion, the molecular mechanisms that program and sustain macrophage TIMP production and MMP activity in AAA disease have not been identified.

Macrophage functional plasticity and gene expression are tightly regulated by transcriptional reprogramming, which is achieved by modifications of chromatin accessibility dictated by the epigenetic landscape. Accumulating evidence suggests that epigenetic regulation of gene expression, through mechanisms, such as histone modification, is a major influence on innate immune cell phenotypes in both normal and pathologic

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conditions.[12–14] Histone methylation is important as it maintains active or suppressed gene expression, depending on the methylation site, and thereby controls downstream protein expression patterns. Prior work by our laboratory and others has identified that epigenetic modifications regulate immune-mediator expression in MΦ both in vitro and in vivo.[14,15] The chromatin-modifying enzyme SET Domain Bifurcated Histone Lysine Methyltransferase 2 (SETDB2), a histone methyltransferase acts to methylate lysine residues on histones in a sequence-specific manner. SETDB2 specifically trimethylates lysine 9 (K9) on histone 3 (H3) (H3K9me3) and keeps chromatin in a conformation where the promoter is not accessible for transcription factor binding, effectively silencing gene transcription.[16] SETDB2 has been reported to be involved in innate and adaptive immunity, proinflammatory responses, and T-cell differentiation through modulation of the expression of NF-κB target genes and type I interferon (IFN) responses.[15,16] From a cardiovascular standpoint, SETDB2 has been shown to be an important regulator of macrophage inflammation and atherosclerosis progression.[17,18] Independently, we have shown that SETDB2 is instrumental to macrophage polarization and gene expression in diabetic wound healing.[17] Despite the importance of histone modification on macrophage function, there remains a paucity of data on epigenetic-based mechanisms that regulate AAA formation.[19,20] In addition, little is known about the upstream mechanisms that regulate SETDB2 in in vivo aortic MΦ.

Herein, we provide experimental evidence that SETDB2 directs macrophage-mediated TIMP expression and aortic aneurysm formation in human tissue samples and 2 well-established murine AAA models [elastase-induced AAAs and angiotensin II (AngII)-induced AAAs]. Mechanistically, during AAA development, macrophage-specific SETDB2 reduces TIMP expression by increasing the repressive histone methylation mark H3K9me3 on *TIMP* gene promoters. Loss of *TIMP* expression leads to unregulated MMP activity, aortic wall degradation, and aneurysmal formation. Mechanistically, SETDB2 expression is regulated by IFN β through the Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) pathway as genetic depletion of the IFN receptor reduced SETDB2 expression and prevented AAA development. Further targeted inhibition of SETDB2 through myeloid-specific genetic depletion of SETDB2 ($Setdb2^{f/f}Lyz2^{Cre+}$) augmented *TIMP* expression as well as decreased MMP activity and prevented AAA expansion. Overall, these findings identify SETDB2 as a critical regulator of monocyte/macrophage-mediated *TIMP* expression and MMP activity during aneurysmal progression and demonstrate translational implications as cell-targeted manipulation of this pathway may reduce AAA development.

METHODS

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contacts, Frank Davis (davisfr@umich.edu) and Katherine Gallagher (kgallag@med.umich.edu).

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Experimental Model and Subject Details

Mice

Mice were maintained in the University of Michigan's pathogen-free animal facility, and all protocols were approved by and in accordance with the guidelines established by the Institutional Animal Care and Use Committee (IACUC). Male mice were used for AAA experiments as detailed in the American Heart Association Council statement.[21] Only male mice were used for these studies as female mice do not adequately develop AAAs.[21] Mouse strains include: C57BL/6J mice maintained on a normal diet (13.5% kcal fat; LabDiet 5001) were purchased at 8 to 10 weeks from The Jackson Laboratory (Bar Harbor). $Setdb2^{f/f}$ was created as published by our laboratory.[15] $Setdb2^{f/f}$ mice were then bred with B6.129P2-Lyz2$^{tm1(Cre)Ifo/J}$ (Lyz2^{Cre}) mice from the Jackson Laboratory to obtain mice deficient in SETDB2 in monocytes, MΦ, and granulocytes.[22] Lyz2^{cre} was chosen for the cell-specific line as this affects myelomonocytic cells. Currently, there is no Cre-transgenic line that is perfectly specific for MΦ.[23] IFN-α/β receptor$^{-/-}$ (Ifnar$^{-/-}$), $Stat3^{f/f}Lyz2^{Cre-}$, and $Stat3^{f/f}Lyz2^{Cre+}$ were obtained from Christiane Wobus, Ph.D. (University of Michigan, Ann Arbor, MI) and maintained in breeding pairs at the Unit of laboratory animal medicine facilities. Animals were housed in a barrier facility on a light:dark cycle of 14:10 hours (ambient temperature of 22°C) with free access to water, food (Lab diet 5001), and bedding (Andersons Lab Bedding Bed o'Cobs combo). Animals underwent all procedures at 8 to 10 weeks of age.

Production and Injection of Adeno-associated Viral Vectors

Adeno-associated virus (AAV) vectors (serotype 8) were produced by the Viral Vector Core at the University of Pennsylvania(https://gtp.med.upenn.edu/core-laboratories-public/vector-core). These AAV vectors contained inserts expressing mouse PCSK9D377Y mutation (equivalent to human PCSK9D374Y gain-of-function mutation). Empty AAV vector (null AAV) was used as a control. AAV vectors were diluted in sterile PBS (200 μl per mouse) and injected intraperitoneally as reported.[24,25] Mice inclusion and randomization were conducted as previously specified. Further, mice had a predefined exclusion from the data analysis if plasma total cholesterol concentrations were <250 mg/dL 3 weeks and <500 mg/dL 6 weeks after PCSK9D377Y. AAV injection.[24] Briefly, mice received injections of AAVs containing either a null insert or a mouse PCKS9 insert expressing D377Y mutation. Immediately after AAV injections, normolipidemic mice were fed a diet containing saturated fat (milk fat 21% wt/wt; Diet #TD.88137, Envigo) for 2 weeks, at which time they underwent implantation of mini osmotic pumps as described below. Body weights were determined before experimentation.

Osmotic Mini Pump Implantation and AngII Infusion

To induce AAAs, 8 to 10-week male C57BL/6J mice were injected with AAV and started on a saturated fat diet as detailed above. After 2 weeks of saturated fat diet feeding, mice were randomized to receive mini osmotic pumps (Model 2004; Alzet) containing AngII (1,000 ng/min per kilogram, Cat# H-1706; Bachem) or saline were implanted subcutaneously in the neck region of anesthetized mice following a protocol described.[26] Briefly, mice were anesthetized in a closed chamber with isoflurane (3%) in oxygen for 2 to 5 minutes until immobile. Each mouse was then removed and taped on a heated (35–37 °C) procedure board with isoflurane (1.0%–1.5%) administered

through the nosecone during minor surgery. Pumps were implanted subcutaneously on the right flank of each mouse, which provided AngII or saline infusion for 28 days. Incisions were closed with surgical staples and postoperative analgesia (buprenorphine, 0.05 mg/kg/12 hours, intraperitoneal) was administered. All AngII groups experienced equivalent elevations of blood pressure. Animal experiments were conducted following the NIH guidelines and were approved by the IACUC of the University of Michigan.

Elastase Treatment Model of Aneurysm Formation

A murine elastase treatment model of AAA formation was used as described by Laser et al[27] In brief, the infrarenal aorta was treated topically with 30 μl elastase reconstituted with normal saline (5 U/mg protein) or 30 μl heat-inactivated elastase (at 90°C for 30 minutes) as a control group. The topical application was accomplished by dropping the elastase on the anterior aorta from a 2 cm height for 5 minutes. Video micrometric measurements of aortic diameters were made in situ before perfusion, after perfusion, and before harvesting the aorta on day 14. Maximum infrarenal aortic diameter and ratio of treated versus untreated section of the aorta were calculated.

Quantification of Aortic Pathologies

For in vivo imaging of the abdominal aorta in mice, 2-dimensional (B-mode) ultrasound images were obtained 27 days after the implantation of osmotic pumps using a VisualSonics Vevo2100 imaging system with a mechanical transducer (MS400) from the University of Michigan Frankel Center for Physiology. The maximum diameter observer in each mouse in the abdominal aortic region was documented. Two independent investigators measured aortic diameters at systole with no significant interobserver or intraobserver variability. At the completion of each murine aneurysm experiment (day 28), mice were deep anesthetized with ketamine (100 mg/kg) and xylazine (20 mg/kg). At termination after blood collection, the right atrium was cut open, and saline was perfused through the left ventricle to remove blood in the aortas. Subsequently, aortas were dissected and placed in either RNA later or 10% neutrally buffered formalin overnight at room temperature. After fixation, periaortic adventitia was removed thoroughly. Maximal outer diameters of suprarenal aortas were measured ex vivo as a parameter for AAA quantification using ImageJ software (National Institutes of Health).

Necropsy was performed for mice that died during AngII infusion. Aortic rupture was defined as the observation of blood clots in either the thoracic cavity (thoracic aortic rupture) or retroperitoneal cavity (abdominal aortic rupture). There was no difference in early rupture between treatment groups and genotypes.

