SUPPLEMENTAL MATERIAL

Aortic Stress Activates an Adaptive Program in Thoracic Aortic Smooth Muscle Cells That Maintains Aortic Strength and Protects Against Aneurysm and Dissection in Mice

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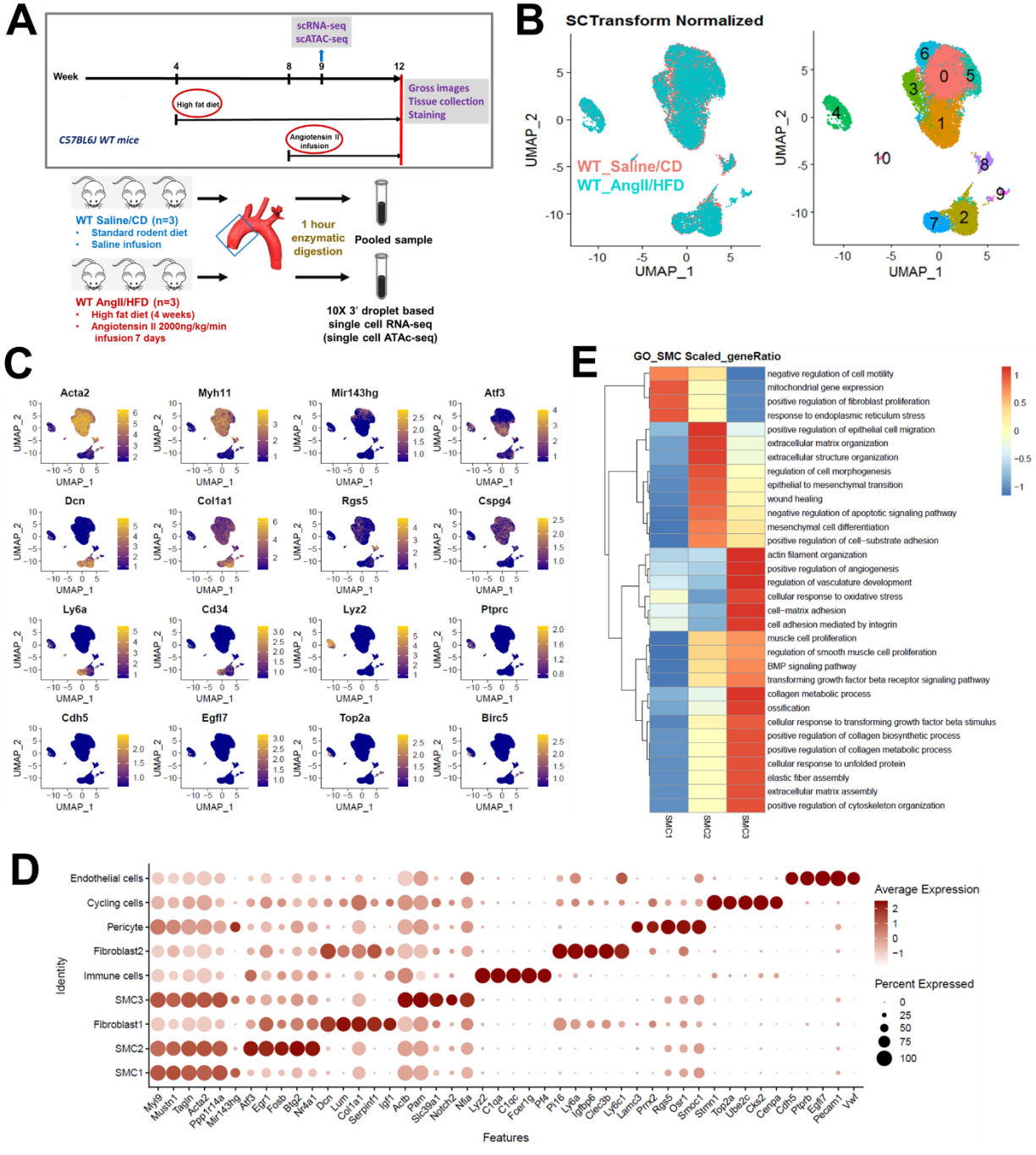
A
scRNA-seq
scATAC-seq
Week
4
8
9
12
High fat diet
Gross images
Tissue collection
Staining
C57BL6J WT mice
Angiotensin II infusion
WT Saline/CD (n=3)
Standard rodent diet
Saline infusion
1 hour enzymatic digestion
Pooled sample
WT AngII/HFD (n=3)
High fat diet (4 weeks)
Angiotensin II 2000ng/kg/min infusion 7 days
10X 3′ droplet based single cell RNA-seq (single cell ATAc-seq)
B
SCTransform Normalized
WT_Saline/CD
WT_AngII/HFD
UMAP_1
UMAP_2
C
Acta2
Myh11
Mir143hg
Atf3
Dcn
Col1a1
Rgs5
Cspg4
Ly6a
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positive regulation of fibroblast proliferation
response to endoplasmic reticulum stress
positive regulation of epithelial cell migration
extracellular matrix organization
extracellular structure organization
regulation of cell morphogenesis
epithelial to mesenchymal transition
wound healing
negative regulation of apoptotic signaling pathway
mesenchymal cell differentiation
positive regulation of cell−substrate adhesion
actin filament organization
positive regulation of angiogenesis
regulation of vasculature development
cellular response to oxidative stress
cell−matrix adhesion
cell adhesion mediated by integrin
muscle cell proliferation
regulation of smooth muscle cell proliferation
BMP signaling pathway
transforming growth factor beta receptor signaling pathway
collagen metabolic process
ossification
cellular response to transforming growth factor beta stimulus
positive regulation of collagen biosynthetic process
positive regulation of collagen metabolic process
cellular response to unfolded protein
elastic fiber assembly
extracellular matrix assembly
positive regulation of cytoskeleton organization
SMC1
SMC2
SMC3
D
Endothelial cells
Cycling cells
Pericyte
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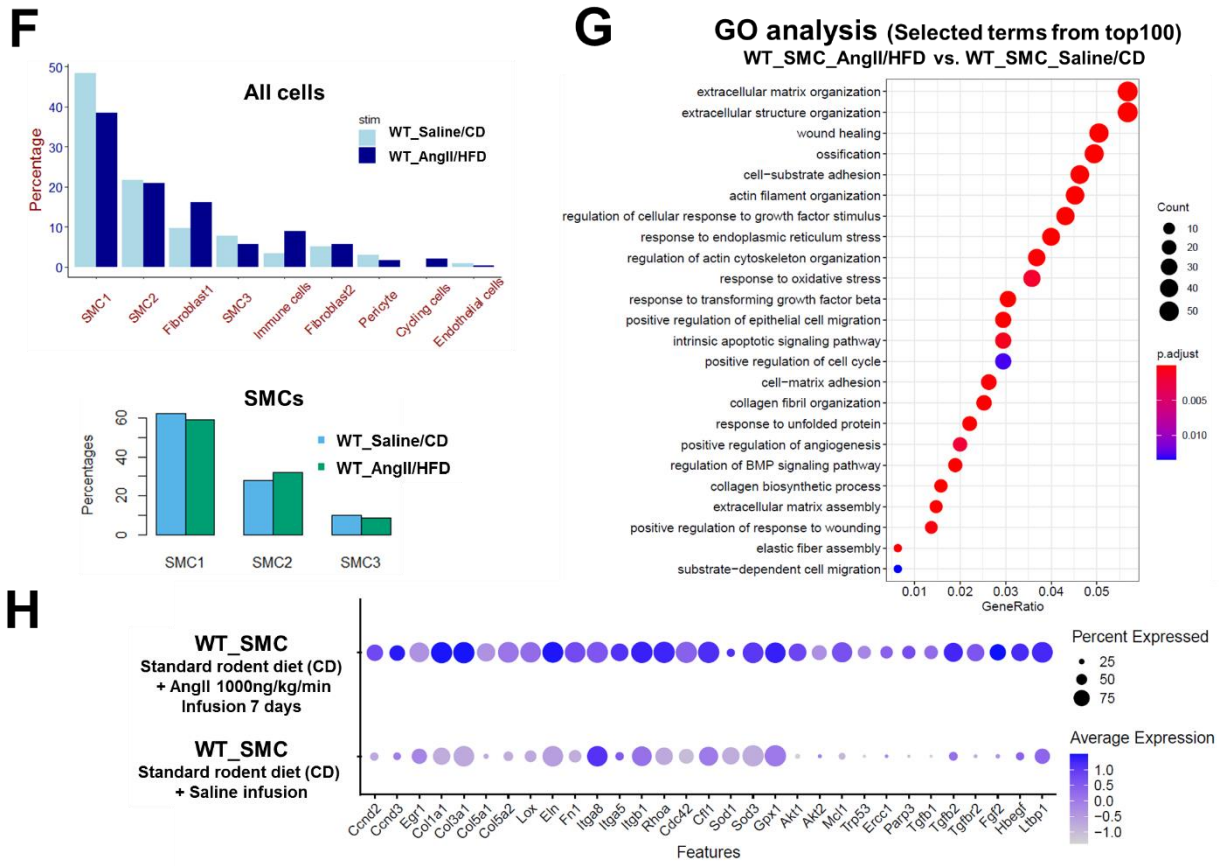
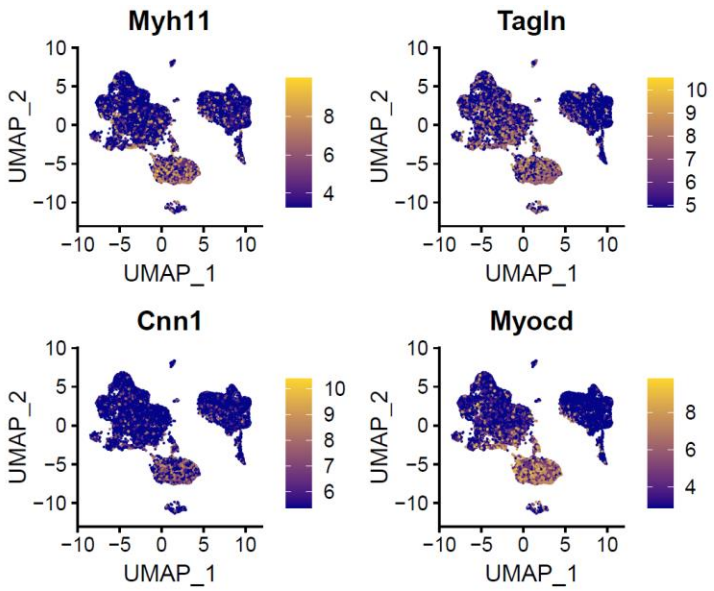
SMC1
Identity
Features
Average Expression
Percent Expressed



