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# Deletion of fibroblast activation protein provides atheroprotection

Sokrates Stein<sup>1,2\*</sup>, Julien Weber<sup>1</sup>, Stefanie Nusser-Stein<sup>1</sup>, Jürgen Pahl<sup>1</sup>, Hui E. Zhang<sup>3</sup>, Shafeeq A. Mohammed<sup>1</sup>, Sara Oppi<sup>1</sup>, Daniel S. Gaul<sup>1</sup>, Francesco Paneni<sup>1,2,4</sup>, Anne Tailleux<sup>5</sup>, Bart Staels<sup>5</sup>, Ferdinand von Meyenn<sup>6</sup>, Frank Ruschitzka<sup>2</sup>, Mark D. Gorrell<sup>3</sup>, Thomas F. Lüscher<sup>1,7</sup>, Christian M. Matter<sup>1,2,\*</sup>

## Affiliations

<sup>1</sup>Center for Molecular Cardiology, University of Zurich, CH-8952 Schlieren, Switzerland.

<sup>2</sup>Department of Cardiology, University Heart Center Zurich, University Hospital Zurich, CH-8091 Zurich Switzerland.

<sup>3</sup>Centenary Institute, The University of Sydney, Faculty of Medicine and Health, Sydney NSW 2050, Australia.

<sup>4</sup>Department of Research and Education, University Hospital Zurich, CH-8091 Zurich Switzerland.

<sup>5</sup>University of Lille, Inserm, CHU Lille, Institut Pasteur de Lille, U1011 - EGID, Lille, France.

<sup>6</sup>Institute of Food, Nutrition and Health, ETH Zurich, 8603 Schwerzenbach, Switzerland.

<sup>7</sup>Cardiology, Royal Brompton & Harefield Hospital Trust and Imperial College London, London SW3 6NP, United Kingdom.

## \*Addresses for correspondence

Sokrates Stein, Center for Molecular Cardiology, University of Zurich, Wagistrasse 12, 8952 Schlieren, Switzerland. E-mail: sokrates.stein@uzh.ch. Phone: +41 44 635 5094.

Christian M. Matter, Center for Molecular Cardiology, University of Zurich, and Department of Cardiology, University Heart Center, University Hospital Zurich. Switzerland. E-mail: christian.matter@usz.ch. Phone: +41 44 255 3871.

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1 **Abstract**

2 **Aims.** Fibroblast activation protein (FAP) is upregulated at sites of tissue remodelling including chronic  
3 arthritis, solid tumours, and fibrotic hearts. It has also been associated with human coronary  
4 atherosclerotic plaques. Yet, the causal role of FAP in atherosclerosis remains unknown. To investigate  
5 the cause-effect relationship of endogenous FAP in atherogenesis, we assessed the effects of  
6 constitutive *Fap* deletion on plaque formation in atherosclerosis-prone *apolipoprotein E* (*ApoE*) or *low-*  
7 *density lipoprotein receptor* (*Ldlr*) knockout mice.

8 **Methods and results.** Using *en face* analyses of thoraco-abdominal aortae and aortic sinus cross-  
9 sections, we demonstrate that *Fap* deficiency decreased plaque formation in two atherosclerotic mouse  
10 models (-46% in *ApoE* and -34 % in *Ldlr* knockout mice). As a surrogate of plaque vulnerability fibrous  
11 cap thickness was used; it was increased in *Fap*-deficient mice, whereas Sirius red staining  
12 demonstrated that total collagen content remained unchanged. Using polarized light, atherosclerotic  
13 lesions from *Fap*-deficient mice displayed increased FAP targets in terms of enhanced collagen  
14 birefringence in plaques and increased pre-COL3A1 expression in aortic lysates. Analyses of the  
15 Stockholm Atherosclerosis Gene Expression (STAGE) data revealed that *FAP* expression was  
16 increased in human atherosclerotic compared to non-atherosclerotic arteries.

17 **Conclusions.** Our data provide causal evidence that constitutive *Fap* deletion decreases progression  
18 of experimental atherosclerosis and increases features of plaque stability with decreased collagen  
19 breakdown. Thus, inhibition of FAP expression or activity may not only represent a promising  
20 therapeutic target in atherosclerosis but appears safe at the experimental level for FAP-targeted cancer  
21 therapies.

22 **Translational Perspective**

23 Fibroblast activation protein (FAP) is upregulated at sites of chronic tissue remodelling including  
24 rheumatoid arthritis and solid tumours. Indeed, depletion of FAP-positive cells inhibits tumour growth  
25 by increasing antitumour immunity. FAP has also been correlated with human coronary plaques,  
26 whereby its causal role remains unknown. Our data provide causal evidence that constitutive *Fap*  
27 deletion decreases progression of experimental atherosclerosis and increases features of plaque  
28 stability. Thus, inhibition of FAP expression or activity may not only represent a novel therapeutic target  
29 for atherosclerosis but appears safe – at the experimental level – for FAP-targeted cancer therapies.

30

## 1 **1. Introduction**

2 Vulnerable plaques pose a central problem in human atherosclerosis. Their rupture may cause  
3 complete thrombotic arterial lumen obstruction, which may lead to myocardial infarction or stroke. A  
4 sufficiently thick fibrous cap ensures plaque stability and a barrier between blood and the prothrombotic  
5 necrotic core within the plaque <sup>1, 2</sup>. Collagenases (e.g. matrix metalloproteinases (MMPs) and  
6 cathepsins) degrade collagen within the fibrous cap, thereby thinning the cap and making them more  
7 prone to rupture <sup>3</sup>. Therefore, extra-cellular matrix-degrading collagenases have emerged as promising  
8 therapeutic targets against rupture of vulnerable plaque <sup>4</sup>. Interestingly, many collagenases involved in  
9 tissue remodelling such as rheumatoid arthritis or tumour expansion play similar roles in atherogenesis  
10 <sup>3, 5</sup>.

11 Fibroblast activation protein (FAP) can be membrane-bound or soluble, and is a constitutively active  
12 serine protease that exhibits dipeptidyl peptidase IV activity and prolyl endopeptidase activity with a  
13 specificity for collagens <sup>6-8</sup>. Matrix turnover is a central event in the pathogenesis of chronic inflammatory  
14 diseases such as rheumatoid arthritis, tumour formation and atherosclerosis. Along these lines, FAP is  
15 expressed by activated fibroblasts in epithelial tumour stroma, arthritis and wound healing, but remains  
16 virtually undetectable in healthy tissues <sup>6, 9-11</sup>. In rheumatoid arthritis, FAP is expressed in SMA-positive  
17 myofibroblasts and associated with matrix metalloproteinase expression <sup>9</sup>. Moreover, a recent study  
18 showed that FAP was increased in cardiac fibrosis, which in turn promotes the development of various  
19 cardiac diseases and heart failure <sup>12</sup>. Interestingly, engineered chimeric (FAP-)antigen receptor (CAR)  
20 T cells could be used to target FAP in cardiac fibrosis; this strategy restored cardiac function in a  
21 hypertensive heart failure model <sup>12</sup>.

22 Previously, we have reported that FAP expression is enhanced in human fibroatheromata versus  
23 plaque-free aortae, and its expression increases upon plaque progression <sup>13</sup>. Although expression of  
24 FAP protein correlated with the macrophage burden in human aortic plaques, colocalization images  
25 revealed that FAP was mainly expressed by smooth muscle cells <sup>13</sup>.

26 Many collagenases involved in chronic tissue remodelling processes such as rheumatoid arthritis and  
27 tumour progression play similar roles in atherogenesis <sup>3, 5</sup>. Yet, the causal role of FAP in atherogenesis  
28 remains unknown. Therefore, we assessed the cause-effect relationship between the loss-of-function

1 of FAP and the phenotype of atherosclerosis by crossbreeding *Fap* knockout mice <sup>14</sup> with  
2 atherosclerosis-prone *apolipoprotein E (ApoE)* or *low-density lipoprotein receptor (Ldlr)* knockout mice.

## 1 **2. Methods**

2

3 **2.1 Mice.** Mice with a germline *Fap* deletion were obtained from the Centenary Institute, Sydney, AU

4 <sup>14, 15</sup> and licensed from Boehringer Ingelheim Pharma GmbH & Co KG. Congenic C57BL6/J *Fap*<sup>-/-</sup> and

5 *Fap*<sup>+/+</sup> mice were kept in a temperature-controlled facility with a 12-h light/dark cycle and free access to

6 normal chow and water. To assess atherosclerosis development, *Fap*<sup>-/-</sup> mice were crossbred to either

7 *Apoe*<sup>-/-</sup> or *Ldlr*<sup>-/-</sup> mice. Of those, male mice were fed a high-cholesterol diet (1.25% total cholesterol,

8 Research Diets) for 12 weeks starting at the age of 8 weeks. After being anesthetised with isoflurane

9 using a gas flow vaporizer (4.5 to 5% isoflurane) for 5 minutes, mice were euthanised by cardiac

10 puncture and exsanguination.

11 **2.2 Study approval.** All animal experiments were approved by the Veterinary Office of the Canton

12 Zurich (animal licenses: 189/2011 and 5263) and all procedures were in accordance to the guidelines

13 from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific

14 purposes.

15 **2.3 FAP activity assay.** The FAP specific enzyme assay was adapted from published assays <sup>11, 16</sup>,

16 using 100 uM Z-Gly-Pro-aminomethylcoumarin (AMC) as substrate, and the prolyl endopeptidase

17 selective inhibitor S-17092 (Sigma-Aldrich #SML0181) at 0.1 uM to ensure FAP specificity.

18 **2.4 FAP proteomics.** Generation of *Fap*-deficient mouse embryonic fibroblasts and cultivation of the

19 cells was described previously <sup>8</sup>. Terminal amine isotopic labelling of substrates (TAILS) and tests of

20 data significance were performed previously <sup>8</sup>. The statistically significant cleavages in collagens were

21 arranged by location in primary structure and the cleavage sites that follow each Gly-Pro, the motif

22 typical of FAP mediated cleavage, were identified.

23 **2.5 Western blot analysis.** 30 mg aortic lysates were pulverized using a steel tissue grinder, and then

24 extracted in lysis buffer (50 mM Tris HCl pH 8.0, 125 mM NaCl, 0.25% NP-40, 0.5 mM

25 phenylmethylsulfonyl fluoride, and protease/phosphatase inhibitor cocktail) for 10 minutes. The

26 supernatant was transferred, and the protein content measured using Bradford reagent. Before

27 running SDS-PAGE, protein lysates were boiled for 5 min at 95°C. Following proteins were used for

28 Western blotting: anti-COL3A1 (22734-1-AP, Proteintech), anti-COL2A1 (sc-52658, Santa Cruz), anti-

29 COL1A1 (PA1-26204, Invitrogen), anti- $\alpha$  tubulin (sc-8035, Santa Cruz), anti-  $\beta$ actin (sc-47778, Santa

30 Cruz), and anti-GAPDH (TA802519, Origene).

1 **2.6 Plasma lipid analysis.** Total cholesterol (TC) and triglyceride (TG) plasma concentrations were  
2 measured using an enzymatic colorimetric assay (Biomerieux). To obtain lipoprotein fractions for fast-  
3 protein liquid chromatography (FPLC) analysis, each individual plasma sample was loaded on a  
4 filtration chromatography column onto a Superose 6 10/300 GL column (GE Healthcare), which allows  
5 separation of the three major lipoprotein classes (VLDLs, LDLs, HDLs and free glycerol) according to  
6 their size. Cholesterol and triglyceride concentrations were continuously measured in the effluent using  
7 an enzymatic colorimetric assay (Biomerieux) and results are expressed as O.D.