Histology/Immunofluorescence

For aortic histology, the aortas were excised as mentioned and fixed in formalin (10%) overnight before embedding in paraffin. Sections (5 μm) were stained with Verhoeff-Van Gieson stain for elastin (Sigma HT25A). Images were captured using the Olympus BX43 microscope and Olympus cell Sens Dimension software. Elastin fragmentation was defined as the presence of free ends in what seems to be an otherwise continuous elastin fiber. Elastin fragmentation was quantified by determining the number of elastin breaks in the whole aortic section at 40× magnification and then averaging the number of breaks across three serial sections of the aorta. Two independent observers blinded to the mouse treatment group were used.

For immunohistochemistry, formalin-fixed, paraffin-embedded tissue slides obtained from patients with AAA and aortic healthy control were heated for 30 minutes at 60 °C, deparaffinized, and rehydrated. Slides were placed in Ph9 antigen retrieval buffer and heated at 95°C for 20 minutes in a hot water bath. After cooling, slides were treated with 3% H_2O_2 (5 minutes) and blocked using 10% goat serum (30 minutes). Overnight incubation (4°C) was then performed using the first antibody at a working concentration. Slides were then washed, and treated with secondary antibody, peroxidase (30 minutes), and diaminobenzidine substrate. Antibody used was human anti-SETDB2 (Applied Biosystems, RRID:AB_10982748). Antibody specificity was verified in our publication.[17] Images were quantified ImageScope software and Image J at 20× magnification.

Peripheral Macrophage Isolation and Magnetic-activated Cell Sorting

Peripheral circulating MΦ were isolated using magnetic-activated cell sorting. Briefly, single-cell suspensions were incubated with fluorescein isothiocyanate-labeled anti-CD3, anti-CD19, anti-NK1.1, and anti-Ly6G (BioLegend) followed by EasySep Mouse Streptavidin RapidSpheres (Stem Cell Technologies 19860). Flow-through was then incubated with EasySep Mouse CD11b Positive Selection Kit (Stem Cell Technologies18970) to isolate the non-neutrophil, non-natural killer (NK) cells, non-lymphocyte, and CD11b+ cells. Of note, we did not negatively select CD11c+ dendritic cells. When indicated, peripheral MΦ were stimulated with/without IFNβ (100 U/mL) (PBL Assay Science, catalog 12400-01). Cells were saved in Trizol (Invitrogen) for quantitative reverse transcription-polymerase chain reaction (PCR) analyses or were processed for chromatin immunoprecipitation (ChIP) as described further.

Cell Culture

Bone marrow-derived MΦ were collected by flushing mouse femurs and tibias at day 28 after AngII or saline infusion with Roswell Park Memorial Institute. Bone marrow-derived MΦ were cultured as detailed.[28] On day 6, the cells were replated, and after resting for 24 hours, they were incubated with or without IFNβ (10 U/mL) (PBL Assay Science, catalog 12400-01). For JAK1, 3 inhibition, cells were treated with 50 nM tofacitinib (Cayman Chemicals) at the time of stimulation with IFNβ. After the appropriate time for stimulation, cells were fixed in paraformaldehyde for ChIP analysis or placed in Trizol (Invitrogen) for RNA analysis.

Chromatin Immunoprecipitation Assay

ChIP assay was performed as described.[28] Briefly, cells fixed in paraformaldehyde were lysed and sonicated to generate 100 to 300 bp fragments. Immunoprecipitated samples were incubated in anti-H3K27trimethyl antibody (Active Motif 39155) or isotype control (rabbit polyclonal IgG) (Millipore) in parallel samples overnight followed by the addition of protein A Sepharose beads (Thermo-Fisher). Bound DNA was eluted and purified using phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation. Primers were designed using the Ensembl genome browser to search the *Timp1*, *Timp2*, and *Timp3* promoter for NF*κB* within the promoter region and then NCBI Primer-BLAST was used to design primers that flank this site. Data are representative of 2 to 3 independent experiments. Primer sequences are available in Supplemental Table (Supplemental Digital Content Table I, http://links.lww.com/SLA/E685).

RNA Analysis

Total RNA extraction was performed using Trizol (Invitrogen) or Trizol LS (for human samples) according to the manufacturer's instructions. RNA was then reversed transcribed to cDNA using iScript (Biorad). PCR was performed with 2X Taqman PCR mix using the 7500 Real-Time PCR System. Primers for *Timp1* (Mm0134136_m1), *Timp2* (Mm00441825_m1), *Timp3* (Mm00441826_m1), murine *Setdb2* (Mm01318752_m1), and human *SETDB2* (HS01126262_m1)18S were used as the internal control. Data were then analyzed relative to 18s ribosomal RNA using the $2^{-\Delta\Delta}$CT methods with values standardized to the control cohort. All samples were assayed in triplicate. The threshold cycle values were used to plot a standard curve. Data are representative of 2 to 3 independent experiments that were compiled in Microsoft Excel and presented using Prism software (GraphPad).

Matrix Metalloproteinase Activity Detection

Total MMP activity was determined in protein extracts from suprarenal aortas with an MMP Assay Kit (catalog No. 112146; Abcam). Protein samples were incubated with 4-aminophenylmercuric acetate for 3 hours at 37°C. Then, the MMP Green substrate was added, and the fluorescence signal was measured in a fluorescent microplate reader (Gemini XPS Spectrofluorometer; Molecular Devices).

Gelatin Zymography

Gelatin zymography was performed as described previously with slight modification. The whole aortic tissue was homogenized and dissolved in Radioimmunoprecipitation assay buffer. Of aortic tissue homogenate, 4.5 μg was then dissolved in zymogram sample buffer (BIO-RED, # 161-0764) and loaded onto zymography gel (EC617S) to run on SDS running buffer for 90 minutes. Gels were then renatured in Zymogram Renaturing Buffer (Invitrogen, # LC2670) for 30 minutes at room temperature and developed in Zymogram Developing Buffer (Invitrogen, # LC2671) for 30 minutes at a 37°C incubator. Fresh developing buffer was then added for 24 hours. The gel was then washed with ddH20 and stained with Simply-Blue Stain (Invitrogen, # LC6060) for 20 minutes, after which it was imaged.

In Situ Zymography

In situ zymography was performed as described.[29] Briefly, 8 to 10 micron sections of frozen tissue samples were cut in optimal cutting temperature compound. Sections were incubated overnight in the dark at room temperature with a 20 μg/mL solution of DQ-gelatin (D-12054 Molecular Probes) diluted in zymogram development buffer (161-0766 BioRad), in the presence or absence of 20 mM EDTA. After 24 hours, slides were washed in PBS and mounted in Vectashield Hardset Mounting Medium with DAPI (VECTASHIELD). Gelatinase activity was visualized as green fluorescence.

Human Tissue

Full-thickness aortic wall tissue specimens were collected from the infrarenal abdominal aorta from patients undergoing open aortic aneurysm repair (n = 19) or open aorto-bifemoral bypass (n = 6). The aneurysmal samples were taken from the midportion of the aneurysmal sac. For control samples, aortic tissue was isolated from patients with atherosclerotic occlusive disease but no history of aneurysmal disease at the time of open aorto-bifemoral bypass. Patient medical comorbidities are represented in Supplemental Tables (Supplemental Digital Content Tables II and III, http://links.lww.com/SLA/E685). All aortic samples were processed for both histology and protein/RNA analyses. For histology, human aortas were placed in formalin (10%) for 24 hours before paraffin embedding. For protein/RNA analysis, specimens were stored at −80°C for future protein and RNA analyses. For single-cell RNA sequencing (scRNA-seq), a second cohort of samples was retrieved from the infrarenal abdominal aorta of patients undergoing open aortic aneurysm repair (n = 4) or open aorto-bifemoral bypass (n = 2). Patient medical comorbidities can be seen in Supplemental Table (Supplemental Digital Content Table II, http://links.lww.com/SLA/E685). These samples were immediately processed as described further. This study was approved by the University of Michigan Institutional Review Board HUM00098915.

Single-cell RNA-sequencing and Bioinformatics Analysis

Generation of single-cell suspensions for scRNA-seq was performed as follows: aortic tissue from aneurysmal and non-aneurysmal controls was harvested at the time of surgical intervention. Briefly, samples were minced, digested in 0.2% Collagenase II (Life Technologies) and 0.2% Collagenase V (Sigma) in plain medium for 1 hour at 37°C, and strained through a 70 μM mesh. The scRNA-seq samples were analyzed by the University of Michigan Advanced Genomics Core on the 10X Chromium system. Libraries were sequenced on the Illumina NovaSeq. 6000 sequencer to generate 151 bp paired-end reads. Data processing, including quality control, read alignment, and gene quantification, was conducted using the 10X Cell Ranger software. Clustered cells were mapped to corresponding cell types by matching cell cluster gene signatures with putative cell-type specific markers. Data availability statement: the scRNA-seq data for this paper is available at GEO accession number GSE166676, as described.[30]

Statistical Analyses

Data were analyzed using GraphPad Prism software version 6. Data are represented as means ± SEM. Shapiro-Wilk test was used to determine the normality of data, the Brown-Forsythe or F test to determine variances, the ROUT test was used to identify outliers in given data sets, and then a parametric or nonparametric test was performed accordingly. Parametric statistical analysis was performed using an unpaired Student *t* test (2-tailed) between 2 groups and 1-way analysis of variance followed by post hoc analysis for analysis of differences between > 2 groups. Nonparametric statistical analysis was performed using the Mann-Whitney *U* test. *P* values <0.05 were considered significant.