Supplemental Figure S1. Integrative Analysis of sc-RNAseq Data and Initial Cluster Identification

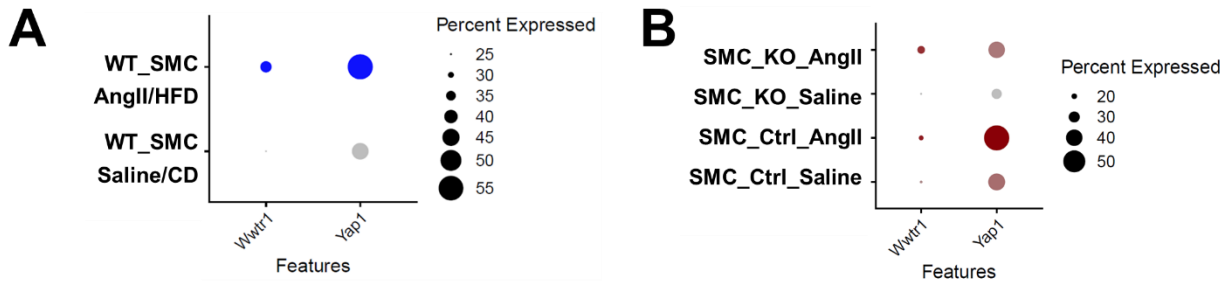
A, Schematic of scRNA-seq performed by using 10x Chromium. Eight-week-old wild-type (WT) mice were fed with a high-fat diet (HFD) for 4 weeks, followed by angiotensin II (2000 ng/kg/min) infusion for 7 days (AngII/HFD). Mice fed with a standard rodent laboratory diet and infused with saline (Saline/control diet [CD]) were used as controls. Ascending aortas from 3 mice per group were isolated and digested, pooled into a single-cell suspension, and sorted into DAPI-live cells. Single cells were subsequently loaded onto 10x Chromium for 3' scRNA-seq. **B**, SCTransform normalization was performed on two single-cell samples, followed by integration analysis of a combined dataset and cluster identification. Cells were projected onto a uniform manifold approximation and projection (UMAP) plot. **C**, The relative expression of

several marker genes is shown in all cells from the combined dataset. Cells were projected onto a UMAP plot. **D**, Dot plot for highest specificity markers of each cell cluster. Dot size represents the percent of cells expressing the denoted gene; color represents the mean normalized expression level within the denoted cluster. **E**, A heat map showing the scaled gene ratio of gene ontology (GO) enrichment terms in smooth muscle cell (SMC) subclusters. **F**, The composition of each cell type and each SMC subcluster is shown in the horizontal bar plot. **G**, Dot plot showing selected gene ontology (GO) enrichment terms (from top100) of differentially expressed genes (DEGs) induced by AngII infusion in all SMCs. **H**, Dot plot showing the similar induction of adaptive genes in the scRNA-seq dataset when WT mice were infused with AngII (1000 ng/kg/min) for 7 days without a HFD.