8 **2.7 Immunohistochemistry.** The heart-aortic root samples were stored in optimal cutting temperature  
9 (OCT) compound at -80°C, and then cut with a cryostat set at -20°C. The samples were then cut from  
10 the left ventricular basis towards the aortic valves. Once reaching the cusps, the specimen was re-  
11 orientated to achieve stringent cross-sectional cutting. 5 µm thick serial cross sections were then  
12 collected at approximately 100 µm intervals on microscopic glass slides until the aortic cusps were no  
13 longer visible. These 5 µm-thick serial cryosections were stained with Oil-red O (ORO), rat anti-CD68  
14 (MCA 1957GA, Serotec), rat anti-CD3 (MCA 500GA, Serotec), rat anti-VCAM-1 (MCA 1129, Serotec),  
15 and rabbit anti-αSMA (D4K9N, Cell Signaling). Means were taken from at least 6 different mice  
16 evaluating at least 6 serial cryosections/tissue from each mouse. Thoraco-abdominal aortae were  
17 fixed with 4% paraformaldehyde and plaques stained with Oil-red O. Collagen content, fibrous cap  
18 thickness, and necrotic core size were analysed by Elastica van Gieson staining. Sirius Red stainings  
19 were performed to visualize collagen content under normal light.

20 **2.8 Second harmonics.** 5-µm-thick serial cryosections from the aortic sinus were used to perform  
21 second-harmonic generation of collagen fibers using two-photon microscopy. Second-harmonic signal  
22 was generated by tuning the Mai Tai Ti:sapphire laser to 880 nm and collecting the resultant tissue  
23 emission at 440 nm.

24 **2.9 Gene expression analysis.** Aortic RNAs were extracted using TRIZOL (Thermo Fisher, no.  
25 15596026). For RT-qPCR, cDNA was generated using the All-in-One cDNA Synthesis kit (Biotool, no.  
26 B24403), and analysed by qPCR using a SYBR Green qPCR Master Mix (Biotool, no. B21202) and the  
27 primers listed in the online supplement (**Supplemental Table S4**). Expression data were normalized to  
28 housekeeping genes, i.e. *Actb*, *B2m* and/or *Ppib* mRNA levels.

1 **2.10 Transcriptomic data analyses.** The raw and/or normalized transcriptomic data from previous  
2 studies are publicly available on Gene Expression Omnibus (GEO; [www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo)) under  
3 the accession number GSE40231 (STAGE<sup>17</sup>) and analysed as described previously<sup>18</sup>. The *tabula muris*  
4 data can be accessed and analysed online (<https://tabula-muris.ds.czbiohub.org>). The single cell-RNA  
5 seq data was obtained from Prof. Qinbo Xu from the King's College London British Heart Foundation  
6 Centre (United Kingdom).<sup>19</sup>

7 **2.11 Statistics.** Data are presented as scatter plots plus mean. Comparison of differences between  
8 two groups was assessed using unpaired two-tailed Student's *t*-tests. A nonparametric Mann-Whitney  
9 test was used for the analysis of the SGH data since the data distribution did not pass the D'Agostino  
10 & Pearson omnibus normality test. Multiple group comparisons were assessed by ANOVA analysis and  
11 Bonferroni post-hoc tests, and multiplicity adjusted P values are displayed for the comparisons.  
12 Differences of  $p < 0.05$  were considered statistically significant. Statistical analyses were performed  
13 using GraphPad Prism 6.



### 1 3. Results

#### 2 3.1 Deletion of *Fap* protects against atherosclerosis

3 To address the causal role of endogenous FAP in atherogenesis, we crossbred *Fap*-deficient mice<sup>15</sup>  
4 with two different atherosclerosis-prone mouse models, i.e. *Ldlr*<sup>-/-</sup> and *Apoe*<sup>-/-</sup> mice<sup>20</sup> (**Supplemental**  
5 **Figure 1**). Deletion of FAP was assessed by genotyping and confirmed by measuring its enzymatic  
6 activity: FAP activity was abolished in both mouse models with constitutive *Fap* deletion (**Figure 1A,**  
7 **B**). We next placed 8-week old male mice on a high-cholesterol diet for 12 weeks and assessed  
8 atherosclerotic plaque development. Deletion of *Fap* diminished formation of atherosclerotic lesions by  
9 approximately one third in *Ldlr*<sup>-/-</sup> *Fap*<sup>-/-</sup> and by about half in *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> mice (**Figure 1C, D**). While  
10 *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> were slightly heavier than control *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> mice, no difference in body weight was  
11 noted in the *Ldlr*<sup>-/-</sup> background (**Supplemental Figure 2**).

12 We next examined whether plasma levels of total cholesterol and triglycerides at the time of harvesting  
13 correlated with the atherosclerosis phenotype. Total cholesterol content did not differ in any of the two  
14 atherosclerotic mouse models, while plasma triglyceride levels were increased in  
15 *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> but not in *Ldlr*<sup>-/-</sup> *Fap*<sup>-/-</sup> mice (**Supplemental Figure 3**). Hematological analyses did not  
16 reveal differences in the number of leukocytes subpopulations, erythrocytes or platelets between  
17 *Ldlr*<sup>-/-</sup> *Fap*<sup>-/-</sup> and *Ldlr*<sup>-/-</sup> *Fap*<sup>+/+</sup> mice (**Supplemental Table 1**). On the other side, *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> had a  
18 reduced amount of blood neutrophils and platelets, and an increased number of monocytes compared  
19 to *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> controls. *Apoe* knockout mice on a high-cholesterol diet display a stronger pro-  
20 inflammatory and atherogenic phenotype compared with *Ldlr*<sup>-/-</sup> mice<sup>20</sup>, which could be related to the  
21 differences observed in leukocyte subpopulations (**Supplemental Table 1**).

#### 22 3.2 *Fap*-deletion increases protective fibrous cap thickness

23 To better characterize the atherosclerotic lesions, we carried out several immunohistochemical  
24 stainings on cross-sections of the aortic sinus of *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> and *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> mice. Along with the  
25 reduced number of thoraco-abdominal lesions, *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> mice displayed reduced content of neutral  
26 lipids in the aortic sinus compared to *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> mice (**Figure 2A and Supplemental Figure 4**).  
27 Interestingly, we did not observe any difference in the amount of the vascular cell adhesion molecule 1  
28 (VCAM-1), macrophages (CD68<sup>+</sup> cells), or T cells (CD3<sup>+</sup> cells) between the two genotypes (**Figure 2B**

1 – D). These findings suggest that the atherosclerotic phenotype is not secondary to a differential  
2 accumulation of immune cells within the lesions.

3 Since FAP is an extra-cellular matrix-degrading collagenase, we performed Elastica van Gieson  
4 stainings to measure the content of collagen and to assess advanced plaque parameters in aortic sinus  
5 cross-sections. Surprisingly, there was no significant difference in the content of collagen in *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup>  
6 <sup>-/-</sup> compared to *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> mice (**Figure 3A**). However, atherosclerotic lesions from *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> mice  
7 displayed larger fibrous caps, whereas the size of the necrotic cores was comparable to *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup>  
8 mice (**Figure 3B-D**), suggesting that *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> mice do not only develop less atherosclerosis, but  
9 their lesions are also less prone to rupture.  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) staining showed a trend for  
10 increased vascular smooth muscle cell content within the atherosclerotic lesions, suggesting that  
11 vascular smooth cells migrated into the plaques to reinforce the fibrous cap (**Supplemental Figure 5**).

### 12 **3.3 Altered collagen organization in aortic sinus lesions from *Fap*-deficient mice**

13 We next performed Sirius red stainings as a alternative method to quantify the collagen content. It  
14 confirmed that there is no difference in collagen content between the genotypes (**Figure 3E**).  
15 Previously, Santos et al. showed that tumours from *Fap*-deficient mice (with a LacZ knockin, *Fap*<sup>lacZ/lacZ</sup>)  
16 display increased collagen birefringence under polarised light in comparison to wildtype mice <sup>21</sup>,  
17 suggesting a reduced organization of the collagen fibers. To investigate a possible change in collagen  
18 organization, we performed second-harmonic generation (SHG) of collagen on aortic sinus lesions  
19 using two-photon microscopy laser microscopy <sup>22, 23</sup>. These analyses revealed that lesions of *Apoe*<sup>-/-</sup>  
20 *Fap*<sup>-/-</sup> mice displayed increased SHG signals compared to *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> mice (**Figure 3F**), confirming  
21 that deletion of FAP indeed augments the deposition of fibrillar collagens in these lesions.

### 22 **3.4 Proteomic analysis demonstrates that FAP cleaves several collagens**

23 To investigate the underlying mechanisms, we first analysed the expression of collagens, extracellular  
24 matrix proteases and fibrosis markers in the aortae. Despite a trend for increases fibrosis markers, we  
25 observed no significant difference of the transcripts at mRNA level (**Supplemental Figure 6**). In order  
26 to understand how FAP induces the observed changes in collagen structure, we drilled down into our  
27 targeted proteomic analyses of cell culture supernatants from *Fap*<sup>-/-</sup> mouse embryonic fibroblasts to  
28 identify potential collagen targets of FAP. We had found by stable isotope labelling with amino acids in  
29 cell culture (SILAC) that the quantities of collagens 3, 5 and 6 differed in the presence of enzymatically

1 active FAP compared to inactive FAP<sup>8</sup>. In addition, terminal amine isotopic labelling of substrates  
2 (TAILS) showed that the major collagen species COL1A1, COL1A2, COL5A2 and COL3A1 are  
3 proteolytic targets of FAP in murine fibroblast cultures. The multiple cleavage sites in these  
4 precollagens are probably generated co-operatively by FAP with other collagenases<sup>8</sup>. However, we  
5 found that many of these cleavage sites followed Gly-Pro, which is prototypical of FAP-induced  
6 hydrolysis, and that most of the greatest increases in collagen fragmentation were coincident with  
7 cleavage after Gly-Pro (**Figure 4A-D**).

### 8 **3.5 Reduced cleavage of pre-COL3A1 in *Fap* knockout mice**

9 To validate the SILAC-TAILS data and verify if collagen degradation could also be affected in the *Fap*-  
10 deficient mice, we analysed the protein expression of the three main collagens in aortic lysates of  
11 *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> and *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> mice. Interestingly, we observed an increase of the precursor of  
12 COL3A1 in *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> compared to *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> aortae (**Figure 4E, F, and Supplemental Figure**  
13 **7A**), suggesting that the precursor cannot be properly cleaved in FAP-deficient mice. Conversely,  
14 COL1A1 and COL2A1 products were not altered between the two genotypes (**Supplemental Figure**  
15 **7C-F**). Noteworthy, there is no known molecular basis for this preference of FAP for COL3 over other  
16 collagen subtypes.

### 17 **3.6 Pathway analyses of FAP-expressing cells supports its role in collagen remodelling**

18 Previously studies showed that FAP is mainly expressed in fibroblast and vascular smooth muscle cells.  
19 Analysis of the transcriptome of targeted aortic fluorescent activated cell sorting (FACS)-isolated aortic  
20 cell available at *Tabula muris*<sup>24</sup> showed and confirmed that FAP is mainly expressed by fibroblasts  
21 (**Supplemental Figure 8**). Since these were aortae from non-atherosclerotic mice, we further analysed  
22 an untargeted single cell RNA-sequencing dataset from aortae of *Apoe* knockout mice<sup>19</sup>. We first  
23 selected all cells displaying *FAP* sequencing counts (>2), and then filtered the top 20 highest expressed  
24 transcripts of each of these cells (**Supplemental Table S2**). Interestingly, pathway analysis shows  
25 enrichment of extracellular remodelling, such as collagen and extracellular fibril organisation,  
26 extracellular matrix and exosome, and collagen binding (**Supplemental Table S3**).

27

28

### 29 **3.7 Increased expression of *FAP* in human atherosclerotic vessels**

1 To evaluate whether the expression of *FAP* could be altered in human atherosclerotic plaques  
2 compared to healthy arteries, we compared the expression of *FAP* in atherosclerotic aortic roots to non-  
3 atherosclerotic mammary arteries from the Stockholm Atherosclerosis Gene Expression (STAGE)<sup>17</sup>  
4 study. Atherosclerotic aortae revealed an increased expression of *FAP* as compared to non-  
5 atherosclerotic mammary arteries (**Figure 4G**). Together, these data suggest that *FAP* expression is  
6 increased in human atherosclerotic plaques and could be a promising target for pharmacologic  
7 inhibition.