RESULTS

TIMP Expression in Abdominal Aortic Aneurysm Development is Regulated by SETDB2

Since ECM degradation due to an imbalance of MMP activity is a pathologic hallmark of aortic dilation,[5,6] we examined the expression of endogenous MMP inhibitors, TIMPs, in murine MΦ using an established AngII-induced murine model.[24] Specifically, male C57BL/6J mice were injected intraperitoneally with a single dose of an AAV vector expressing the mouse D377Y gain-of-function proprotein convertase subtilisin/kexin type 9 (PCSK9), which resulted in sustained hypercholesterolemia as described.[24] After this, mice were fed a saturated fat enrich diet for 6 weeks and received either saline or AngII infusion (1000 ng/min/kg) for the last 4 weeks. The use of a saturated fat diet and AngII infusion to

induce AAAs recapitulates important clinical risk factors including dyslipidemia and hypertension. We then isolated in vivo infiltrating monocyte/MΦ [CD11b$^+$ (CD3$^-$CD19$^-$Nk1.1$^-$Ly6G$^-$)] by cell sorting on day 28 from mice infused with AngII or saline given that the predominance of inflammatory MΦ within the aortic wall during AAA develop arise from infiltrating monocyte/MΦ rather than resident tissue MΦ that populate the aorta during development.[31] *Timp1*, *Timp2*, and *Timp3* expression were significantly reduced in in vivo MΦ in AngII-induced AAAs in comparison to controls (Fig. 1A and Supplemental Digital Content Figs. 1A, B, http://links.lww.com/SLA/E686). This was also associated with an increase in ECM degradation and elastin fragmentation in AngII-induced AAAs (Fig. 1B).

Given the decreased gene expression of multiple *TIMPS* in murine aneurysmal aortic tissue, we examined the expression of *SETDB2*, a methyltransferase associated with gene repression, in both the murine AngII-induced and elastase-induced AAA models. This demonstrated *SETDB2* expression was increased in both aneurysmal tissue and in vivo MΦ in the AngII-induced and elastase-induced AAA murine model compared with non-aneurysmal controls (Figs. 1C, D, and Supplemental Digital Content Fig. 1C, http://links.lww.com/SLA/E686). SETDB2 has been shown to act through the H3K9me3-mediated mechanism on gene promoters. As a methyltransferase, increased SETDB2 methylates the H3K9 site, which renders gene promoters inaccessible to transcription factor binding, resulting in gene suppression. To examine the transcriptional effects of SETDB2 in aortic tissue, ChIP was performed on aortic tissue from AngII-induced AAAs compared with those from saline-infused controls. Utilizing primers for the NFκB binding sites on the *TIMP1*, *TIMP2,* and *TIMP3* promoters, we identified that H3K9me3 was increased at the NFκB binding sites in the AngII-induced AAA aortic tissue (Fig. 1E). Collectively, these results suggested that upregulation of SETDB2 may play a causative role for aberrant *TIMP* expression and protease activity in murine AAA development.

Human Aortic Single-cell Transcription Profiling Reveals Elevated SETDB2 and Inflammatory Pathway Expression in Myeloid Cells

To translate our murine findings to human disease and to further characterize the structural and infiltrating cells within the aortic wall, we examined the presence of SETDB2 in human aortic tissue samples isolated from infrarenal AAAs and atherosclerotic aorto-iliac occlusive controls. We found expression of *SETDB2* was markedly increased in AAA tissue samples compared with atherosclerotic control aortic tissues (Fig. 2A, Supplemental Digital Content Table II, http://links.lww.com/SLA/E685). Immunostaining showed that SETDB2 was highly expressed in the aortic media and adventitia of diseased aortas (Fig. 2B). Next, to further characterize the impact of *SETDB2* expression in structural and infiltrating cells within the aortic wall, we analyzed scRNA-seq analysis on a cohort of patients undergoing abdominal aortic surgery (Supplemental Digital Content Table III, http://links.lww.com/SLA/E685).[30] Cluster analysis using the uniform manifold approximation and projection technique identified 21 different cell clusters present in the aortic tissue. We attributed clusters to their putative identities and hierarchical similarities by differentially expressed gene signatures (Fig. 2C). Given our murine findings, we sought to determine whether SETDB2 contributes to the gene expression and pathway alterations in AAA monocyte/macrophage. As such, we screened for alterations in known epigenetic enzymes in AAA MΦ/monocytes and found that *SETDB2* expression was markedly elevated in the macrophage and monocyte clusters in human AAA tissue in comparison to control samples (Fig. 2D) with minimal expression in smooth muscle cells and T and B-lymphocyte subclusters, confirming our findings from the murine model. On analysis of SETDB2$^+$ MΦ/monocytes compared with SETDB2$^-$ MΦ/monocytes from human AAA samples, gene ontology analysis and differential gene expression analysis demonstrated that multiple chromatin and transcription regulatory complexes were elevated in SETDB2$^+$ monocytes in comparison to SETBD2$^-$ monocytes. In addition, pathways instrumental to cell-cell adhesion and the extracellular space as well as gene expression of *TIMP1* and *TIMP2* were substantially altered in SETDB2$^+$ monocytes further confirming the association among SETDB2, histone regulation, and ECM organization (Fig. 3E and Supplemental Digital Content Table IV, http://links.lww.com/SLA/E685). Taken together, these results suggest human AAAs are characterized by a significant upregulation of the histone methyltransferase SETDB2 in monocytes that likely leads to alterations in transcriptional regulation and extracellular protease activity.

Myeloid-specific Deletion of SETDB2 Augments *TIMP* Expression and Prevents Aortic Wall Degeneration

SETDB2-mediated epigenetic modifications in monocytes/MΦ altered histone regulatory complexes and gene expression, hence, we investigated the translational potential of SETDB2 inhibition in regulating AAA formation and protease activity. As shown in the schematic in Figure 3A, we examined the effects of macrophage-specific genetic deletion of SETDB2 on AAA development in a murine model. To define the cell-specific function of SETDB2 in MΦ during AAA development, we uniquely generated a myeloid-specific *Setdb2*-deficient mouse. The $Setdb2^{flox/flox}Lys2^{Cre+}$ ($Setbd2^{-/-}$MΦ) and $Setdb2^{flox/flox}Lys2^{Cre-/-}$ [wild-type (WTMΦ)] control mice showed no phenotypic defects at baseline. $Setdb2^{-/-}$MΦ and WTMΦ mice underwent AngII-induced AAA induction with temporal monitoring of aortic dilation. $Setdb2^{-/-}$MΦ mice were less susceptible to AngII-induced AAA formation with a significantly decreased maximum abdominal aortic diameter and AAA incidence (Figs. 3B–D). This was accompanied by the preservation of aortic architecture in the $Setbd2^{-/-}$MΦ mice who displayed decreased elastin fragmentation compared with WTMΦ after AngII infusion (Fig. 3E). There was no difference in aortic rupture or ascending aortic diameter (Supplemental Digital Content Fig. 2, http://links.lww.com/SLA/E686).

To determine the transcriptional effects of macrophage-specific SETDB2 deficiency on *TIMP* gene expression in vivo MΦ were sorted from our $Setdb2^{-/-}$MΦ and WTMΦ mice after 28 days of saline or AngII infusion. We found a significant reduction in H3K9me3 levels on *TIMP1, TIMP2*, and *TIMP3* gene promoters in $Setdb2^{-/-}$MΦ mice after AngII infusion in comparison to WTMΦ (Fig. 3F). This was associated with a substantial increase in *TIMP*1 to 3 gene expression in vivo MΦ from $Setdb2^{-/-}$MΦ mice (Fig. 3G). To further determine the functional impact of altered *TIMP*1 to 3 expressions, we analyzed MMP activity within the aortic wall and observed decreased MMP activity in $Setdb2^{-/-}$MΦ mice after AngII infusion in comparison to WTMΦ mice that underwent AngII-induced AAA induction (Figs. 3H, I and Supplemental Digital Content Fig. 3A, http://links.lww.com/SLA/E686). These data identify that SETDB2 is important in myeloid cells for AAA development and that in vivo macrophage *TIMP* gene expression and protease activity is controlled, at least partly, by SETDB2.