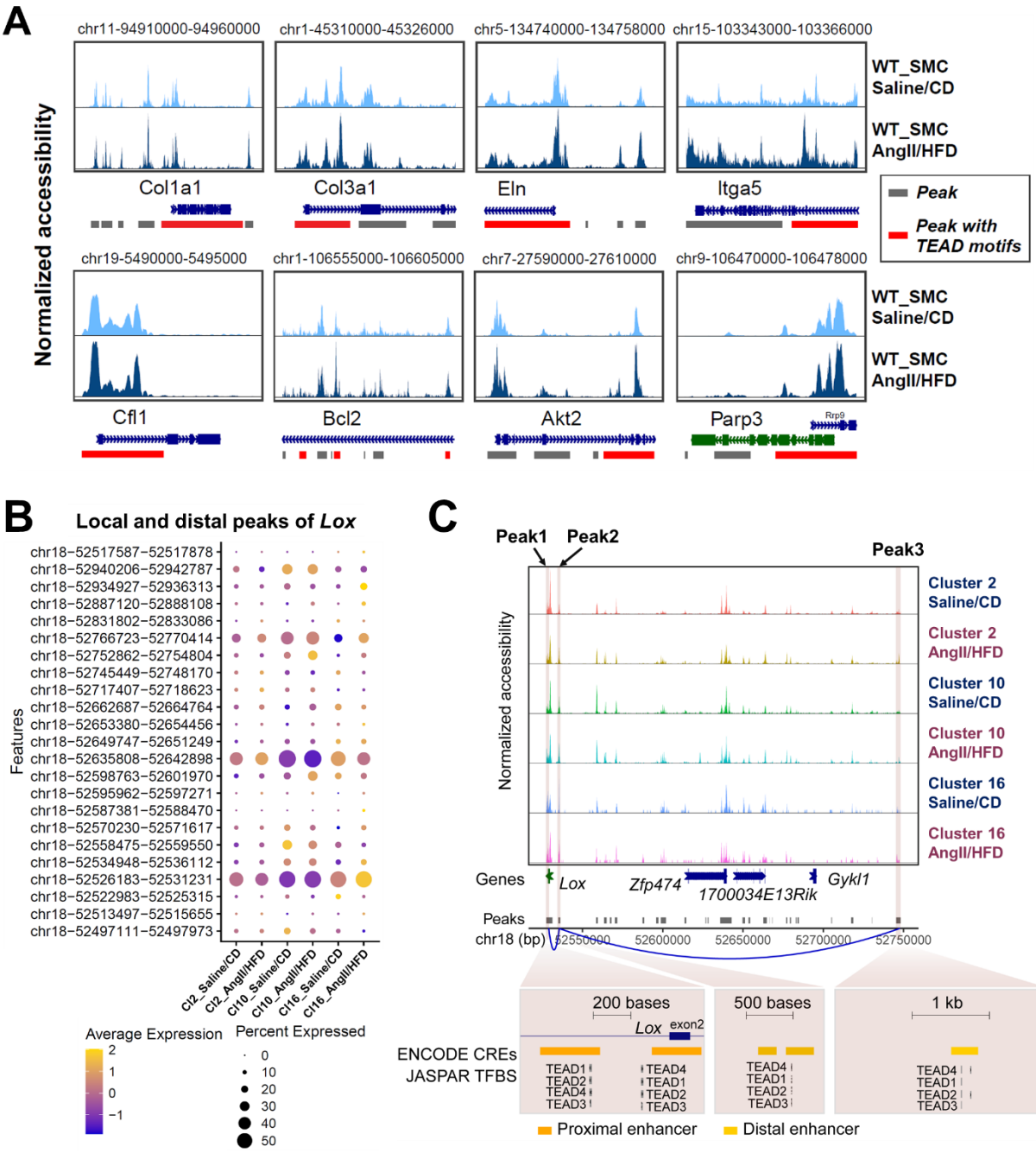
Supplemental Figure S2. Smooth Muscle Cell (SMC) Clusters Were Identified by Gene Activity of SMC Marker Genes in scATAC-seq

Feature plots showing the gene activity of SMC marker genes.



Supplemental Figure S3. Expression level of *Yap1* and *Taz* (*Wwtr1*) in SMCs

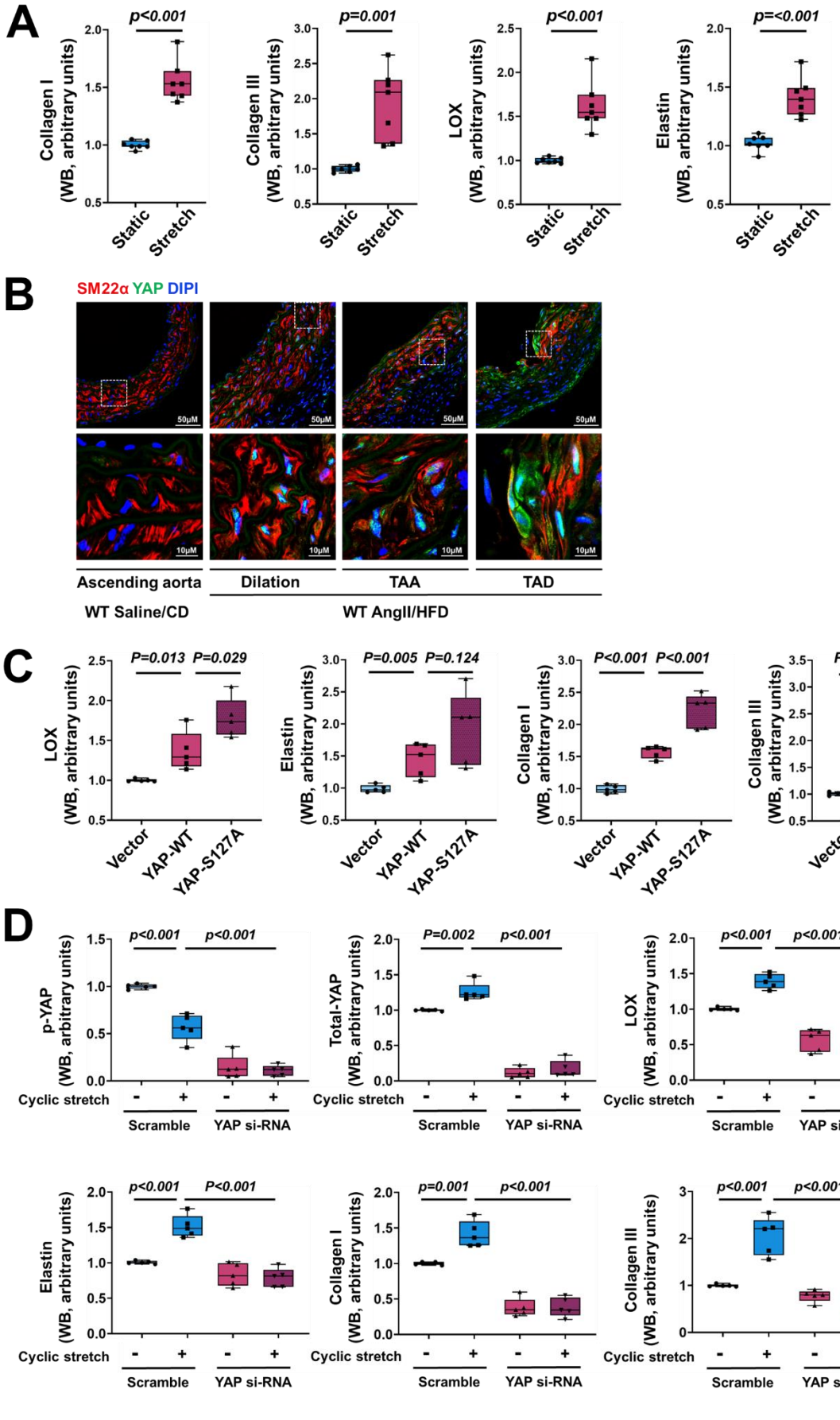
A-B, Dot plot showing the expression level of Yap1 and Taz (Wwtr1) in all SMCs (A, WT scRNA-seq dataset; B, SMC-specific YAP knockout [KO] dataset). Dot size represents the percent of cells expressing the denoted gene; color represents the mean normalized expression level within the denoted cluster.