#### 1 4. Discussion

2 In a previous study, we demonstrated that FAP expression is increased in human fibroatheromata  
3 versus plaque-free aortae, and that its expression positively correlates with the stages of plaque  
4 progression<sup>13</sup>. Moreover, soluble FAP cleaves  $\alpha$ 2-antiplasmin, which enhances its activity and  
5 incorporation into fibrin clots, thus promoting coagulation<sup>25</sup>. Surprisingly, the levels of circulating FAP  
6 are reduced in patients with coronary artery disease or stroke<sup>26-29</sup>, and suggest that tissue resident and  
7 circulating FAP are independent markers of cardiovascular diseases<sup>30</sup>.

8 The current study provides the following novel findings (**Figure 5**):

9 1) Constitutive genetic *Fap* loss-of-function in atherosclerosis-prone *Apoe* or *Ldlr*-deficient mice  
10 decreases plaque formation that is independent of plasma lipid levels. Moreover, the content of plaque  
11 VCAM-1, macrophages, and T cells was not different between the groups.

12 2) *Fap* deletion increases features of plaque stability with increased fibrous cap thickness. Analyses of  
13 aortic roots demonstrated that atherosclerotic lesions from *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> mice harbour thicker fibrous  
14 caps.

15 3) Plaque matrix analyses display increased SHG signals compared to *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> mice. The  
16 enhanced SHG signal suggests that the deletion of *Fap* increases deposition of fibrillar collagens in the  
17 lesions.

18 Using a targeted proteomic analyses of cell culture supernatants from *Fap*<sup>-/-</sup> mouse embryonic  
19 fibroblasts that express enzymatically active FAP or inactive FAP, we showed that FAP cleaves major  
20 collagens species at several sites, including COL3A1. When analysing COL3A1 protein expression in  
21 aortic lysates, we detected reduced cleavage of pre-COL3A1 in *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> compared to *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup>  
22 mice. Notably, in a recent study with acromegaly patients, surgical or medical treatment of the pituitary  
23 somatotroph tumour markedly reduced the levels of FAP compared to untreated subjects. These effects  
24 were closely associated with a reduction in biomarkers of collagen turnover<sup>31</sup>.

25 The exact mechanisms by which impaired COL3A1 expression and/or processing affect collagen fibril  
26 organization is not yet well known. In the first *Col3a1* knockout study, Rudolph Jaenisch's group  
27 demonstrated that collagen SHG imaging of fibroblast-derived matrices showed that *Col3*-deficient  
28 fibroblasts produced a more aligned and fibrillar matrix than wild-type cells, and that COL3A1 is crucial

1 for collagen I fibrillogenesis <sup>32</sup>. Therefore, changes in COL3A1 processing have an impact on the  
2 formation of fibrillar collagens.

3 Moreover, the vascular (type IV) form of the Ehlers-Danlos Syndrome, which is caused by mutations in  
4 the *COL3A1* gene, leads to an impaired fibrillar collagen disposition <sup>32-36</sup>. Both exon-skipping or  
5 missense mutations in the *COL3A1* loci can disrupt the collagen triple helix. These malformed helices  
6 are then degraded or accumulate in intracellular compartments, and hence not secreted into the  
7 extracellular matrix. As a consequence, the reduction in COL3A1 caused by the mutations alters the  
8 size and structure of collagen fibrils <sup>34</sup>. Nevertheless, the precise mechanisms by which mutant COL3A1  
9 causes dermal and vascular fragility remain largely unexplored.

10 Taken together, our current mouse and published human data <sup>13</sup> demonstrate that endogenous pro-  
11 oncogenic FAP promotes atherosclerosis development. Interestingly, depletion of FAP-positive cells  
12 inhibits tumour growth by increasing antitumour immunity <sup>21</sup>, and small antagonists and blocking  
13 antibodies for FAP are already being applied in oncology <sup>37</sup>. Along these lines, it will not only be exciting  
14 to further test the impact of FAP inhibition on atherosclerosis in the experimental and clinical setting,  
15 FAP targeting may also comprise a promising cardio-oncological treatment avenue.

16 Interestingly, a recent study suggest that FAP also plays a central role in cardiac fibrosis, which  
17 promotes the development of various cardiac diseases and heart failure <sup>12</sup>. The authors showed that  
18 engineered chimeric FAP-antigen receptor T cells can be used to reduce experimental cardiac fibrosis  
19 and restore cardiac function using an angiotensin II/phenylephrine-induced hypertensive heart failure  
20 mouse model <sup>12</sup>.

21 Thus, FAP appears an attractive therapeutic target for fibrotic heart failure and atherosclerosis worth  
22 being further investigated in the clinical arena.

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8

9 **Conflict of Interest**

10 None declared.

11

12 **Author Contributions**

- 13 • Christian Matter initiated the study.
- 14 • Sokrates Stein and Christian Matter designed the experiments and co-supervised the  
15 study.
- 16 • Julien Weber, Stefanie Nusser-Stein, Jürgen Pahla, Shafeeq Mohammed, Sara Oppi,  
17 Daniel S. Gaul, and Sokrates Stein performed experiments and data analyses.
- 18 • Hui E. Zhang and Mark D. Gorrell performed the SILAC-mass spec analysis.
- 19 • Ferdinand von Meyenn provided support with the scRNA-seq analyses.
- 20 • Anne Tailleux and Bart Staels performed lipoprotein analyses.
- 21 • Sokrates Stein, Christian Matter, and Mark D. Gorrell wrote the manuscript.
- 22 • Francesco Paneni, Frank Ruschitzka and Thomas F. Lüscher edited the manuscript.
- 23 • Christian Matter, Sokrates Stein, Frank Ruschitzka and Thomas F. Lüscher obtained  
24 funding for the study.

25

26 **Data Availability Statement**

27 The data underlying this article is either publicly available (transcriptomic data) or will be shared on  
28 reasonable request to the corresponding author.

29

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## Figures legends

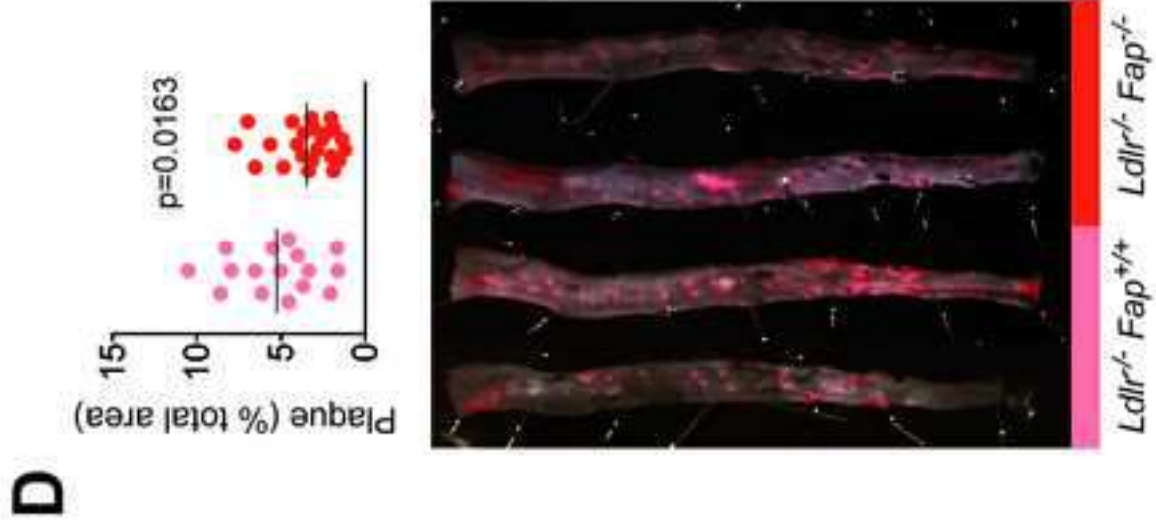
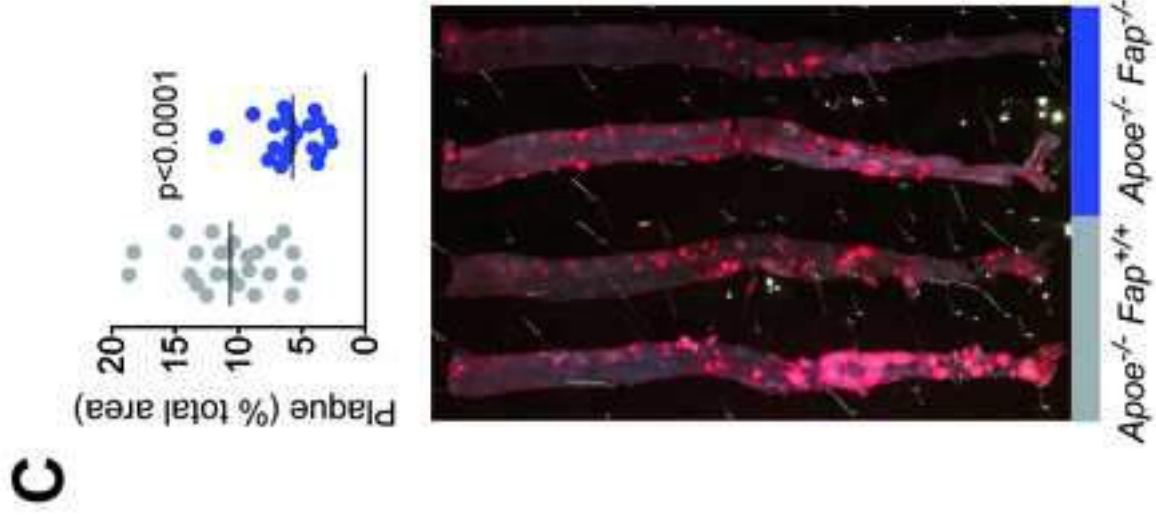
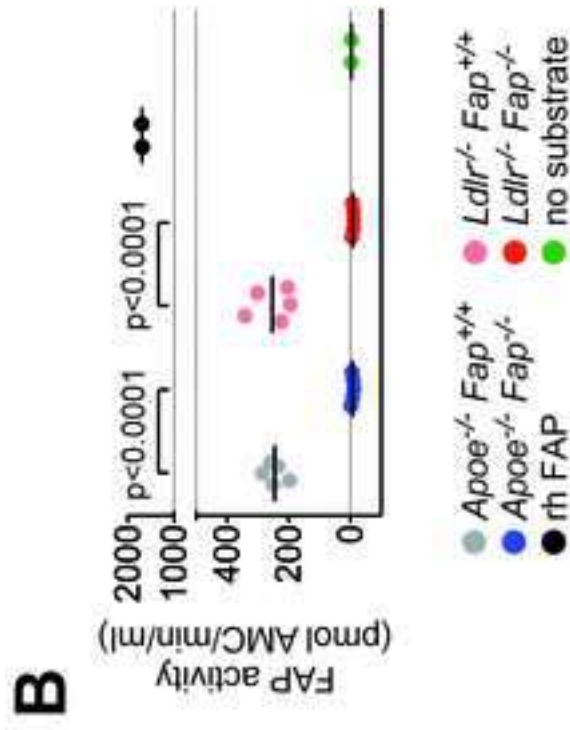
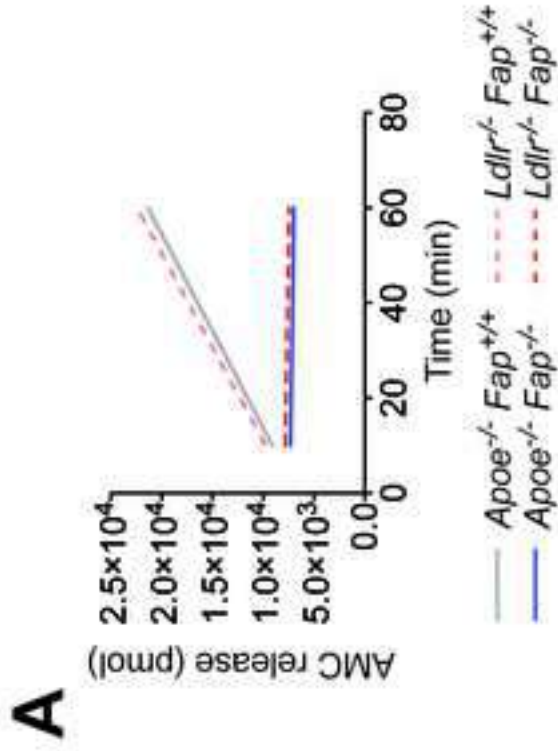
**Figure 1. Constitutive *Fap* deletion reduces atherosclerosis development in mice.** (A, B) FAP activity assay performed with plasma obtained from indicated mouse lines. (A) FAP activity as assessed by the FAP target 3144-aminomethylcoumarin (AMC) release curve. (B) Quantification of the AMC release over time.  $n = 5$  per genotype,  $n = 2$  for controls. rhFAP, recombinant human FAP. (C, D) Quantification and representative images of atherosclerotic lesions in thoraco-abdominal aortae stained with Oil-red O in the indicated genotypes.  $n \geq 16$  per genotype. Scatter blots with means. (Adjusted) P values are indicated in the figures (99.9% confidence interval). Unpaired two-tailed Student's *t*-test (C, D); ANOVA analysis and Bonferroni post-hoc test (B).