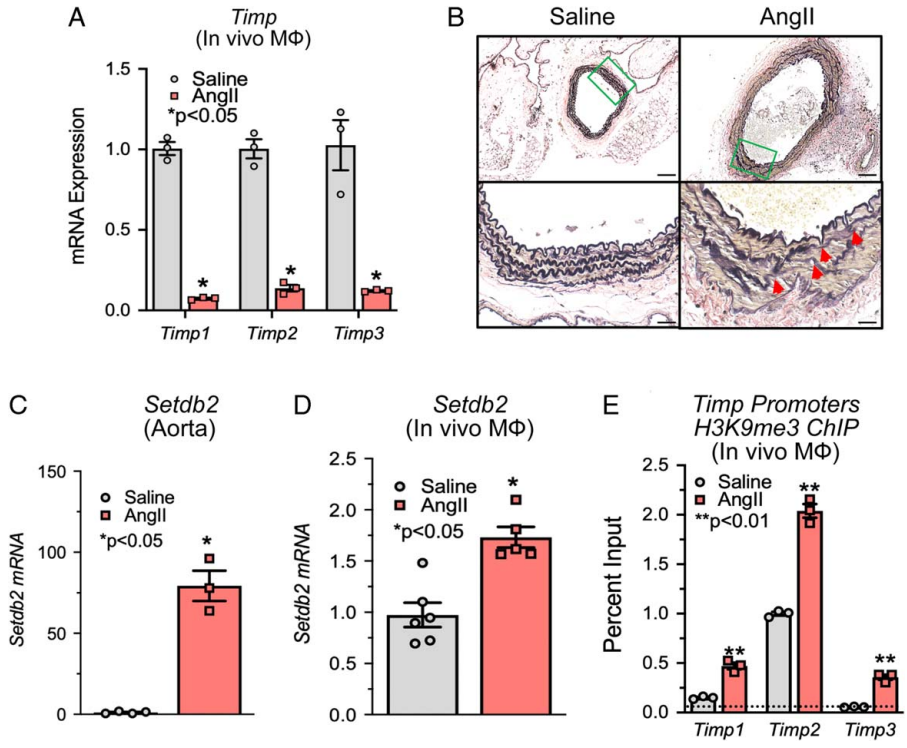

FIGURE 1. SETDB2 is an increased murine AAA MΦ and increases the repressive H3K9 trimethylation on *TIMP* gene promoters. A, Male C57BL/6J mice were injected intraperitoneally with an AAV containing mouse PCSK9D377Y and fed saturated fat diet for 6 weeks. Mice were infused with saline or AngII (1,000 ng/min/kg) for 4 weeks. qPCR analysis of *TIMP1*, *TIMP2*, and *TIMP3* isolated from in vivo MΦ [CD11b$^+$(CD3$^-$CD19$^-$Nk1.1$^-$Ly6G$^-$)] of mice exposed to saline or AngII for 28 days (n = 3/group run in triplicate). *$P < 0.05$, **$P < 0.01$ for Welch *t* test. B, Representative Verhoeff-Van Gieson elastin staining of abdominal aortic sections at 10× and 40× showing disrupted aortic structure in AngII mice compared with saline control mice; scale bar is 50 μm or 10 μm in Verhoeff-Van Gieson stain; arrows represent elastin fragmentation. C and D, qPCR analysis of *SETDB2* isolated from aortas or MΦs [CD11b$^+$(CD3$^-$CD19$^-$Nk1.1$^-$Ly6G$^-$)] in mice infused with either saline or Ang II for 28 days (n = 3–4/group run in triplicate). *$P <$ 0.05 for Mann-Whitney *U* test. E, ChIP analysis for H3K9me3 at *TIMP1*, *TIMP2*, and *TIMP3* promoters was performed (n = 3/group run in triplicate). For all ChIP experiments, isotype-matched IgG was run in parallel. Dotted line represents isotype-matched control. **$P < 0.01$ for Mann-Whitney *U* test. qPCR indicates quantitative PCR.

IFNβ/JAK/STAT3 Regulate *SETDB2* Expression Resulting in Increased H3K9me3 at NFκB Binding Sites on *TIMP* Gene Promoters in Monocytes/Macrophages

IFN-I serve as cell signaling molecules that bind to cell surface receptors and ultimately phosphorylate tyrosine kinases inducing specific transcription factors to activate inflammatory genes.[32] Although IFN-I has been well-studied in viral disorders and autoimmune diseases, very little is known about the role of IFN-I in both normal and pathologic vascular remodeling.[33–35] Prior investigations have demonstrated that IFN-Is are increased in both human tissue samples and murine models of AAAs. Further, downstream IFN-I signaling pathways, JAK1/STAT3 pathway, have also been shown to be highly upregulated in human AAA tissue samples[35] and administration of an anti-IFN receptor 1 antibody attenuated AAA development in a murine model.[33,36] It has recently been suggested in diabetic cutaneous MΦ that transcriptional regulation of SETDB2 by IFNβ and STAT signaling may drive inflammatory responses.[17] IFNβ has been recently shown to be correlated with advanced age and aortic aneurysmal disease progression,[37] however, the mechanisms, by which IFNβ and JAK/STAT signaling contribute to SETDB2 regulation in aneurysmal development, remain undefined.

Our results support altered levels of IFNβ in the AngII-induced and elastase-induced AAAs (Supplemental Digital Content Fig. 1D, http://links.lww.com/SLA/E686). To determine whether IFNβ stimulation regulated *SETDB2* expression in MΦ, we sorted MΦ and stimulated them ex vivo with IFNβ (100 U) for 6 hours and examined *SETDB2* gene expression. In MΦ, *SETDB2* was significantly increased after IFNβ stimulation (Fig. 4A). To confirm that SETDB2 regulated *TIMP* expression, ChIP analysis was conducted for H3K9me3 on the NFκB binding sites on *TIMP*1 to 3 gene promoters demonstrating a significant increase in repressive H3K9me3 after IFNβ stimulation (Fig. 4B). This also corresponded with a significant decrease in *TIMP*1 to 3 expressions (Fig. 4C). To confirm that loss of IFNβ signaling decreased macrophage *SETDB2* expression, we isolated MΦ from *Ifnar*$^{-/-}$ mice and *Ifnar*$^{+/+}$ controls. After 6-hour IFNβ stimulation, *SETBD2* was significantly reduced in the *Ifnar*$^{-/-}$ MΦ compared with matched controls suggesting that IFNβ signaling alters SETDB2 expression in MΦ (Fig. 4D). IFN-I signals through the JAK/STAT pathway to promote gene transcription during viral infection, however, the