Supplemental Figure S4. Chromatin Accessibility at the Locus of *Lox* and Other Adaptive Genes Identified in Smooth Muscle Cell (SMC) Clusters and Changes in Response to Aortic Stress

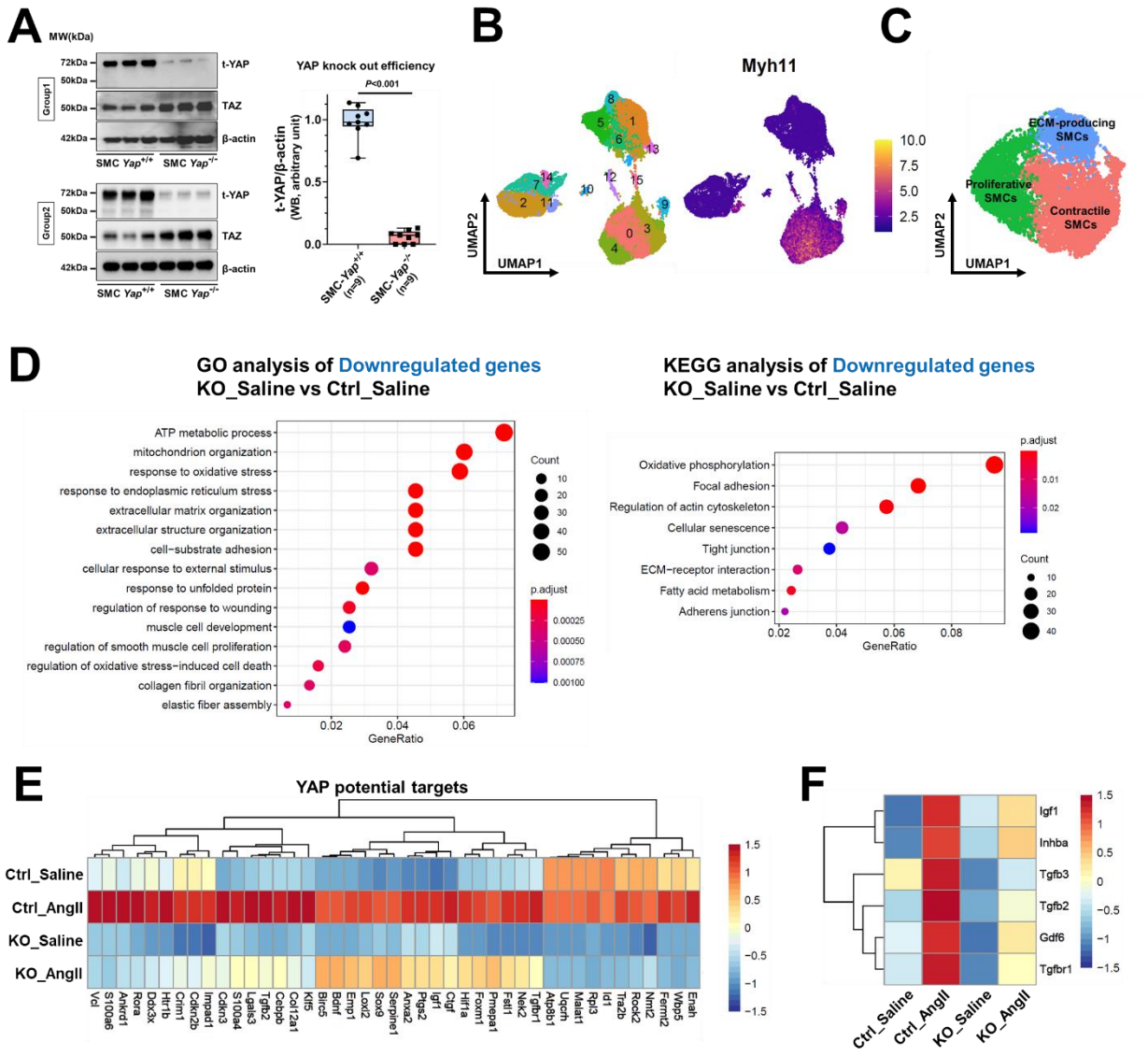
A, Accessibility and detected peaks in the chromatin region of 8 selected adaptive genes. Accessibilities shown for SMCs under different conditions. Peaks harboring TEAD motifs are

highlighted in red. **B**, Dot plot showing the local and distal peak changes of the *Lox* gene in SMCs. **C**, Chromatin accessibility and regulatory potential of *Lox* and its upstream genomic region. The upper panel shows the mean frequency of sequenced DNA fragments for 3 SMC clusters under different conditions. The gene and peak locations are aligned at the bottom. Three regions are highlighted, and the ENCODE CRE annotation and JASPAR TFBS information for each highlighted region are provided. ENCODE stands for The Encyclopedia of DNA Elements, CRE stands for cis-regulatory element, and TFBS stands for transcription factor binding site.