**Figure 2. *Fap* deficiency diminishes accumulation of neutral lipids in atherosclerotic plaques of *Apoe*<sup>-/-</sup> mice without affecting macrophages and T cells.** (A) Quantification of neutral lipids and representative images of aortic sinus cross-sections with Oil-red O. (B-D) Quantification of immunohistochemical stainings and representative images of VCAM-1 (B), CD68 (C), and CD3 (D) in cross-sections from the aortic sinus.  $n \geq 8$  per genotype (4 cross-sections per mouse). Black bars = 400  $\mu\text{m}$ , orange bars = 40  $\mu\text{m}$ . Black arrows, examples of CD3-positive cells. Scatter blots with means, unpaired two-tailed Student's *t*-test.

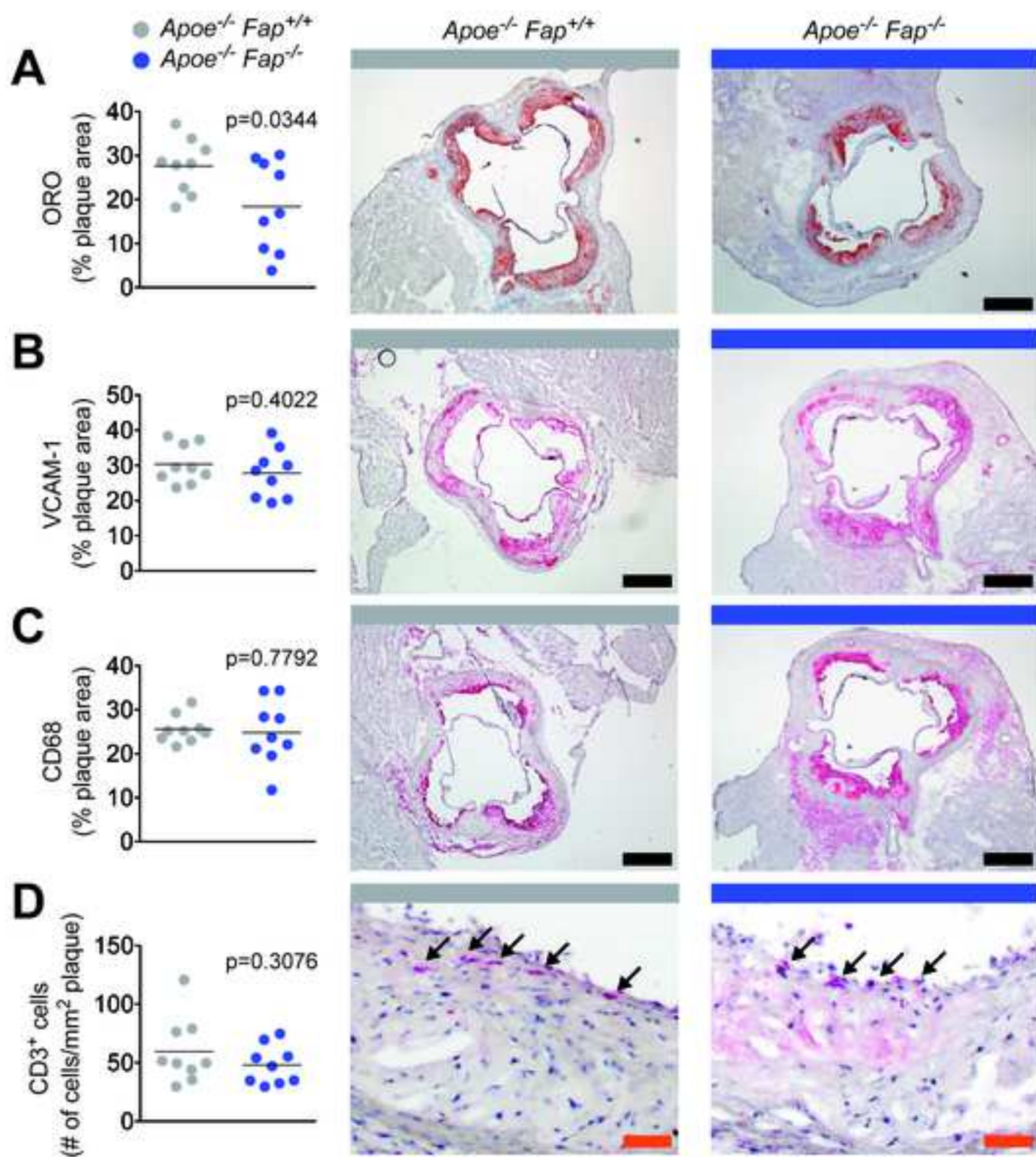
**Figure 3. *Fap* deletion alters collagen structure in atherosclerotic lesions.** (A-D) Quantification of total collagen content (A), necrotic core size (B), fibrous cap thickness (C), and (D) representative images of aortic sinus cross-sections stained with Elastica van Gieson (EvG). Green line, fibrous cap; dashed green line, necrotic core.  $n = 7$  *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> mice,  $n = 9$  *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup>. (E) Quantification of collagen content and representative images of aortic sinus cross-sections stained with Sirius Red and visualized under normal light.  $n = 7$  *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> mice,  $n = 9$  *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup>. (F) Representative images of second harmonics generation (SHG) and quantification of fibrillar collagen in aortic sinus cross-sections.  $n = 9$  *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> mice,  $n = 6$  *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup>. Black and white bars = 400  $\mu\text{m}$ , orange bars = 100  $\mu\text{m}$ . Scatter blots with means, unpaired two-tailed Student's *t*-test (A-E); nonparametric Mann-Whitney test (F).

**Figure 4. Cleavage events in collagens revealed by proteomics in the secretomes of mouse embryonic fibroblasts that express active FAP compared with inactive FAP.** (A-D) Log transformed fold-change of neo-N-terminal peptides is shown for individual cleavage sites and arranged by amino acid position for COL1A1 (A), COL1A2 (B), COL3A1 (C), and COL5A2 (D). Neo-N-terminal peptides resulting from cleavage after Gly-Pro are represented by coloured squares, whereas other cleavage sites are plotted as black circles. A fold change  $\geq 0.58$  represents increased peptide abundance of  $\geq 50\%$  in the presence of FAP activity. A fold change  $\leq -0.58$  represents decreased peptide abundance of  $\geq 50\%$  in the presence of FAP activity. Mean and standard deviation of data that reached significance (limma statistic with  $p < 0.05$ ). (E, F) COL3A1 immunoblots from aortae from *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> and *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> mice. Pre, precursor COL3A1; cleaved, cleaved COL3A1 fibers.  $n = 4$  per genotype. (G) Expression of *FAP* in biopsies of non-atherosclerotic mammary arteries (Mammary) and atherosclerotic aortic roots (Athero) (raw data, GSE40231). Unpaired two-tailed Student's *t*-test (F, G).

**Figure 5. Graphical abstract.** Scheme demonstrating how the deletion of FAP protects against atherosclerosis development.