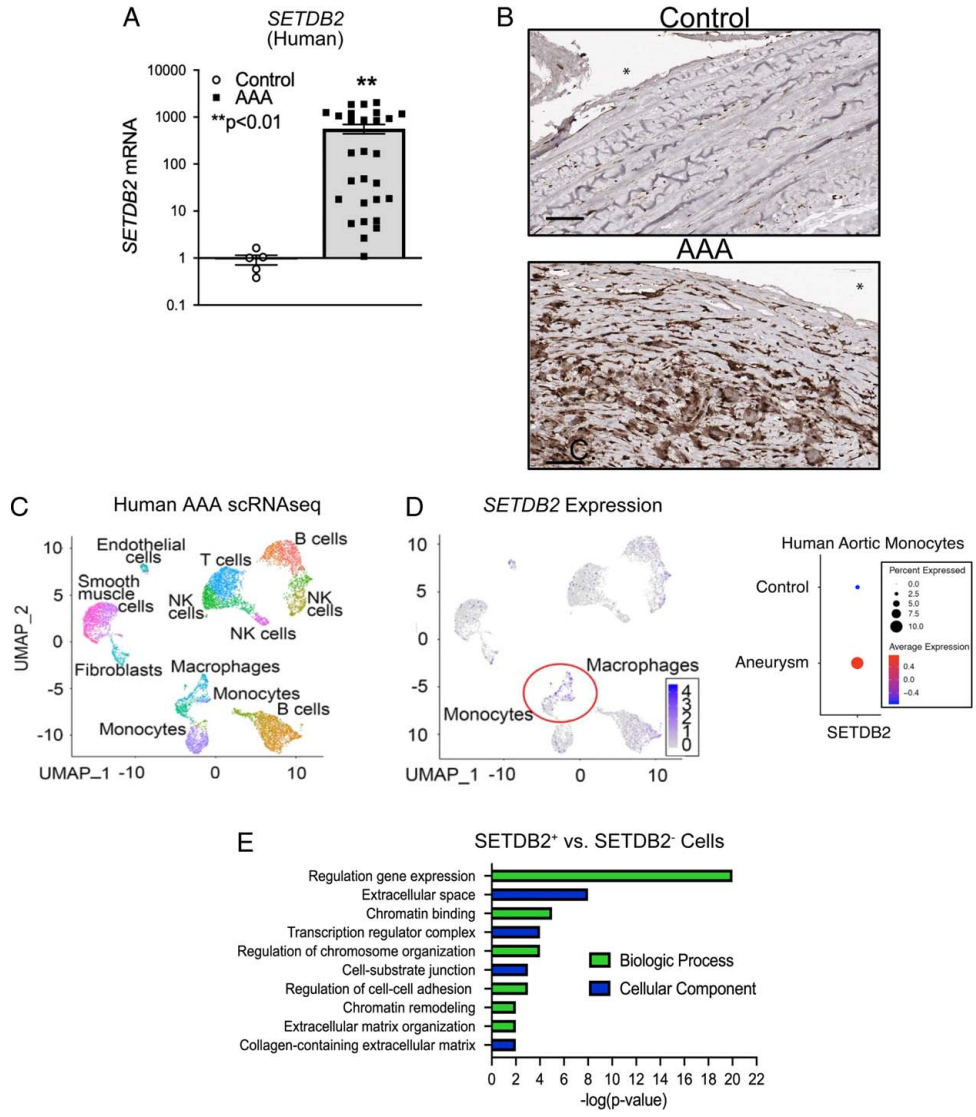

FIGURE 2. Human aortic single-cell transcription profiling reveals elevated SETDB2 and ECM organization pathways in infiltrating monocyte/MΦ. A, Aortic tissue from patients with AAA (n = 19) and atherosclerotic controls (n = 6) was collected. No statistical differences were found between groups with respect to sex, age, or comorbid conditions. *SETDB2* gene expression was measured by qPCR with a log scale. *$P < 0.05$ by Welch *t* test replicated twice. B, Immunohistochemistry was performed for SETDB2 in human control and AAA samples. Representative slides are shown at 40× and the scale bar is 60 μm. C, Cluster analysis using the UMAP technique of single-cell sequencing from human AAA (n = 4) and nonaneurysmal (n = 2) samples revealed 21 distinct cell clusters (representative). D, Feature plots displaying the single-cell gene expression of *SETDB2* across cell clusters. E, Gene ontology biological process or cellular component enrichment analysis of differentially expressed genes SETDB2⁺ versus SETDB2⁻ cells. The combined score metric corresponds to the *P* value (2-tailed Fisher exact test) multiplied by the *Z*-score of the deviation from the expected rank, and *q* values determined by Benjamini-Hochberg correction. qPCR indicates quantitative PCR; UMAP, uniform manifold approximation and projection.

role of IFN-I/JAK/STAT in AAA pathogenesis remains undefined. We, thereby, isolated MΦ and stimulated them with IFNβ (100 U) with or without the JAK1 inhibitor, tofacitinib (50 nM). *SETDB2* was significantly decreased in MΦ treated with the JAK1 inhibitor after stimulation with IFNβ (Fig. 4E). To further confirm that tofacitinib impacted AAA development in vivo, mice underwent AngII-induced AAA induction with and without tofacitinib treatment (20 mg/kg intraperitoneal injection 3 times weekly) and temporal monitoring of aortic dilation was conducted. Tofacitinib therapy significantly decreased AAA development (Supplemental Digital Content Fig. 3B, http://links.lww.com/SLA/E686). Given the prior findings implicating STAT in AAA tissue, we further investigated JAK/STAT3 downstream signaling, we analyzed *SETDB2* expression in MΦ from *Stat3*$^{-/-}$ mice and matched controls (*Stat3*$^{+/+}$). *Stat3*$^{-/-}$ mice demonstrated decreased *SETDB2* expression in response to IFNβ stimulation (Fig. 4F). Taken together, these results suggest that the increased SETDB2 responsible for the TIMP regulation during AAA development may be altered through upstream therapeutic manipulation of the IFNβ/JAK1/STAT3 pathway.

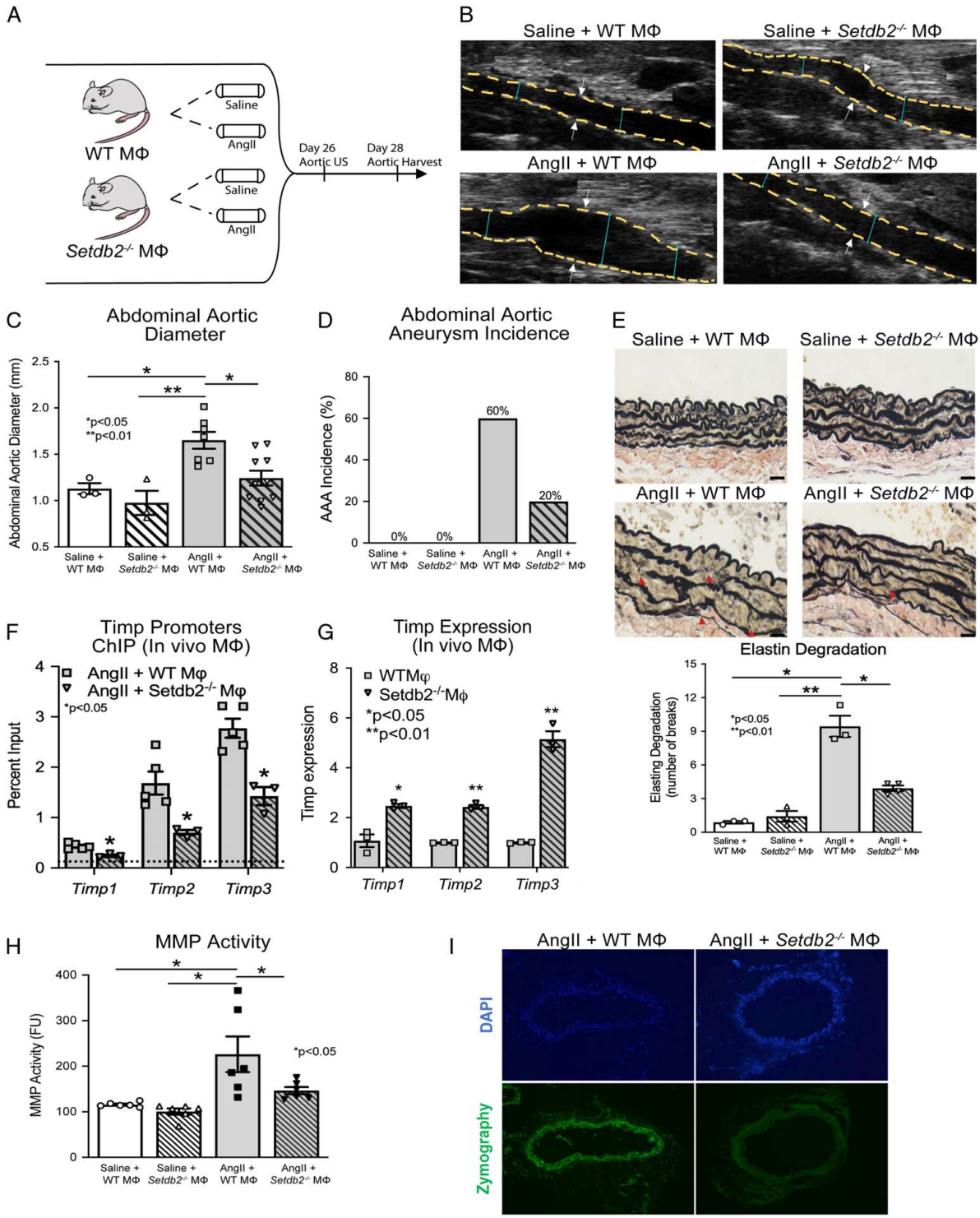

FIGURE 3. AAA formation is inhibited in macrophage-specific SETDB2-deficient mice. A, Experimental design of macrophage-specific SETDB2 deficiency in murine AAA model. WT (WT MΦ) mice and mice with macrophage-specific SETDB2 deficiency (*Setdb2*$^{-/-}$ MΦ) were fed a high-fat diet for 6 weeks and infused with saline or AngII infusion (1,000 ng/min/kg) for 4 weeks. B, Representative ultrasound images of the abdominal aorta at day 28 in WT MΦ or *Setdb2*$^{-/-}$ MΦ after either saline or AngII infusion. Dotted line represents the aortic contour and the arrows represent the aortic wall diameter. C and D, Maximal abdominal aortic diameter and aneurysm incidence as determined by ultrasound measured by 2 observers in WT MΦ or *Setdb2*$^{-/-}$ MΦ infused with either saline or AngII (n = 6 in saline-infused cohorts and 20 in AngII-infused cohorts). *$P < 0.05$; **$P < 0.001$ by ANOVA with Newman-Keuls multiple comparison test. Data are presented as the mean ± SEM. E, Representative Verhoeff-Van Gieson elastin staining of abdominal aortic sections showing a preserved aortic structure in *Setdb2*$^{-/-}$ MΦ + AngII compared with WT MΦ + AngII mice; scale bar is 200 μm in Verhoeff-Van Gieson stain; arrows represent elastin fragmentation. Average number of elastin fragmentation per high power field. F, ChIP analysis for H3K9me3 at *TIMP1*, *TIMP2*, and *TIMP3* promoter was performed on MΦ [CD11b^{+}(CD3^{-}CD19^{-}Nk1.1^{-}Ly6G^{-})] isolated from AngII + WT MΦ and *Setdb2*$^{-/-}$ MΦ at day 28 (n = 5 mice/group pooled and run in triplicate). For all ChIP experiments, isotype-matched IgG was run in parallel. Dotted line represents isotype-matched control. **$P < 0.01$ for Mann-Whitney *U* test. G, *TIMP1*, *TIMP2*, and *TIMP3* expression were measured by qPCR in in vivo MΦs isolated from WT MΦ or *Setdb2*$^{-/-}$ MΦ after AngII-infused mice on day 28 (n = 3/group run in triplicate). *$P < 0.05$, **$P < 0.01$ by Mann-Whitney *U* Test. H, MMP activity was measured in protein abdominal aorta extracts by fluorometry and measured in fluorometric units. Data represent the mean ± SEM from n = 6 animals per group. Statistical analysis was performed by 1-way ANOVA (Newman-Keuls post hoc test). *$P < 0.05$. I. MMP activity in abdominal aortic sections by in situ zymography. Representative images are shown with green signal corresponding to active MMPs; blue signal corresponding to DAPI staining for cell nuclei. ANOVA indicates analysis of variance; qPCR, quantitative PCR.