A
p<0.001
Collagen I (WB, arbitrary units)
p=0.001
Collagen III (WB, arbitrary units)
p<0.001
LOX (WB, arbitrary units)
p=<0.001
Elastin (WB, arbitrary units)
Static
Stretch
B
SM22α YAP DIPI
50μM
10μM
Ascending aorta
Dilation
TAA
TAD
WT Saline/CD
WT AngII/HFD
C
P=0.013 P=0.029
LOX (WB, arbitrary units)
P=0.005 P=0.124
Elastin (WB, arbitrary units)
P<0.001 P<0.001
Collagen I (WB, arbitrary units)
P<0.001 P=0.039
Collagen III (WB, arbitrary units)
Vector
YAP-WT
YAP-S127A
D
p<0.001 p<0.001
p-YAP (WB, arbitrary units)
P=0.002 p<0.001
Total-YAP (WB, arbitrary units)
p<0.001 p<0.001
LOX (WB, arbitrary units)
p<0.001 P<0.001
Elastin (WB, arbitrary units)
p=0.001 p<0.001
Collagen I (WB, arbitrary units)
p<0.001 p<0.001
Collagen III (WB, arbitrary units)
Cyclic stretch
Scramble
YAP si-RNA

Supplemental Figure S5. Western Blot Analysis and Densitometric Quantification of Extracellular Matrix (ECM) Proteins in the Response of Human Aortic Smooth Muscle Cells to Cyclic Stretch

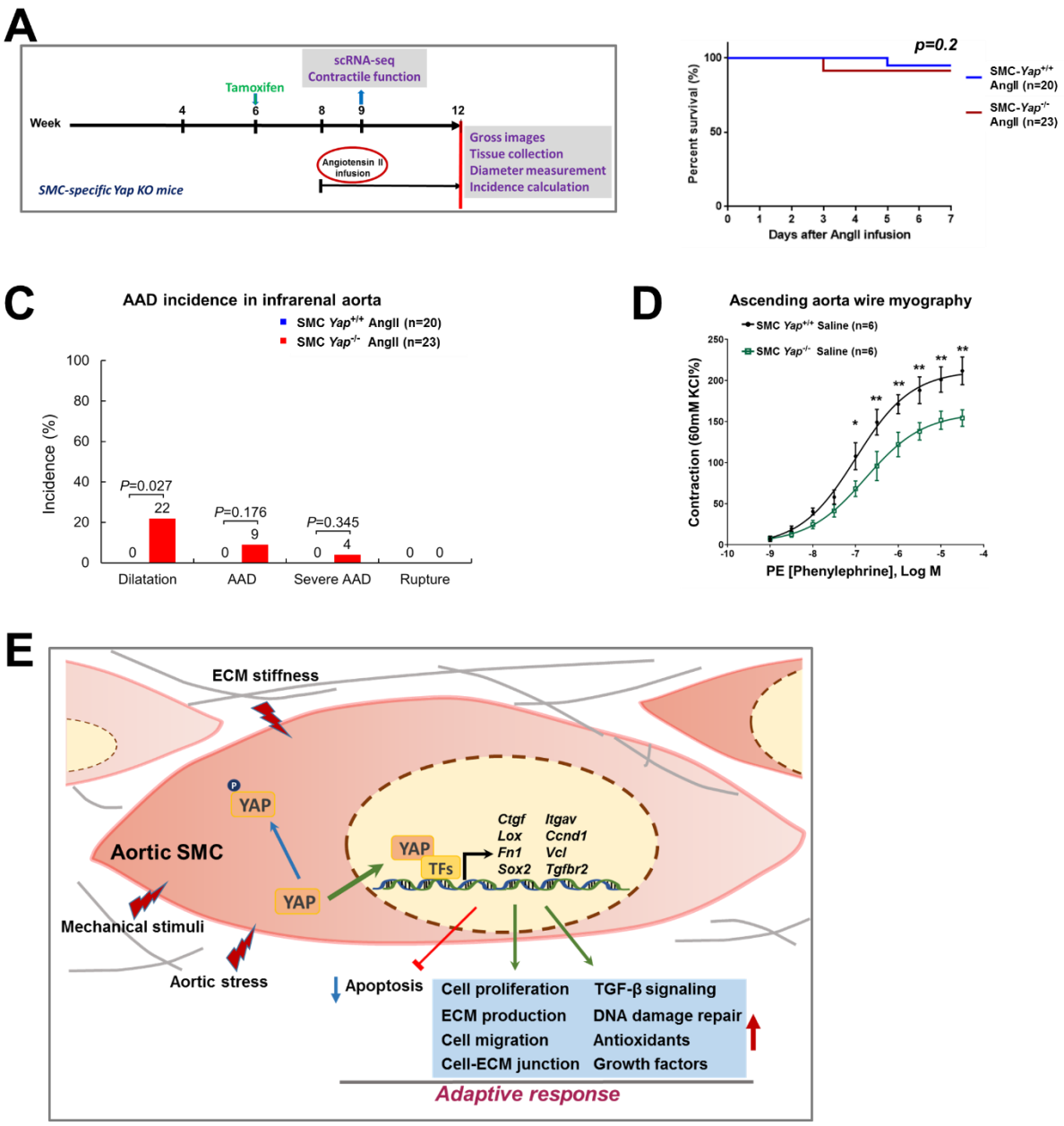
A, Box and whisker plots showing the quantification of the Western blot shown in Figure 5B. **B**, Representative immunofluorescence staining images showing the nuclear translocation of YAP induced by AngII infusion in WT mice. **C**, Box and whisker plots showing the quantification of Western blots shown in Figure 5F. **D**, Box and whisker plots showing the quantification of Western blots shown in Figure 5G. A paired Student's *t*-test was used in (**A**). One-way analysis of variance (ANOVA) with the Bonferroni post hoc test was used for pairwise comparisons in (**C**). Two-way analysis of variance (ANOVA) with the Bonferroni post hoc test was used for pairwise comparisons in (**D**). Data are shown as box and whisker plots with the first quartile, minimum, median, third quartile, and maximum.