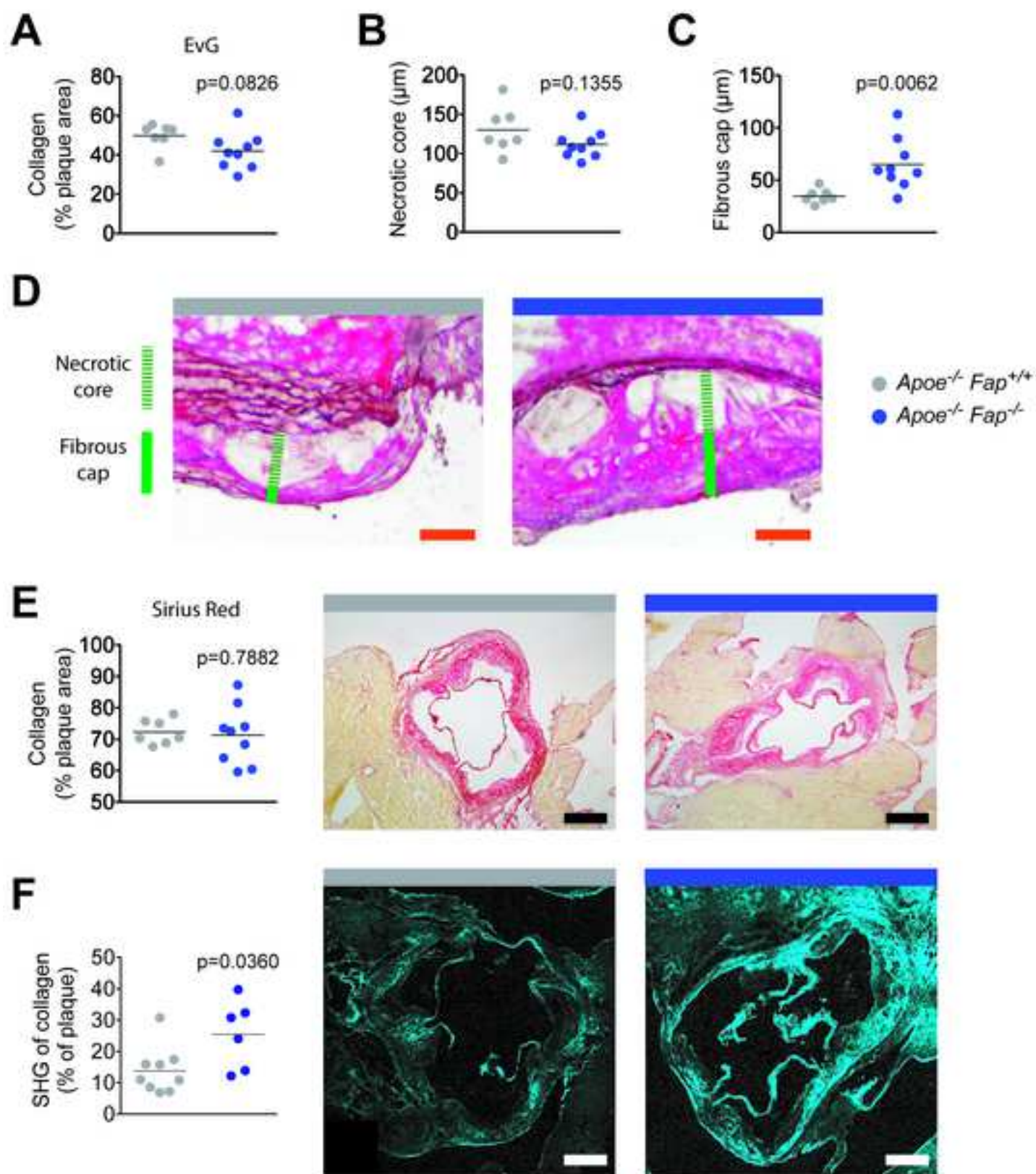
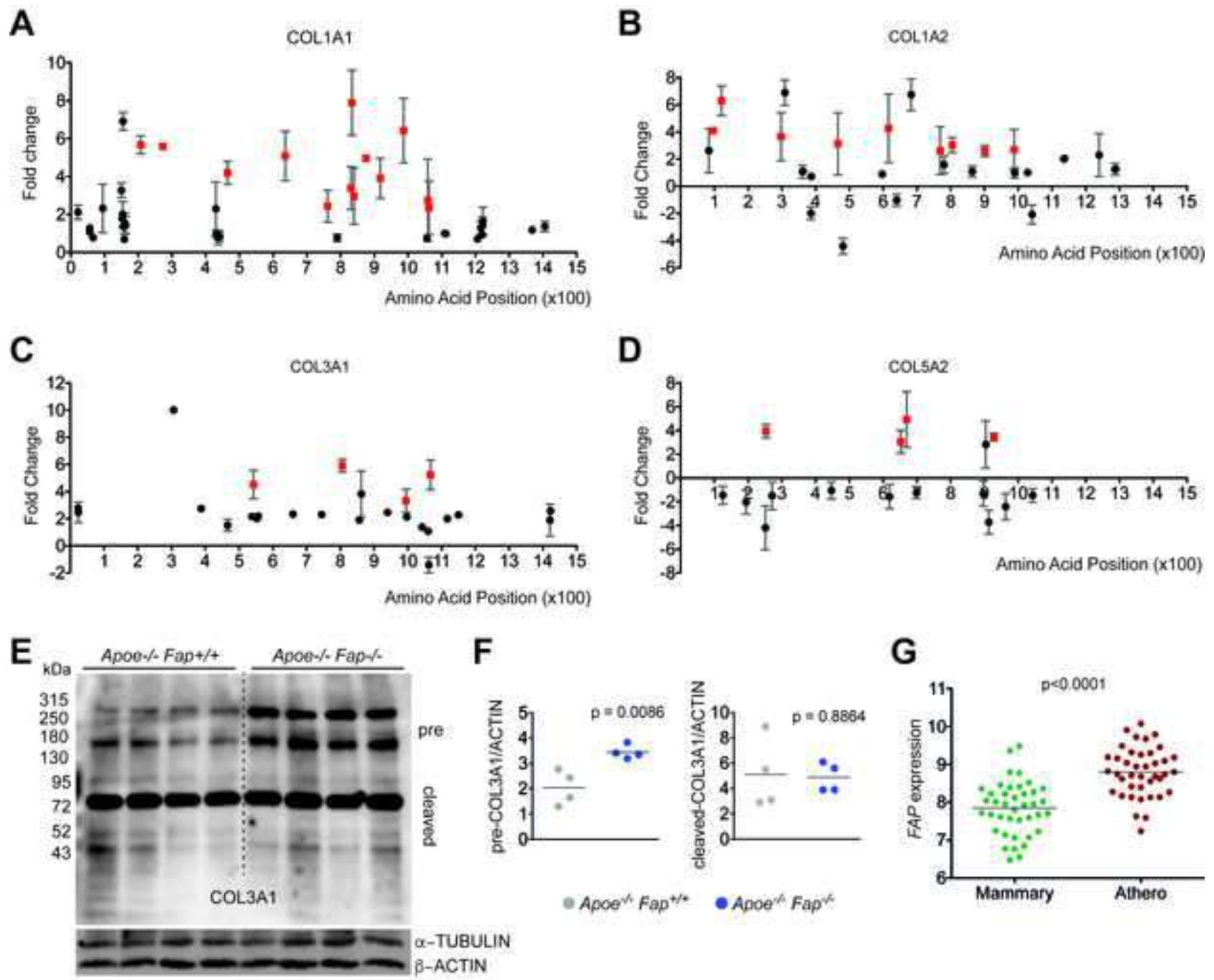
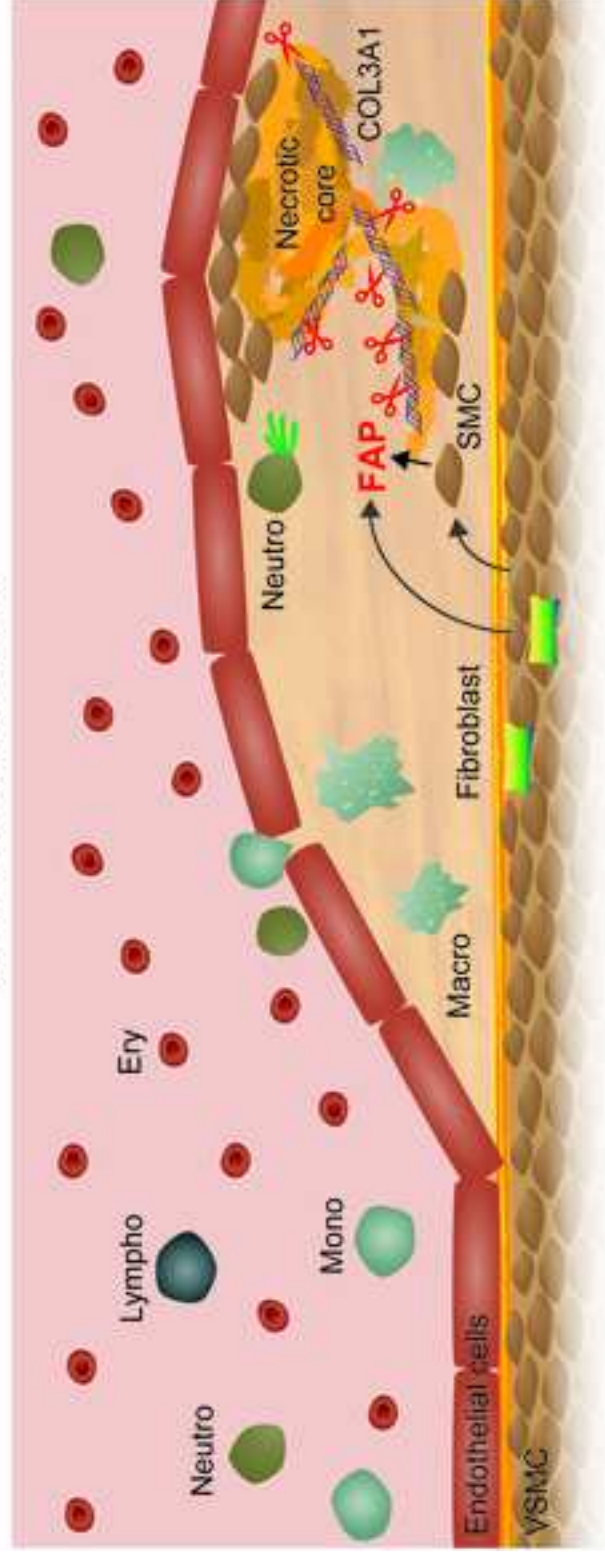