DISCUSSION

Our results have uncovered dynamic mechanisms that bridge the imbalance of protease-mediated ECM degradation and natural inhibitors of proteases during AAA expansion. Further, we have identified the epigenetic chromatin modifying enzyme, SETDB2, as an instrumental signal in perpetuating the aortic wall degradation (Fig. 5). Herein, using human AAA tissue samples and 2 murine models (AngII-induced AAAs and elastase AAA model), we identified that the epigenetic enzyme SETDB2 is a crucial regulator of *TIMP* expression and subsequent MMP activity. Mechanistically, IFNβ signals through the JAK/STAT3 pathway to increase SETDB2, which colocalizes to NFκB binding sites on *TIMP*1 to 3 gene promoters, where it leads to inciting the repressive histone methylation mark H3K9me3, thereby inhibiting *TIMP* gene expression. In addition, single-cell transcriptomics demonstrated human monocytes and tissue MΦ have increased *SETDB2* expression resulting in the activation of multiple chromatin regulatory and ECM pathways. Ultimately, manipulation of this pathway, using a macrophage-specific genetic model (*Setdb2*$^{flox/flox}$*Lys2*$^{Cre+}$) increased *TIMP* expression, limited ECM protease activity, and decreased AAA formation.

MΦ present in aortic tissue arise from either hematopoietic progenitor cell proliferation/differentiation or mobilization of splenic monocytes.[38,39] These recruited MΦ serve to promote AAA formation, in part, through increased proteolytic enzyme production that weakens the aortic wall. The main class of proteolytic enzymes associated with impaired aortic tissue integrity is the MMPs. These enzymes degrade elastin, collagen, and the ECM allowing for negative remodeling and loss of vascular smooth muscle cells. Although some papers have reported how altered MMP activity is critical for the regulation of vascular remodeling during development, within AAA formation MMP activity has consistently been demonstrated to be pathologic.[40,41] MMPs are regulated at the level of mRNA expression, pro-enzyme activation, and inhibitory action of TIMPs. These endogenous naturally occurring MMP inhibitors have a protective effect against the development of aneurysms as they inhibit the proteolytic activity of MMPs by forming protein complexes with their specific pairs.[42,43] The molecular mechanisms that program and sustain macrophage gene expression profiles in AAA disease and allow dysregulation of MMP/TIMPs have not previously been explored. Hence elucidating the precise mechanisms responsible for how MΦ regulate proteolytic enzyme production will allow for the development of more precise and targeted therapeutics.

Prior investigations by our group and others have begun to explore the role of epigenetics on macrophage-mediated inflammation in human disease,[17,20,44,45] however, few studies have looked at epigenetic regulation of macrophage function in the setting of aortic pathologies. Initial investigations found histone deacetylases were increased in AAA tissues from humans and AngII-infused mice. Further, inhibition of class I or class IIa histone deacetylases improved survival and decreased AAA formation in mice however the cell-specific mechanisms were not fully investigated and the genes these deacetylases regulated were unknown.[46–48] More recent studies have attempted to provide cell-specific information by analyzing immune cell subsets within AAA disease. Especially, the isolation of regulatory T lymphocytes (FOXP3^{+} CD4^{+} CD25^{+}) from human AAA tissue had reduced acetylation on histone 3 and increased histone deacetylase enzymes compared with healthy controls but failed to correlate changes in histone acetylation to regulatory T-lymphocyte function.[49,50] In contrast to histone acetylation, the role of histone methylation has not been extensively investigated in AAA disease. Specific to this, our group recently published on the role of an epigenetic enzyme histone demethylase, JMJD3, on macrophage-mediated inflammation in AAAs, however, this work did not examine the role of other epigenetic enzymes or macrophage regulation of proteases.[20] In the current investigation, our human single-cell sequencing identified SETDB2 to be significantly increased in MΦ from AAA tissue and that SETDB2 adds a repressive H3K9me3 mark onto the TIMP promoters in monocyte/MΦ resulting in decreased *TIMP* expression and unregulated MMP activity. Further, mice with macrophage-specific deletion of SETDB2 demonstrated decreased AAA formation and improved TIMP/MMP ratios, consistent with alterations in the protease levels and decreased elastin degradation. SETDB2 has recently been shown to be important to the regulation of macrophage inflammation in a variety of disease states including in atherosclerosis;[17,18] however, no prior publication has examined the role of SETDB2 in AAA MΦ or in any disease state with respect to protease function. Our findings suggest the SETDB2-mediated regulation of TIMP/MMP imbalance in

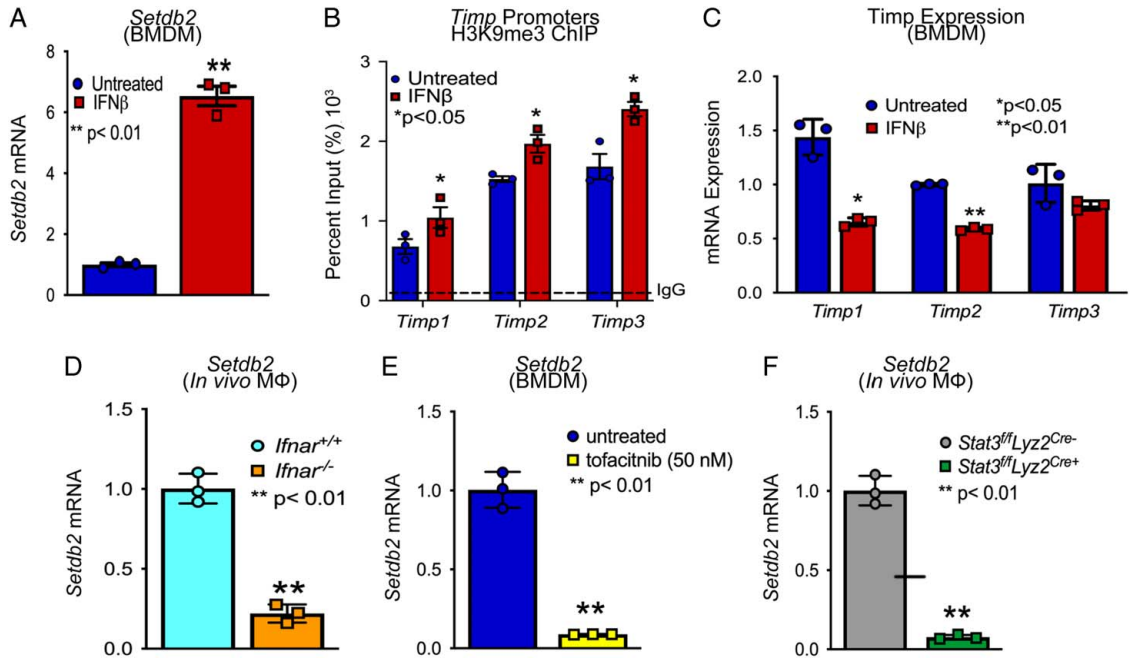

FIGURE 4. IFNβ/JAK/STAT3 pathway induced Setdb2/H3K9me3 on TIMP promoters in MΦ. A, *SETDB2* expression in BMDM treated ex vivo with IFNβ for 8 hours (n = 6 mice per group). B, ChIP analysis of BMDMs from controls treated ex vivo with IFNβ (100 U) for 6 hours and analyzed for H3K9me3 at the NF-κB binding site on the *TIMP1, TIMP2, and TIMP3* promoters (n = 6 mice per group). C, TIMP1, TIMP2, and TIMP3 expressions in BMDM treated ex vivo with IFNβ for 8 hours (n = 6 mice per group). *P < 0.05. D, qPCR analysis of *SETDB2* was conducted in MΦ [CD11b$^+$(CD3$^-$CD19$^-$Nk1.1$^-$Ly6G$^-$)] isolated from *Ifnar*$^{-/-}$ and littermate controls (*Ifnar*$^{+/+}$) (n = 3 mice/group run in triplicate). **P < 0.01 for Mann-Whitney U test. E, *SETDB2* expression in BMDMs treated with IFNβ +/− tofacitinib (JAK inhibitor; 50 nM) (n = 3 mice per group). **P < 0.01 for Mann-Whitney U test. F, qPCR analysis of *SETDB2* was conducted in MΦ [CD11b$^+$(CD3$^-$CD19$^-$Nk1.1$^-$Ly6G$^-$)] isolated from *Stat3*$^{f/f}$*Lyz2*$^{Cre-}$ and littermate controls (*Stat3*$^{f/f}$*Lyz2*$^{Cre+}$) (n = 3 mice/group run in triplciated). **P < 0.01 for Mann-Whitney U test. BMDM indicates bone marrow-derived macrophage. qPCR indicates quantitative PCR.

the aortic wall that degrades the structural integrity of the aorta may be a more influential driver of aortic aneurysmal dilation than SETDB2-mediated regulation of inflammation. In addition, from a translational standpoint, it is well-established clinically that diabetic patients have a reduced prevalence of AAAs and a slower AAA growth rate of established aneurysms.[51–53] However, the mechanism behind the negative association between AAAs and diabetes remains unknown. We have demonstrated that SETDB2 is profoundly reduced in MΦ from diabetic patients and multiple diabetic animal models resulting in altered macrophage function during wound healing.[17] As such, a potential driver behind the reduction in AAA incidence seen in diabetic patients is a decrease in SETDB2 expression and an alteration of TIMP/MMP balance in the aortic wall in favor of the maintenance of the aortic wall architecture. However, additional preclinical and clinical investigations focusing only on diabetic patients are warranted to further establish this mechanistic link.