Supplemental Figure S6. Integrative Analysis of scRNA-seq and Differentially Expressed Gene Analysis of *Myh11*+ Smooth Muscle Cells (SMCs) in Four Groups of Mice

A, Western blot analyses of YAP expression in ascending aortas dissected from various SMC-*Yap*$^{+/+}$ mice and SMC-*Yap*$^{-/-}$ mice (n=9). Data quantification indicated the efficient deletion of *Yap*. Data are shown as box and whisker plots with the first quartile, minimum, median, third quartile, and maximum. **B**, A uniform manifold approximation and projection (UMAP) plot showing all cells colored according to the 16 cell clusters. **C**, Feature plot showing the relative

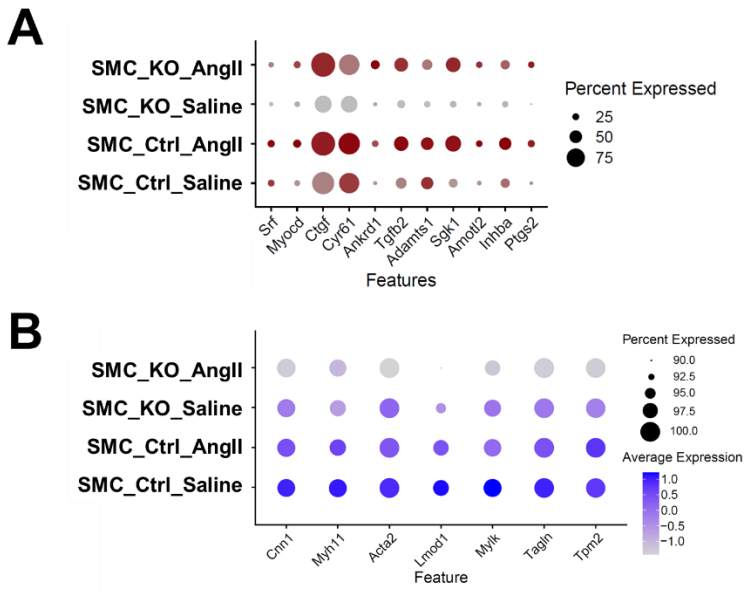
expression of the *Myh11* gene in all cells. **D**, Dot plots for the top enriched biologic process GO terms and KEGG pathways of significantly downregulated genes (logFC<-0.3 & FDR<0.05) in SMCs of SMC-$Yap^{-/-}$ saline-treated mice compared with SMCs of SMC-$Yap^{+/+}$ saline-treated mice. **E**, Heat map showing the mean mRNA abundance of potential downstream gene targets of YAP in SMCs. Gene mRNA abundance was increased significantly in AngII-infused SMC-$Yap^{+/+}$ mice (Ctrl_AngII) compared with saline-infused SMC-$Yap^{+/+}$ mice (Ctrl_Saline), although this induction was compromised in AngII-infused SMC-$Yap^{-/-}$ (KO_AngII) mice. **F**, Heat map showing the mean mRNA abundance of selected TGF-β signaling genes in SMCs. SMC-specific *Yap* deficiency in AngII-infused SMC-$Yap^{-/-}$ mice (KO_AngII) compromised the expression of aortic stress–induced TGF-β signaling genes.



Supplemental Figure S7. Increased Incidence of Aortic Aneurysm and Dissection (AAD) in AngII-infused SMC-*Yap*$^{-/-}$ Mice

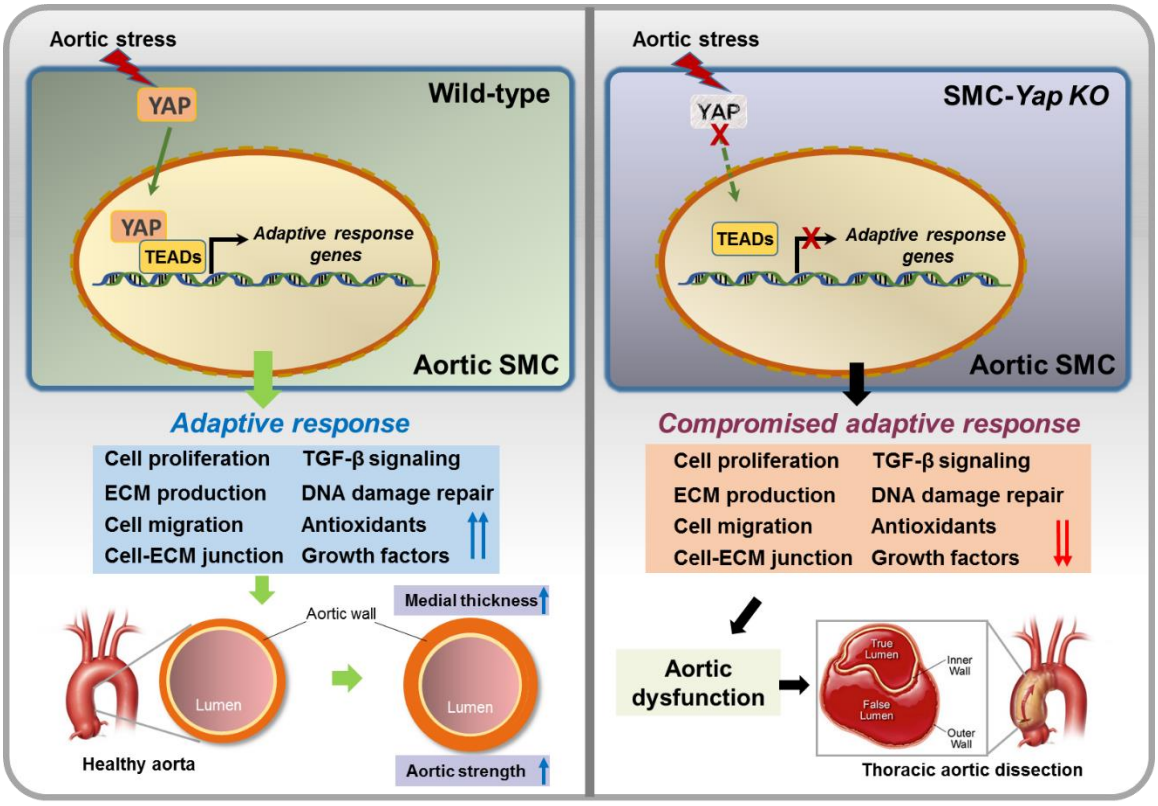
A, Schematic of the experimental design and timeline for generating SMC-*Yap*$^{-/-}$ mice. **B**, The survival rate of AngII-infused SMC-*Yap*$^{-/-}$ mice compared with AngII-infused SMC-*Yap*$^{+/+}$ mice. **C**, The incidence of AAD in the infrarenal segment was not significantly difference between

AngII-infused SMC-*Yap*$^{-/-}$ mice and AngII-infused SMC-*Yap*$^{+/+}$ mice. **D**, Wire myography analysis of ascending thoracic aortic rings showing that the contractile response to phenylephrine was reduced significantly in saline-infused SMC-*Yap*$^{-/-}$ mice compared with saline-infused SMC-*Yap*$^{+/+}$ mice, starting from Log -7M. *P<0.05, **P<0.01. Data are presented as the mean ± standard deviation of the mean. **E**, Schematic diagram showing that stress-activated YAP translocates into the nucleus and binds with transcription co-factors to regulate downstream adaptive response genes.