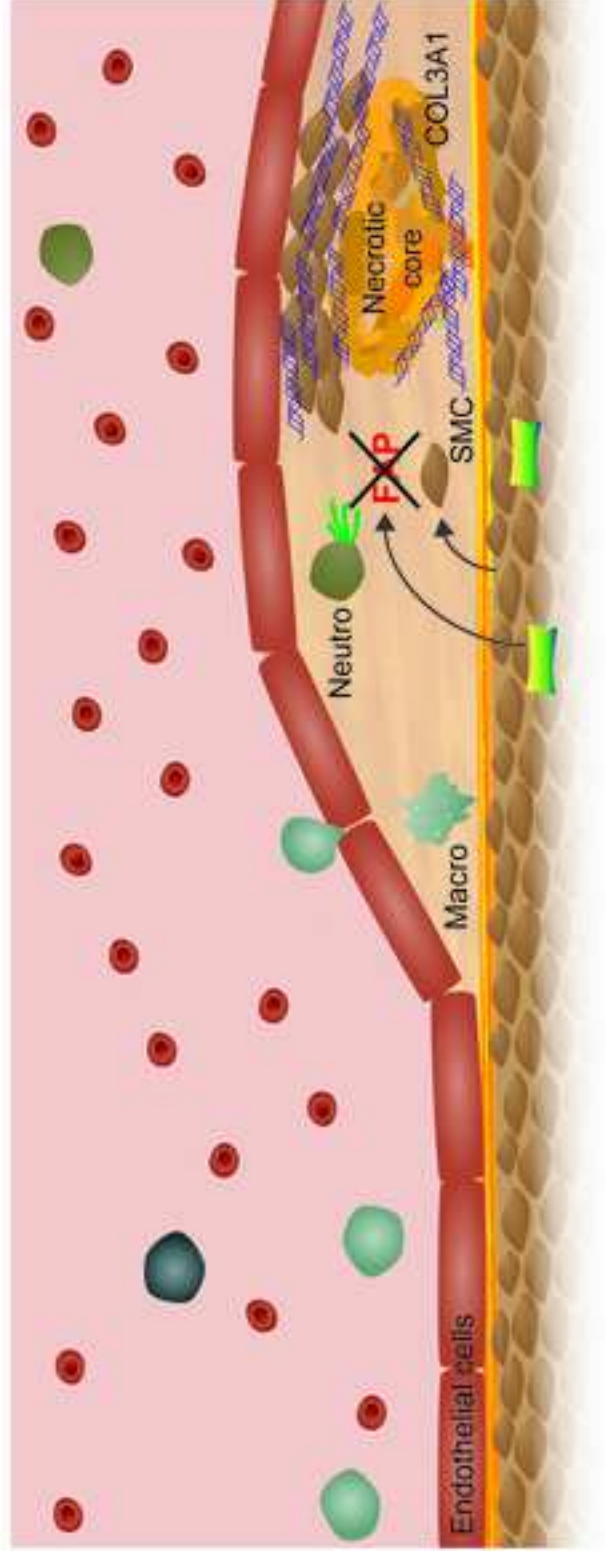
Figure 4



### Normal endogenous FAP

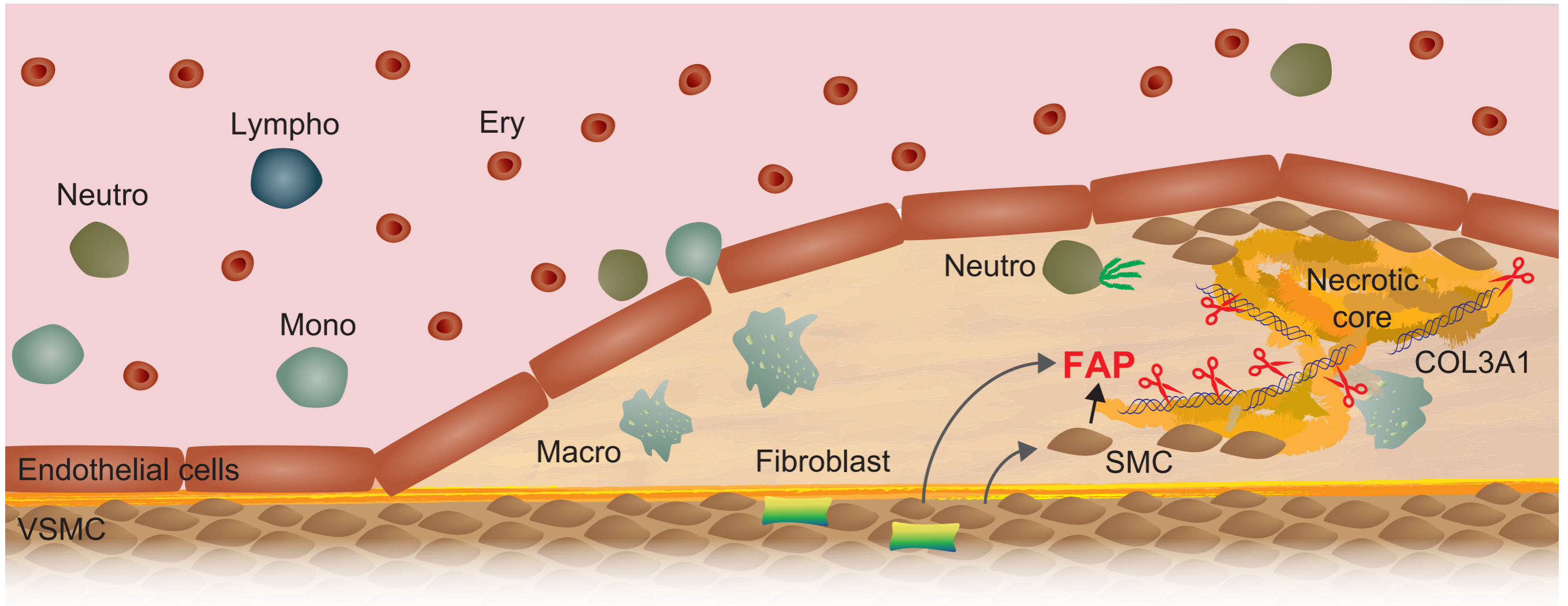


### Constitutive FAP deletion

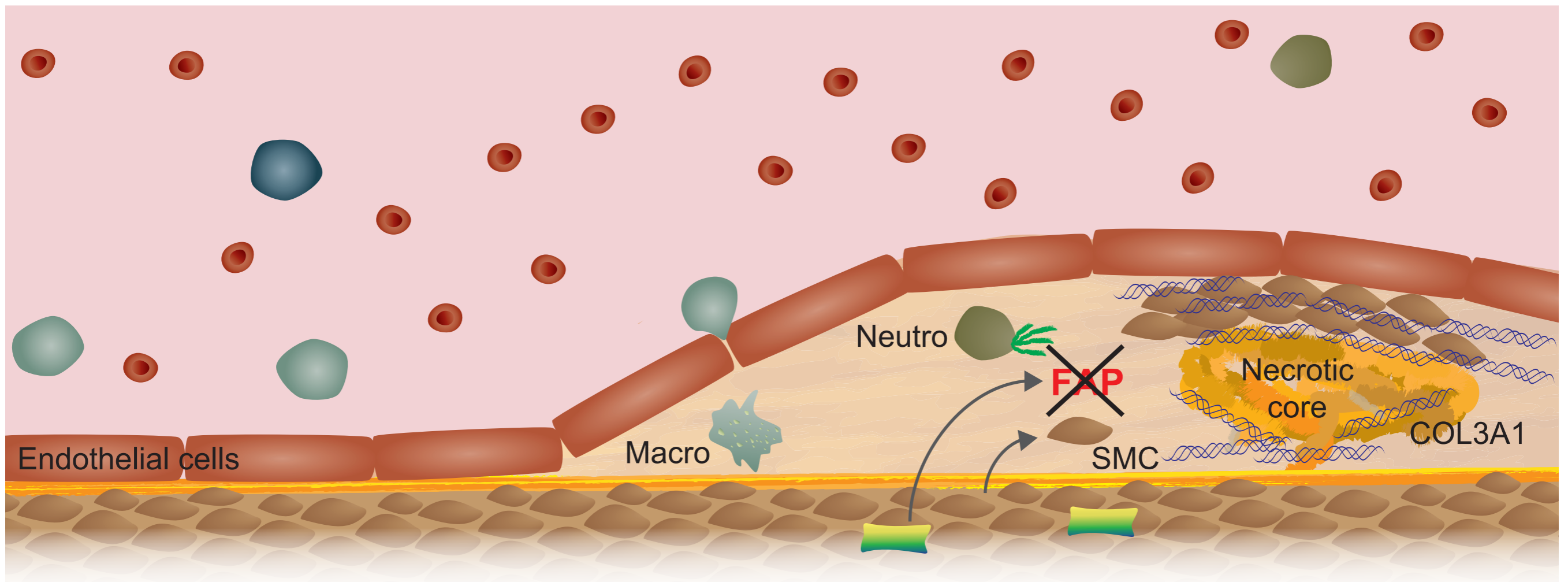


More pre-COL3A1, thicker fibrous caps, and less atherosclerotic lesions

### Normal endogenous FAP

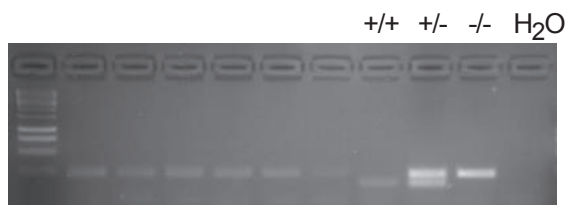
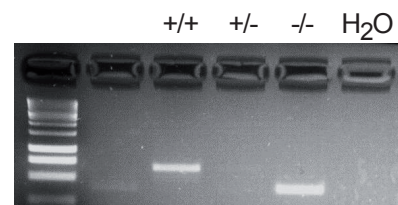
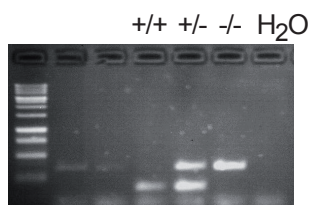
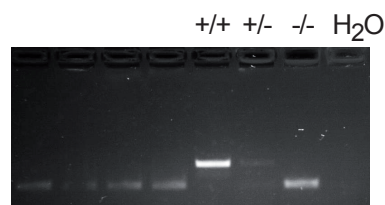


### Constitutive FAP deletion



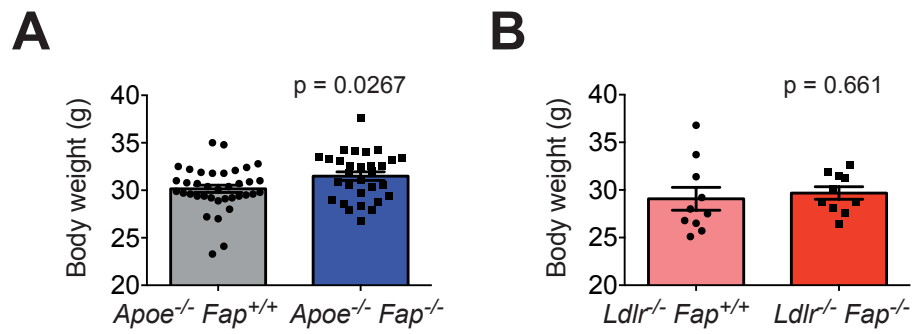
More pre-COL3A1, thicker fibrous caps, and less atherosclerotic lesions

## Supplemental Information for

**Deletion of fibroblast activation protein provides atheroprotection**Contact e-mail: [sokrates.stein@uzh.ch](mailto:sokrates.stein@uzh.ch) and [christian.matter@usz.ch](mailto:christian.matter@usz.ch)**Figure S1****A** *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> mice*Apoe* primers:*Fap* primers:**B** *Ldlr*<sup>-/-</sup> *Fap*<sup>-/-</sup> mice*Ldlr* primers:*Fap* primers:

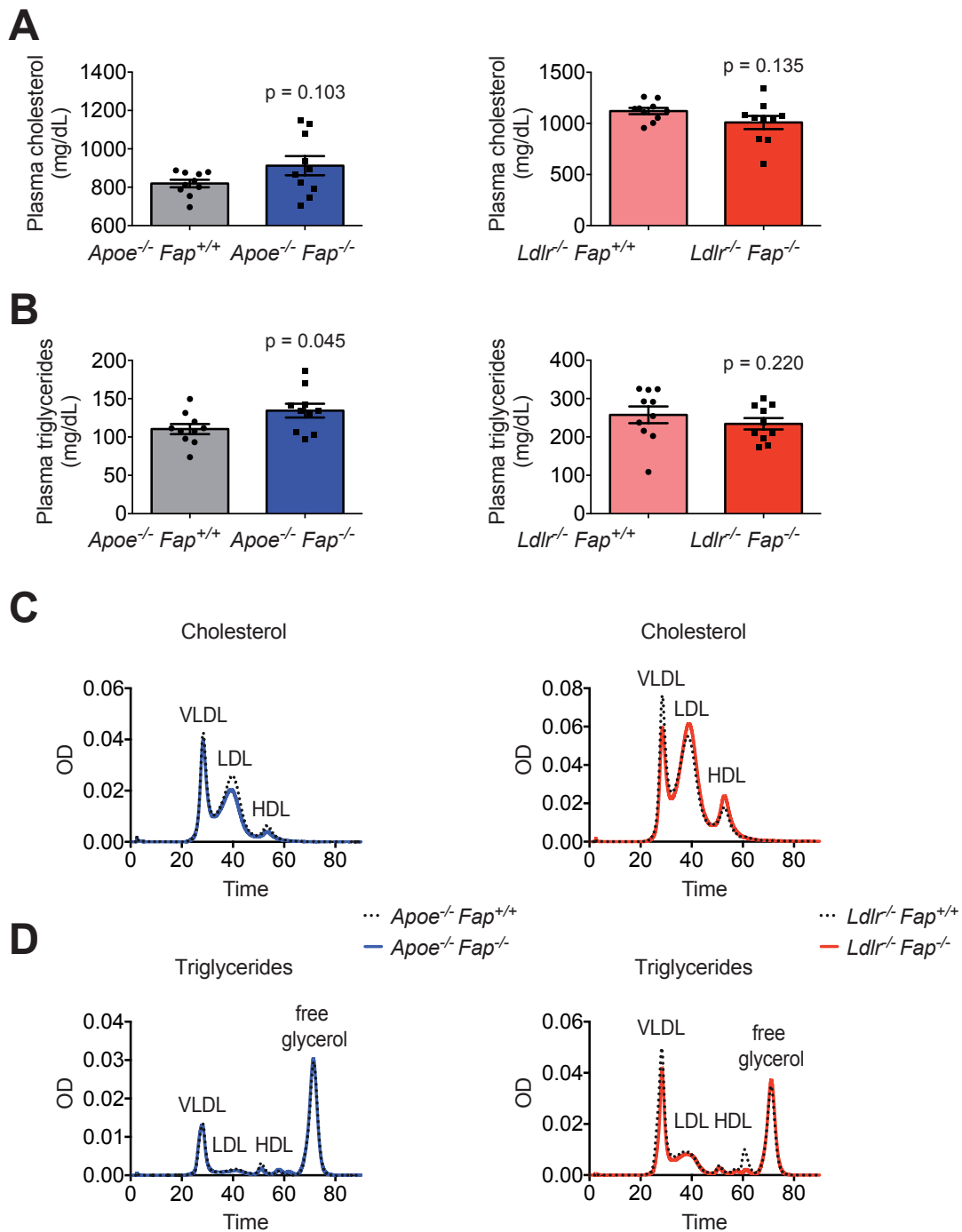
**Figure S1.** Validation of the genotype of *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> and *Ldlr*<sup>-/-</sup> *Fap*<sup>-/-</sup> mice and the corresponding controls. (A, B) PCR validation of (A) *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> and *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> mice and (B) *Ldlr*<sup>-/-</sup> *Fap*<sup>+/+</sup> and *Ldlr*<sup>-/-</sup> *Fap*<sup>-/-</sup> mice.