Presently, there are no pharmacological inhibitors of SETDB2, however, we examined the upstream signaling pathway whereby JAK/STAT signaling increases SETDB2 in aortic MΦ. Multiple clinical trials have attempted to investigate medical therapy for AAA disease with the recent N-TA^{3}CT trial investigating the utility of doxycycline, a broad MMP inhibitor, on the reduction of aortic dilation. Unfortunately, within the N-TA^{3}CT, doxycycline failed to prevent AAA progression.[54] This lack of clinical impact of doxycycline on AAA disease despite multiple promising preclinical investigations was likely related to the inability of doxycycline to inhibit MMP activity in comparison to placebo controls in the serum of N-TA^{3}CT participants. In contrast to doxycycline therapy that broadly targets MMPs, within this manuscript, we chose to target cell-specific SETDB2 epigenetic modifications within MΦ to prevent AAA dilation. As MΦ exhibit different functional phenotypes during tissue repair,[55] the ability to modulate macrophage

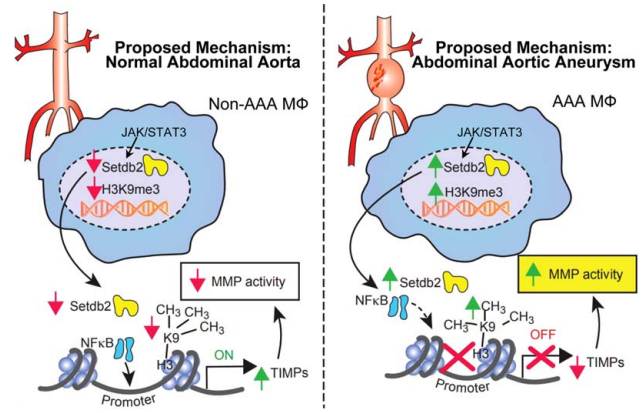

FIGURE. 5. Schematic of SETDB2-mediated regulation of macrophage *TIMP* expression and MMP during AAA development.

phenotype at a particular time after injury is an attractive therapeutic strategy. Indeed, epigenetic therapies have been shown to be effective in the treatment of cancer with drugs resulting in dose-dependent inhibition of cell proliferation, invasion, and cell migration.[56–58] More recently, epigenetic therapies have are beginning to be examined in cardiovascular disease.[14,59] For example, epigenetic therapies for atherosclerosis are currently undergoing phase 3 clinical trials, suggesting that methods that alter epigenetic enzymes show promise in controlling cardiovascular disease.[60] Despite these preclinical and ongoing trials for cardiovascular disease, the role of JAK/STAT3 signaling and SETDB2 inhibition in AAA development has not been vigorously investigated. Prior preclinical studies focusing on STAT3 pharmacological and genetic inhibition have yielded conflicting results demonstrating both inhibition and progression of the aneurysmal disease as well as limited translational ability due to the low oral bioavailability of pharmacological STAT3 inhibitors.[61] Our results demonstrate SETDB2 and potential inhibition with tofacitinib may be an attractive therapeutic target, either in peripheral monocytes or aortic tissue MΦ, due to its capacity to decrease vascular remodeling.

Although this study provides mechanism(s) behind important advances related to dysregulated macrophage function in AAA development, there are several limitations to this study. Regarding the human samples, these are taken from patients with AAA that meet repair criteria and thus represent the end stages of the disease. It is possible that SETDB2 is less relevant during the earlier stages where AAAs are developing and the elastin is less fragmented. Second, within our myeloid-specific SETDB2 murine model, we utilized the Lyz2^{Cre} system, which we have demonstrates efficiently depletes SETDB2 in myeloid cell population.[17] We acknowledge that gene expression profiles among monocytes/MΦ, neutrophils, and dendritic cells overlap due to their close lineage relationship. As such, there is no Cre-transgenic line that is perfectly specific for MΦ.[23] However, the contributions of neutrophils and dendritic cells for AAA development have been shown to be minor in comparison to macrophage pathophysiology.[6,31,62] Lastly, we recognize that other epigenetic enzymes or vascular cell types may regulate aberrant macrophage function during AAA development as well.[63]

CONCLUSIONS

Our study provides important insight into how JAK/STAT signaling regulates SETDB2 in aortic MΦ, which results in increased protease activity that ultimately increases AAA development. Increased SETDB2 in MΦ increases the repressive histone methylation mark H3K9me3 on the TIMP gene promoters, which alters the MMP/TIMP imbalance and leads to adverse vascular remodeling and aortic dilation. Targeting the JAK/STAT/SETDB2 pathway in a cell-specific manner will allow us to modulate macrophage-mediated protease activity in aortic tissue and may lead to decreased AAA formation and rupture.

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DISCUSSANT

Dr. Ronald Dalman (Stanford, CA)

Thank you, and thanks to the audience and the program committee for asking me to comment on this fine paper. I am honored to be the first to congratulate Dr. Davis and colleagues for their in-depth investigation of epigenetic regulation of macrophage differentiation and activation in AAA pathogenesis. They have presented elegant experimental work with conclusions well supported by multiple layers of complementary modeling and human evidence, exactly as we have come to expect from this research group.

To understand the impact of this work, however, we need to consider the long history of failure in identifying putative inhibitory agents for AAA disease progression. These include strategies using platelet inhibition, immune activator modulators, renin-angiotensin system inhibitors, other antiproteolytic agents, antibiotics, and antioxidants, just to name a few.

This field in general suffers from the ready, fire, aim syndrome; the pursuit of target inhibition mechanisms, candidate inhibitors identified either through murine modeling or adopted from potentially related conditions, such as atherosclerosis or, in

this case, wound healing, without direct evidence of relevance to human AAA disease.

Statins are remarkably effective at reducing all-cause Cardiovascular mortality, but they do not inhibit aneurysm progression or prevent rupture, and neither does doxycycline, an agent trialed specifically because of its MMP inhibitory activity. Interest in MMP modulation as an inhibitory strategy for AAA management diminished after the failure of the N-TA(3)CT trial, a level 1 clinical trial published in JAMA demonstrating no difference in aneurysm growth rates between doxycycline and placebo groups.

AAA trials are challenging for multiple reasons. I have done a few, but the N-TA(3)CT trial results cast a long shadow on the translational relevance of MMP inhibition in AAA management, so the first question, is there a Mendelian randomization study or similar evidence suggesting that genetic variation in *SETDB2* expression, or *TIMP* expression activity in general, are related to human aneurysm disease risk? The diabetic connection that you mentioned in the manuscript is intriguing, and you have nicely demonstrated increased expression in human AAA tissue harvested at surgery, but given the discouraging track record of MMP inhibition and translational application for this problem, more evidence is needed.

Secondly, ECM proteases are not exclusively produced by activated tissue MΦ, and proteases other than metalloproteases likely promote aortic ECM degradation as well. Cystine and serine proteases, for example, are known to be involved. Do similar epigenetic mechanisms influence the balance of proteolytic activity considered more broadly?

Finally, you recently identified the histone demethylase JMJD3, also regulated by IFN-B through JAK/STAT pathways as another potential regulatory target for this problem. JMJD3 induces NF-kappa B-mediated inflammatory gene transcription in infiltrative MΦ. Pharmacologic or genetic inhibition of macrophage-specific activity was able to prevent AAA development and rupture in similar modeling experiments to what you present today. Please comment on the relative significance and translational potential of JMJD3 versus SETDB2 as inhibitory strategies for aneurysm inhibition, and are you considering other epigenetic mechanisms as well?

Congratulations on a landmark contribution to this evolving field. When added to the rapidly expanding lexicon of AAA pathogenesis research, this work provides hope that a medical cure, if you will, for AAA disease will be discovered in our professional lifetimes.

Thank you to the program committee for asking me to comment on this fine manuscript.

Response From Frank M. Davis

Thank you, Dr. Dalman, for your very insightful questions, and you have long been a pioneer in this field, so I appreciate your reviewing our manuscript.