Supplemental Figure S8. Expression level of Myocardin-related Signaling Genes and contractile genes in SMCs

A, Dot plot showing the expression level of myocardin-related signaling genes in all SMCs of the scRNA-seq dataset from SMC-specific *YAP* KO mice. **B**, Dot plot showing the expression level of SMC contractile genes in all SMCs of the scRNA-seq dataset from SMC-specific *YAP* KO mice. Dot size represents the percent of cells expressing the denoted gene; color represents the mean normalized expression level within the denoted cluster.



Supplemental Figure S9. Schematic Diagram Showing the Experimental Approach of Our Study

Schematic diagram showing the experimental approach used in this study, as well as the key findings. The integrative analysis of single-cell RNA sequencing (scRNA-seq) and single-cell sequencing assay for transposase-accessible chromatin (scATAC-seq) revealed that aortic stress and mechanical stimuli activated the mechanical sensor smooth muscle cell (SMC) YAP, which, through its co-transcription factors (TFs), triggered a comprehensive induction of adaptive genes associated with cell proliferation, cell migration, extracellular matrix (ECM) production and organization, cell-matrix focal adhesion, anti-oxidants, DNA repair, and cell survival. This adaptive response is critical for maintaining aortic strength and homeostasis and protecting the aorta from destruction, dissection, and rupture. ER, endoplasmic reticulum; ROS, reactive oxygen species.

EXPANDED MATERIALS AND METHODS

Single Live-cell Suspension Preparation

For each sample, the ascending aortas of 3 mice were pooled together in ice-cold Hanks' balanced salt solution (HBSS, without Ca^{2+} and Mg^{2+}) (Gibco, Waltham, MA) with 10% foetal bovine serum (FBS). Aortas were cut into small pieces by microsurgical scissors and then moved into enzyme cocktail prepared with 3 mg/ml collagenase type II (LS004176, Worthington Biochemical Corp., Lakewood, NJ), 0.15 mg/ml collagenase type XI (H3506, Sigma Corp., Kanagawa, Japan), 0.1875 mg/ml elastase lyophilized (LS002292, Worthington), 0.24 mg/ml hyaluronidase type I (H3506, Sigma), and 2.38 mg/ml 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, H4034, Sigma) in HBSS (with Ca^{2+} and Mg^{2+}) (14025092, Thermo Fisher Scientific, Waltham, MA). Tissues were digested in a 37 °C water bath for 1 to 2 hours and dissociation was examined under a microscope. Cell suspensions were collected by using a 40-μm cell strainer (CLS431750-50EA, Corning, Inc., Corning, NY), centrifuged at 300 g for 10 minutes, and resuspended in HBSS (without Ca^{2+} and Mg^{2+}) (14175095, Thermo Fisher) with 5% FBS, followed by incubation on ice for 30 minutes. Cells were then stained by using a live and dead cell kit (L3224, Thermo Fisher) and were submitted for flow cytometry (BD Biosciences) to collect live singlet cells. The living cell rate was determined with a microscope by using trypan blue (T8154, Sigma Corp., Kanagawa, Japan) staining.

Single-cell RNA Sequencing (scRNA-seq): Cell Clustering and Identification

FASTQ sequence data were processed to obtain a UMI count in each cell by using "Cell Ranger." A mouse reference genome (mm10) was used for gene alignment. Subsequent scRNA-

seq analysis was performed in R package "Seurat" (version 3.0.0).[75] Cells with low quality (<200 or >5000 genes per cell and >10% mitochondrial genes in the cell) were filtered out. Data were normalized and scaled within each sample, and canonical correlation analysis was performed to integrate data across samples. Deconvolution of the cell population was performed by the dimensional reduction and shared nearest neighbour (SNN) modularity clustering algorithm. We defined the cell clusters based on a set of highly expressed differential/conservative/cell marker genes. Smooth muscle cell (SMC) clusters were defined according to the high expression levels of SMC marker genes (*Acta2, Myh11, Mylk*). Macrophage clusters were defined according to the high level of macrophage marker genes (*Cd68, Adger1, F13a1*) and the absence of SMC genes. Differentially expressed genes (DEGs) in each cluster across different conditions were identified by using the "FindMarkers" function in Seurat. Within each cluster, DEGs between two groups of cells were identified by using a Wilcoxon rank-sum test. Adjusted p-values were calculated on the basis of the Bonferroni correction by using all features in the dataset. Genes with a P_val_adj <0.05 were considered DEGs. Gene ontology (GO) analysis was performed by using an R package "ClusterProfiler."

Differential Gene Expression Analysis and Function Annotation

Differential gene expression between groups of cells (e.g. SMCs) and overall differential gene expression between each condition were identified by using the "Find Markers" function in Seurat.[75] Differential gene expression within each cluster between each condition was determined by using WaVE-EdgeR.[76,77] Data were applied to a zero-inflated negative binomial model by using R package "ZINB-WaVE", followed by differential analysis with a moderated F-test by using R package "EdgeR". Genes with a threshold of an absolute fold change >3 and a

false discovery rate (FDR) <0.05 were considered as differentially expressed genes (DEGs). FDR was adjusted by using the Benjamini-Hochberg correction. GO and KEGG analysis was performed by using the associated packages "clusterProfiler" in R.[78] Genes were submitted for a one-sided Fisher's exact test to find enriched GO categories, and p-values were adjusted by using the Benjamini-Hochberg procedure.

Module (Composite) Score

The module score for a particular list of genes expressed in each cluster was calculated by using the function "AddModuleScore" in Seurat. To avoid bias by selecting only certain genes, set lists such as all collagen genes, or, all integrin genes, were compiled for use in the cluster analyses. All genes in each list were used in the module score unless they were not expressed in any of the clusters.

Correlation Analysis

To identify correlated genes for a particular gene, we performed correlation analysis for the gene of interest within each cluster of interest by using Spearman's correlation in R.

Processing of Single-cell Sequencing Assay for Transposase-accessible Chromatin (scATAC-seq) Data

The raw fastq data for each sample were submitted to Cell Ranger ATAC for read filtering and alignment, barcode counting, identification of transposase cut sites, detection of accessible chromatin peaks, and cell calling by using "cellranger-atac count." The outputs from two runs of cellranger-atac count were further analyzed by using "cellranger-atac aggr" for the normalization

of input runs to the same median fragments per cell and the detection of accessible chromatin peaks. For alignment, we used mm10 as reference genome.

ScATAC-seq Cluster Identification

The peaks called by cellranger-atac aggr were binarized and submitted to R packages Signac (version 1.1.0) and Seurat for dimensional reduction and clustering analysis. Briefly, only cells past peak_region_fragments >3000, peak_region_fragments <100,000, pct_reads_in_peaks >15, blacklist_ratio <0.025, nucleosome_signal <10, and TSS.enrichment >2 were kept for further analysis. Latent semantic indexing (LSI) was performed with peaks of remaining cells by using the function RunTFIDF and RunSVD of Signac to reduce dimension. To further remove the batch effect, we performed the integration by using Seurat function "FindIntegrationAnchors" and "IntegrateData." We then used these integrated reduced dimensions as input into a Seurat object, and major clusters were identified by using Seurat's SNN graph clustering FindClusters at a resolution of 0.6. These same reduced dimensions were used as input into Seurat's 'RunUMAP' with default parameters for visualization.