**Figure S2**



**Figure S2.** Body weight of atherosclerotic mice lacking *Fap*. **(A, B)** Body weight of **(A)** *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> and *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> mice and **(B)** *Ldlr*<sup>-/-</sup> *Fap*<sup>+/+</sup> and *Ldlr*<sup>-/-</sup> *Fap*<sup>-/-</sup> mice that were fed a high-cholesterol diet for 12 weeks. Data are represented in scatter plots and bars  $\pm$  standard error; unpaired two-tailed Student's *t*-test.

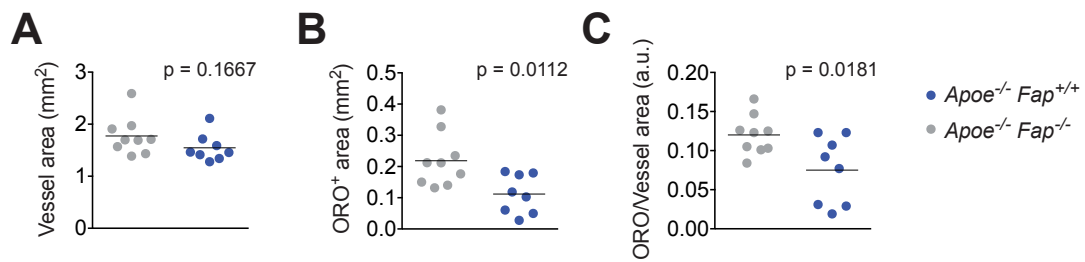
**Figure S3**



**Figure S3.** Plasma lipid levels in atherosclerotic *Fap*-deficient mice. **(A, B)** Total plasma cholesterol and **(B)** triglyceride levels in  $Apoe^{-/-} Fap^{+/+}$  versus  $Apoe^{-/-} Fap^{-/-}$  mice as well as in  $Ldlr^{-/-} Fap^{+/+}$  and  $Ldlr^{-/-} Fap^{-/-}$  mice.  $n = 10$  per genotype. **(C, D)** Cholesterol and **(B)** triglycerides levels in lipoprotein subfractions of  $Apoe^{-/-} Fap^{+/+}$  versus  $Apoe^{-/-} Fap^{-/-}$  mice as well as in  $Ldlr^{-/-} Fap^{+/+}$  and  $Ldlr^{-/-} Fap^{-/-}$  mice.  $n = 1$  pool from 10 mice per genotype. Data are represented in scatter plots and bars  $\pm$  standard error (A and B) or plotting individual values on a XY graph. unpaired two-tailed Student's *t*-test **(A, B)**.

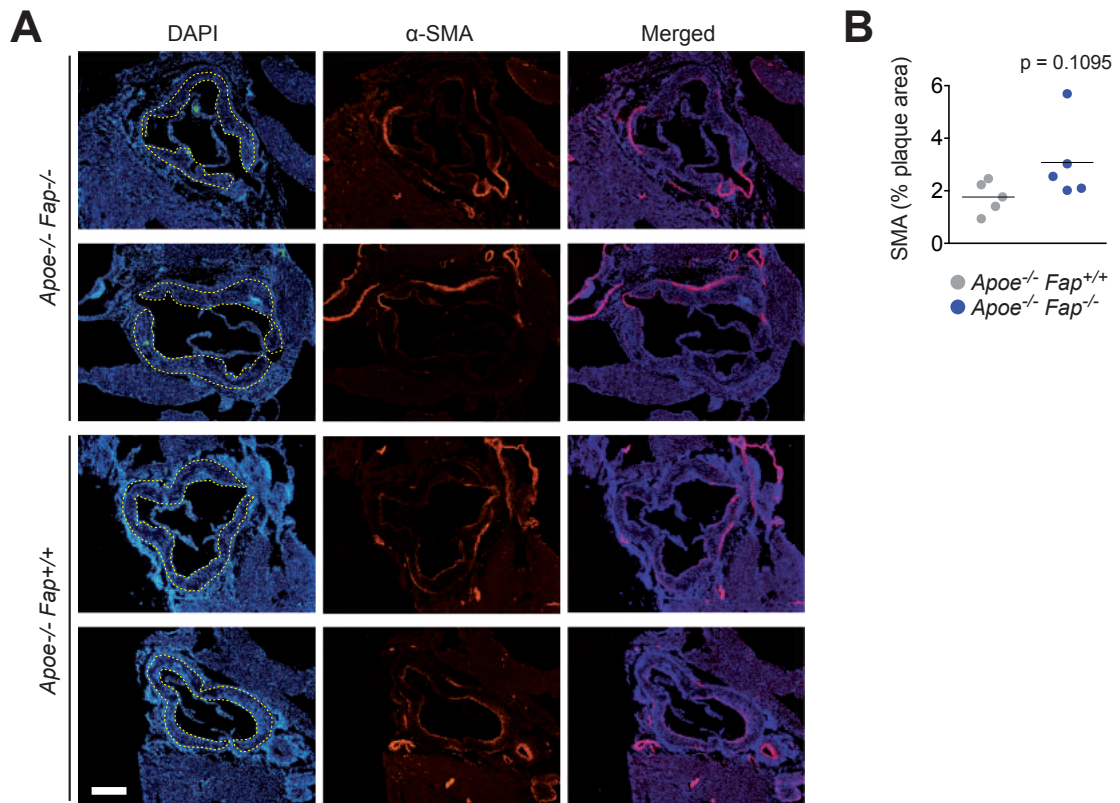


**Figure S4**



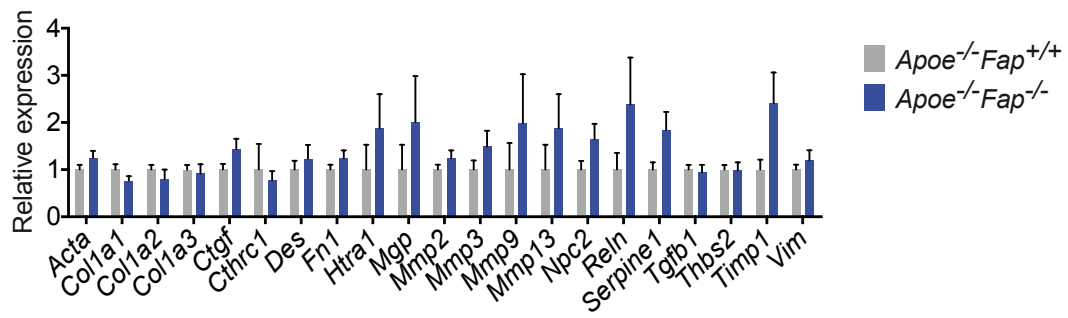
**Figure S4.** Aortic sinus plaque analysis. (A) Entire aortic sinus from *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> versus *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> mice. (B) Oil-red O-positive area in aortic sinus of *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> versus *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> mice. (C) Oil-red O-positive area relative to vessel area in aortic sinus of *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> versus *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> mice. n = 9 *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup>, n = 8 *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> mice. Data are represented in scatter plots with means; unpaired two-tailed Student's *t*-test.

**Figure S5**



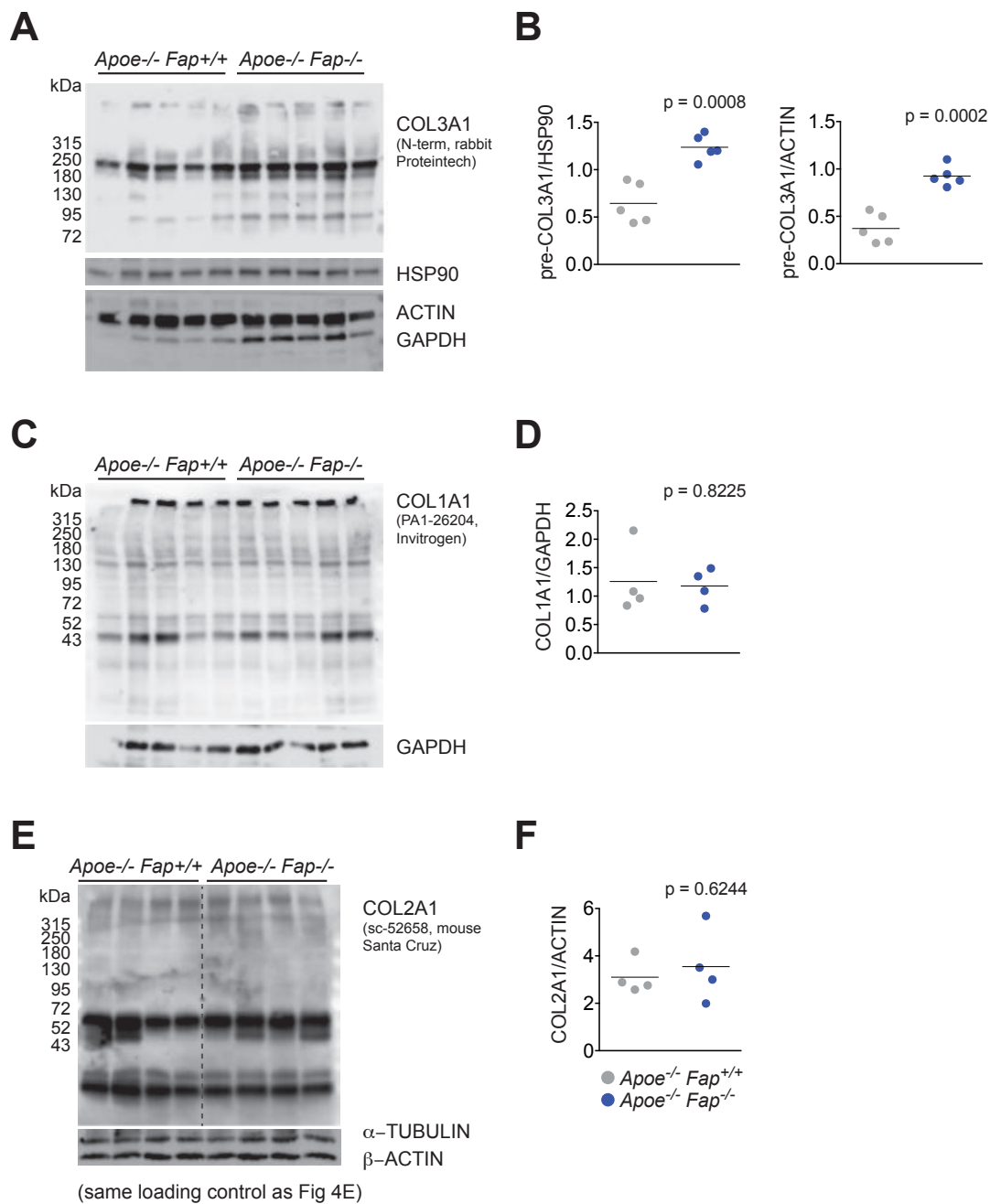
**Figure S5.** *Vascular smooth muscle cell staining.* (A, B) Representative images (A) and quantification (B) of intra-plaque  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) staining in cross-sections from the aortic sinus. The plaque area is marked in yellow dotted lines in the DAPI images.  $n \geq 5$  per genotype (4 cross-sections per mouse). White bar = 400  $\mu$ m. Data are represented in scatter plots with means; unpaired two-tailed Student's *t*-test.