Regarding your first question about GWAS data in terms of any role of SETDB2 in GWAS data, currently, there is been a handful of GWAS studies that have looked at aortic aneurysm prevalence, both in Europe as well as here within the United States, and the GWAS data thus far has not shown any SETDB2 or epigenetic enzyme that correlates with AAA. With that being said, it has shown differences in more cholesterol-based gene expressions, but as you correctly pointed out, modification of that with the statins has not shown any change in aortic aneurysms.

Your second question about MMP inhibition and its translational potential, I agree that the doxycycline trial conducted here in the United States was, unfortunately, a negative trial and did not show that doxycycline prevented AAAs despite promising research in animal-based models. That being said, doxycycline is a broad antibiotic and the MMP inhibitor administered to humans was not successful, so we are hoping that by being more cell-specific, we could be able to intervene in that.

You also correctly pointed out that although this paper specifically looks at MMPs and TIMPs and their overall interaction, we did not look at cysteine and serine proteases. Those have been shown by other research groups to potentially contribute to aortic aneurysm development, but at this time we have not looked at epigenetic regulation of those proteases.

And last, you had mentioned that JMJD3 in one of our recent papers looking at a separate epigenetic enzyme and its role within macrophage inflammation showing that pharmacological and genetic inhibition prevented aortic aneurysms. In this paper, we look at a separate enzyme, SETDB2. Right now, I do not have a good answer for you, about which one has the better translational potential, and I am hoping throughout my career we can get better answers for you on that. I think both epigenetic enzymes contribute to different mechanisms within aortic aneurysm development and designing the optimal translational therapy to inhibit epigenetic enzymes will be a subject of ongoing work.

Dr. Vikas Dudeja (Birmingham, AL)

Vikas Dudeja, University of Alabama at Birmingham. Congratulations, Frank, on this excellent work. I have 2 questions, so typically, epigenetic changes affect multiple genes by affecting multiple sites on the DNA. So in your ChIP assay, did you look at what else gets immunoprecipitated, or in other words, what other sites are being acquired and being involved with the SETDB2?

Second thing is have you looked at the inducible inhibition of this enzyme? So you have used the inducible LysM model. Tamoxifen inducible LysM-cre model is available. Have you considered using it to see if, once the aneurysm develops, inhibition of this enzyme can reverse or at least prevent progression?

Response From Frank M. Davis

Yeah, that is a great point. Yeah, I will take your second question first. We have not looked at inducible LysM-Cre deletion, and I think ultimately that is where things need to go because, in humans, they do not come to us before they have an aneurysm. We have to prevent it. They come with an already established aneurysm, and we somehow have to prevent that growth but not stop the initiation of it, so that is definitely an area we could pursue in terms of inducible LysM-Cre, so that is something maybe we can talk about afterward, but I do appreciate your insight there.

And then in terms of your other question about H3K9 trimethylation in SETDB2, we only specifically looked at it in TIMPs. We did not do a broad histone modification array just because of the limited tissue aspects we were able to do, but it is something you could pursue.

Dr. Elliot Chaikof (Boston, MA)

Congratulations on a very nice study. As you think about targeting SETDB2, are there any pharmacodynamic biomarkers you could use, circulating monocytes, do they express any changes in SETDB2?

As a second question, angiotensin-induced models are used for the study of aortic aneurysms but actually represent a model of aortic dissection. Have you observed changes in SETDB2 in human specimens from patients with an aortic dissection?

Response From Frank M. Davis

Great questions, so in terms of your biomarker for SETDB2, to my knowledge, there is no specific blood-based biomarker that is able to tell whether SETDB is high or low, and that is something I could look into more, but I have not seen that at all published in the literature.

In terms of your question about our modeling of aortic aneurysms in the Ang-2 model, that does produce dissecting aortic aneurysms. We also looked at it in the last ACE model, and I did not produce that data here, but it is in the manuscript, and we also found similar results as an elevated SETDB and knockout of SETDB2 prevents aortic aneurysms. So far in the human patient population, we did not look at SETDB2 in dissections, especially in single-cell sequencing. We just have not been able to accumulate enough of those patient samples to be able to adequately look at that and investigate that.

Dr. David Soybel (White River Junction, VT)

David Soybel, White River Junction. Before I ask my questions, I just want to assure Dr. Freischlag and my vascular colleagues I am not planning on repairing an aneurysm anytime soon. So I really like this idea, which I do not think has quite enough currency, that it is the persistent inflammatory state, the macrophage is kind of locked into this M1 phase, and as you are aware, this whole field of resolution biology starts with the lipoxygenase pathway and resolvins, and I am wondering first of all if you have thought about the relationship of that pathway to the formation of aneurysms, how you incorporate it into your model here, and second of all, also do you have a sense for what locks the macrophage lineage into that pathway in the setting of an aneurysm?

Response From Frank M. Davis

Yeah, 2 great questions. Your first question regarding resolvins, we specifically in our laboratory have not looked at the role of resolvins in activating epigenetic enzymes. There have been other laboratories, mainly Dr. Upchurch's laboratory at the University of Florida, which have looked aggressively into resolvins and resolvin-D and their role in aortic aneurysms but have not linked those to epigenetics to my knowledge, so that is something that I guess could be pursued in the combination of the laboratory.

In terms of your question about what locks the MΦ into this role, that is the million-dollar question. I do not have an answer for you in terms of once it is primed and activated with an epigenetic, why is it always locked, and we have seen in other disease pathways, such as diabetes that hyperglycemia also induces epigenetic changes that lock it in. In terms of how to make that macrophage more fluid and able to transition out of that stage, I think that is something we are pursuing, but I do not have a good answer for you yet.

Thank you for your good questions.

Dr. Ai-Xuan Holterman (Chicago, IL)

University of Illinois. My comment has to do with several previous remarks about alternative mechanisms for AAA development to the one presented because as you know, there is a link between MMP and TIMP with inflammation and fibrosis, and particularly, TIMP as a profibrogenic factor. As TIMP expression is upregulated according to your data, your specimens do not show histological evidence of collagen deposition and fibrosis despite the upregulation of TIMP. I do not know if you have done an immunoassay for collagen or other assays for fibrosis activities, but it gets to the question that there might be some other mechanisms in AAA generation.

Response From Frank M. Davis

Yeah, great question. In terms of our sample, both in the animal samples and our human AAA samples, we do not see a significant upregulation and fibrosis around the adventitial layer in either of our animal models, so I can not really add a lot of credence to kind of what your question asks because I do not have that data.

Dr. Scott Lemaire (Houston, TX)

Great work, Frank. My first question relates to the opportunity to add single-cell Assay for transposase-accessible chromatin with sequencing (ATAC-seq) analyses so you can directly test chromatin accessibility in the human samples and also the opportunity to do single-cell analysis in the mouse models. Would you comment on whether you are doing single-cell ATAC-seq to directly demonstrate changes in chromatin accessibility in the different phenotypes?

Response From Frank M. Davis

Great question, yeah, we are pursuing single-cell ATAC-seq as well as spatial sequencing in our human AAA samples. I have not pursued single-cell ATAC-seq in our mouse aneurysm samples. Part of it is the processing and the limited tissue, so you have got to get enough pooled cohort to do that, and I know your lab has done an aggressive amount of single-cell sequencing in aortic aneurysms as well.

Dr. Scott Lemaire (Houston, TX)

My second question gets back to the mouse model and the issue Dr. Chaikof mentioned. The AngII infusion model is an imperfect model, I think, for infrarenal AAAs. First, it does not affect the infrarenal aorta, and second, it features a dissection phenotype and extensive thoracic aneurysm development. So, two adjacent questions would be one, are you looking at SETDB2 in other models of infrarenal aneurysms, such as the elastase perfusion model, to study it in a nondissection model? And two, are you looking at the ascending aortas in these mice? I think there would be a lot of interest in that, and perhaps collaborating with Bo Yang and his group at Michigan and getting ascending aortic tissue would afford an opportunity to examine ascending aortas in both patients and animals.

Response From Frank M. Davis

Two great questions. For your elastase question, yes, we have looked at it in the elastase AAA model, and it shows the same findings of SETDB2 upregulation and inhibition with the absence of SETDB2. We did not look at it in the calcium chloride model, which is the third model of AAAs.

In terms of ascending aortic pathology, in our Ang-2 model, we do not see differences in the SETDB2 WT versus SETDB2 macrophage-specific knockout in the ascending aneurysm. We have not looked at ascending aortic human aneurysmal tissue to look at variations in SETDB2, so I can only comment on the mouse aspect from that standpoint.

Dr. Scott Lemaire (Houston, TX)

Great work.

Response From Frank M. Davis

Thank you.

Dr. Keith Lillemoe (Boston, MA)

I am sure everybody in the audience asks, "Why is he up there talking about this? He knows nothing about aortic aneurysms and even less about epigenetic regulation," but as a tease for the business meeting, I would like to let everybody know that Dr. Davis is the 2023 to 2025 awardee of the ASA Foundation Fellowship, and as you can see, it is been a good investment. I am sure we will see his papers in the future here, and congratulations, Dr. Davis.

Response From Frank M. Davis

Thank you, Dr. Lillemoe. Thank you to the ASA for their support, too.