ScATAC-seq Analysis of Gene Activity

Cis analysis through peaks in the gene body and promoter was performed to generate gene activity scores to infer the cell type of clusters and integrate them with the scRNA-seq data. The gene body region was extracted from R package EnsDb.Mmusculus. v79. The 2000 base pairs upstream were considered the promoter region. The gene activity score matrix was generated by the function "FeatureMatrix" of Signac and was further added to Seurat object for cluster-based analysis and visualization. Differentially activated genes (DAG) were identified with the

"FindMarkers" function of "Seurat" using the "LR" method. Basically, a logistic regression framework was used to construct a logistic regression model predicting group membership based on each feature individually. This was compared with a null model by using a likelihood ratio test. P-values were adjusted by using the Benjamini-Hochberg correction.

Gene activity scores were also calculated by using the R package Cicero. First, the co-accessibility of peaks in the genome were estimated to predict cis-regulatory interactions. The Cicero gene activity score was then calculated by the accessibility of a promoter and its associated distal peaks.

Integration of scATAC-seq Data with scRNA-seq Data

The scATAC-seq gene activity score calculated by using peaks in the gene body and promoter region was used for integration with scRNA-seq data. The functions "FindTransferAnchors" and "TransferData" of Seurat were used to infer the cell type of scATAC-seq clusters by using scRNA-seq clusters.

ScATAC-seq Motif Analysis

Cluster or cell type special accessible peaks were identified by using the function "FindAllMarkers" of Seurat. Aortic stress–dependent accessibility-altered peaks were identified by using Fisher's exact test with R function "fisher.test". Peaks with an odds ratio greater than 2 or less than 0.5 and a p-value less than 0.05 were considered as differential accessible peaks. The peaks (either cluster special or differential peaks) were then submitted to Cistrome Toolkit (http://dbtoolkit.cistrome.org/) to identify putative transcription factors (TFs) that may bind at those peaks by integration with all available mouse chromatin immunoprecipitation sequencing

data in the database. Alternatively, the peaks (either cluster special or differential peaks) were analyzed by using the function “FindMotifs” of Signac to identify the overrepresented motifs.

Global TF activity was measured by using R package chromVAR. Motifs associated with variability in chromatin accessibility between cells were identified. The deviation scores obtained by using chromVar analysis were used for identifying differentially active motifs between clusters or treatments.

Table S1 Major Resources Table

Mouse breeding: C57BL/6J wild-type mice (Stock No. 000664), *Myh11-CreERT2* mice (Stock No. 019079), and yes-associated protein (*Yap*)-floxed mice (Stock No. 027929) were ordered from The Jackson Laboratory. This *Yap* floxed strain was maintained on a mixed C57BL/6-129 genetic background. Upon arrival, the mice were backcrossed to C57BL/6J (Stock No. 000664) for at least 7 generations.

	Vendor or Source	Breeding Strategy	Background Strain
Parent - Male	In house	*Yap* f/f x *Myh11-CreERt2* +/-	C57BL/6J
Parent - Female	In house	*Yap* f/f x *Myh11-CreERt2* -/-	C57BL/6J

Mouse Models (in vivo studies)

Mouse Model	Vendor or Source	Sex
Yap f/f x *Myh11-CreERt2* +/-	In house	Male
Yap f/f x *Myh11-CreERt2* -/-		
C57BL/6J	In house	Male and female

Mouse Housing Conditions

	Mouse Housing Conditions	Note
Set temperature range	21 ± 1 °C	
Set humidity range	50%	
Light : dark cycle	12 hours: 12 hours	
Water	RO Water	
Standard feed	Standard rodent laboratory diet (Mouse Diet, No. 5015, LabDiet, St. Louis, MO)	
Experimental feed	Western diet (D12108C, Research Diets Inc., New Brunswick, NJ)	
SPF	Yes	

Reagents/Kits/Commercial Service

Reagents	Vendor or Source	Catalog #
Angiotensin II	Sigma-Aldrich	A2900
Osmotic mini pump	ALZA Scientific	Model 2004
In Situ Cell Death Detection Kit, Fluorescein	Sigma-Aldrich	11684795910

EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 488 dye	Invitrogen	C10337

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration
Western blot analysis			
Anti-YAP (D8H1X)	Cell Signaling	14074	1:1000
Anti-YAP (63.7)	Santa Cruz	sc-101199	0.5 µg/ml
Anti-Phospho-YAP (Ser127) (D9W2I)	Cell Signaling	13008	1:1000
Anti-DYKDDDDK Tag (D6W5B)	Cell Signaling	14793	1:1000
Anti-LOX	Abcam	ab-31238	1:1000
Anti-Elastin (E-11)	Santa Cruz	sc-166543	0.8 µg/ml
Anti-Collagen I	Abcam	ab-260043	1 µg/ml
Anti-Collagen III	Abcam	ab-7778	1 µg/ml
Anti-SM22-α	Abcam	ab-10135	0.5 µg/ml
Anti-β-actin	Santa Cruz	sc-47778	0.04 µg/ml
Anti-rabbit IgG, HRP-linked antibody	Cell Signaling	7074	1:5000
Anti-mouse IgG, HRP-linked antibody	Cell Signaling	7076	1:5000
Immunostaining			
Anti-SM22-α	Abcam	ab-10135	1 µg/ml
Anti-YAP (D8H1X)	Cell Signaling	14074	1:500
Anti-YAP (63.7)	Santa Cruz	sc-101199	2 µg/ml
Anti-Histone H3 (phospho S10)	Abcam	ab-5176	1 µg/ml
Normal mouse IgG	Santa Cruz	sc-2025	2 µg/ml
Normal rabbit IgG	Invitrogen	# 31235	2 µg/ml
Normal goat IgG	Invitrogen	# 02-6202	2 µg/ml
Alexa Fluor™ 568 donkey anti-goat IgG (H+L)	Invitrogen	A11057	2 µg/ml
Alexa Fluor™ 568 donkey anti-mouse IgG (H+L)	Invitrogen	A10037	2 µg/ml
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Alexa Fluor™ 568 donkey anti-rabbit IgG (H+L)	Invitrogen	A10042	2 μg/ml

HRP, horseradish peroxidase; H+L, heavy chain and light chain.

Table S2 **TEAD Motif-enriched Adaptive Genes** (provided in a separate Excel file)

Table S3 **YAP-based ChIP-seq All Peaks Annotation** (provided in a separate Excel file)