**Figure S6**



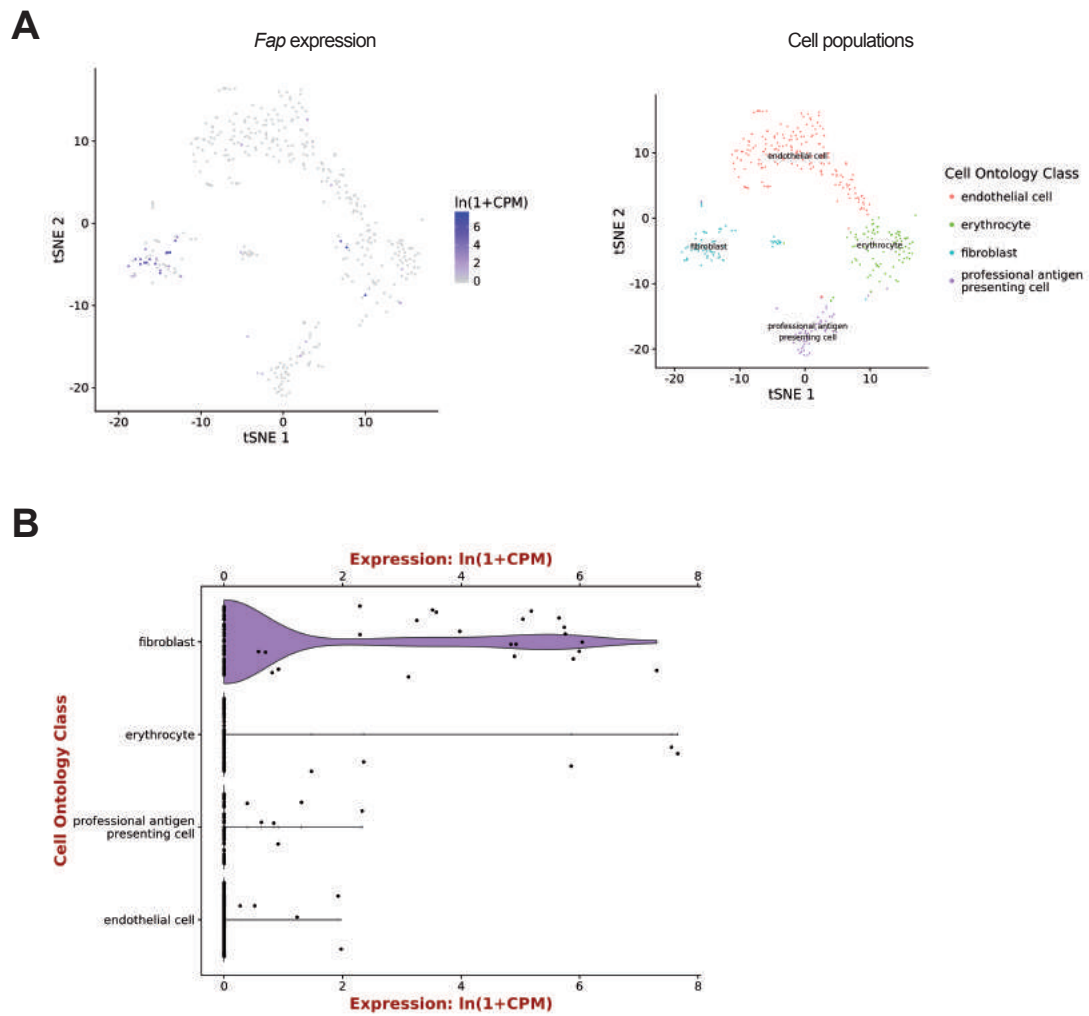
**Figure S6.** Expression levels of extracellular matrix modulators. mRNA expression of collagens, matrix metalloproteinases, and markers of fibrosis in aortae from *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> versus *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> mice. n = 6 per genotype. Data are represented in bar graphs with means and standard errors.

**Figure S7**



**Figure S7.** *COL13A1*, *COL1A1* and *COL2A1* protein levels in atherosclerotic *Fap*-deficient mice. (**A**, **B**) *COL3A1* immunoblots (**A**) from aortae from *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> and *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> mice and their quantification (**B**). n = 5 per genotype (**C**, **D**) *COL1A1* immunoblots (**C**) from aortae from *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> and *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> mice, respectively. n = 5 per genotype. (**D**) Quantification of *COL1A1* expression (n = 4 per genotype; the two external lanes were excluded). (**E**, **F**) *COL2A1* immunoblots (**E**) from aortae from *Apoe*<sup>-/-</sup> *Fap*<sup>-/-</sup> and *Apoe*<sup>-/-</sup> *Fap*<sup>+/+</sup> mice and their quantification (**F**). n = 4 per genotype. Data are represented in scatter plots with means; unpaired two-tailed Student's *t*-test.

**Figure S8**



**Figure S8.** *Fap* expression in aortae of 3-month-old C57BL/6JN mice. **(A)** *Fap* expression in different aortic cell populations as described on the right panel. **(B)** Quantification of *Fap* expression in corresponding vascular cells. Graphs were directly exported from the *Tabula muris* dataset<sup>24</sup>.

**Table S1.** Hematological analyses.

	WBC	RBC	PLT	Neutrophils		Lymphocytes		Monocytes		Eosinophils		Basophils	
	number 10 <sup>9</sup> /L	number 10 <sup>12</sup> /L	number 10 <sup>3</sup> /μL	%	number 10 <sup>3</sup> /μL	%	number 10 <sup>3</sup> /μL	%	number 10 <sup>3</sup> /μL	%	number 10 <sup>3</sup> /μL	%	number 10 <sup>3</sup> /μL
<i>Apo<sup>e</sup>-/ Fap<sup>+/+</sup></i> (n=10)	6.593 ± 0.5620	9.190 ± 0.1246	1891 ± 164.2	52.20 ± 2.401	3.502 ± 0.4123	39.18 ± 2.422	2.549 ± 0.2428	1.290 ± 0.2442	0.0790 ± 0.01130	2.790 ± 1.143	0.1630 ± 0.05727	0.4000 ± 0.06325	0.0260 ± 0.003712
<i>Apo<sup>e</sup>-/ Fap<sup>-/-</sup></i> (n=12)	6.234 ± 0.7441	9.234 ± 0.2201	1480 ± 118.2	28.86 ± 1.881	1.694 ± 0.1213	61.47 ± 1.883	3.926 ± 0.6243	2.833 ± 0.2076	0.1767 ± 0.0255	3.700 ± 0.6370	0.2275 ± 0.04432	0.3750 ± 0.02787	0.02333 ± 0.002562
P value	0.7138	0.8704	0.0511	< 0.0001	0.0002	< 0.0001	0.0707	< 0.0001	0.0038	0.4760	0.3764	0.7051	0.5508
<i>Ldl<sup>-/ Fap<sup>+/+</sup></sup></i> (n=9)	7.557 ± 0.9580	9.794 ± 0.1407	1208 ± 104.4	17.09 ± 1.656	1.199 ± 0.1184	74.19 ± 1.708	5.714 ± 0.8037	2.711 ± 0.4118	0.1967 ± 0.02867	2.422 ± 0.3800	0.1911 ± 0.03642	0.4667 ± 0.03333	0.03444 ± 0.005031
<i>Ldl<sup>-/ Fap<sup>-/-</sup></sup></i> (n=10)	9.163 ± 0.4785	9.808 ± 0.09269	1414 ± 140.5	13.50 ± 0.7397	1.241 ± 0.09821	79.31 ± 1.316	7.252 ± 0.3676	2.750 ± 0.4342	0.2560 ± 0.04190	1.580 ± 0.4685	0.1442 ± 0.05537	0.4500 ± 0.03727	0.0420 ± 0.005121
P value	0.14	0.9356	0.2644	0.056	0.786	0.028	0.0894	0.9492	0.2691	0.1866	0.499	0.7452	0.3091

WBC, white blood cell; PLT, platelets; RBC, red blood cells; unpaired two-tailed Student's *t*-test.

**Table S2.** Selection of the highest transcripts in cells expressing *Fap* in atherosclerotic *Apoe* aortae.

CATCCAC AGATCCT GT-1	CCTACAC TCCAATG GT-1	GTATTCT CATGCTA GT-1	GTGCATA GTATCGC AT-1	CACACTC GTTTAGG AA-1	CCTTCGA TCCACTC CA-1	CTTGGCT GTCAAGC GA-1	GACGCGT GTATCTG CA-1	GCTCTGT AGCGTCA AG-1	GGACAAG GTAAATA CG-1	TGCCCTA CAAGCCG TC-1	TGGCGCA CAAGGAC TG-1	TGTGGTA AGGCGAC AT-1
<i>Col3a1</i>	<i>Dcn</i>	<i>Mgp</i>	<i>Gsn</i>	<i>Gsn</i>	<i>Dcn</i>	<i>Gsn</i>	<i>Gsn</i>	<i>Cyt11</i>	<i>Gsn</i>	<i>Dcn</i>	<i>Dcn</i>	<i>Gsn</i>
<i>S100a6</i>	<i>Gsn</i>	<i>Cst3</i>	<i>Dcn</i>	<i>S100a6</i>	<i>Gsn</i>	<i>Dcn</i>	<i>S100a6</i>	<i>Cst3</i>	<i>Dcn</i>	<i>Tmsb4x</i>	<i>Tmsb4x</i>	<i>Dcn</i>
<i>Tmsb4x</i>	<i>Rps27</i>	<i>Dcn</i>	<i>Tmsb4x</i>	<i>Dcn</i>	<i>Ftl1</i>	<i>Tmsb4x</i>	<i>Dcn</i>	<i>Rpl41</i>	<i>Mgp</i>	<i>Rpl41</i>	<i>Rpl41</i>	<i>Mfap5</i>
<i>Col1a2</i>	<i>Rpl41</i>	<i>Igfbp7</i>	<i>Rpl41</i>	<i>Ftl1</i>	<i>S100a6</i>	<i>B2m</i>	<i>Ftl1</i>	<i>Gsn</i> <i>31100790</i>	<i>Igfbp7</i>	<i>S100a6</i>	<i>Gsn</i>	<i>S100a6</i>
<i>Fn1</i>	<i>Mfap5</i>	<i>Lum</i>	<i>Mfap5</i>	<i>Tmsb4x</i>	<i>Ifitm3</i>	<i>Ftl1</i>	<i>Cst3</i>	<i>15Rik</i>	<i>Mt1</i>	<i>Mfap5</i>	<i>Ftl1</i>	<i>Rpl41</i>
<i>Dcn</i>	<i>Rps19</i>	<i>Rpl41</i>	<i>Rps27</i>	<i>Rpl41</i>	<i>Rpl41</i>	<i>S100a6</i>	<i>Col3a1</i>	<i>Mia</i>	<i>Ftl1</i>	<i>Col1a2</i>	<i>Cxcl1</i>	<i>Mt1</i>
<i>Mfap5</i>	<i>Ftl1</i>	<i>Cfh</i>	<i>Rpl37a</i>	<i>Mt1</i>	<i>B2m</i>	<i>Rpl41</i>	<i>Rpl41</i>	<i>Rps19</i>	<i>Rpl41</i>	<i>Rpl35</i>	<i>S100a6</i>	<i>Igfbp5</i>
<i>Postn</i>	<i>Rpl37a</i>	<i>Ftl1</i>	<i>Ftl1</i>	<i>Fn1</i>	<i>Penk</i>	<i>Ifitm3</i>	<i>Tmsb4x</i>	<i>Rpl37a</i>	<i>Hbb-bs</i>	<i>Ftl1</i>	<i>Rps27</i>	<i>Mt2</i>
<i>Rpl41</i>	<i>Rps3a1</i>	<i>Gsn</i>	<i>S100a6</i>	<i>Rpl35</i>	<i>Mt2</i>	<i>Tnfaip6</i>	<i>Rpl37a</i>	<i>Rps6</i>	<i>B2m</i>	<i>Tmsb10</i>	<i>Mt1</i>	<i>Tmsb4x</i>
<i>Serpinh1</i>	<i>Rpl35</i>	<i>Tmsb4x</i>	<i>Rpl35</i>	<i>Rps19</i>	<i>Tmsb4x</i>	<i>Rps27</i>	<i>B2m</i>	<i>Rps5</i>	<i>Ifitm3</i>	<i>Gsn</i>	<i>Rpl37a</i>	<i>Rps27</i>
<i>Gsn</i>	<i>Rpl32</i>	<i>Bgn</i>	<i>Rps19</i>	<i>Rpl37a</i>	<i>Rarres2</i>	<i>Rpl10</i>	<i>Igfbp7</i>	<i>Rps4x</i>	<i>Tmsb4x</i>	<i>Rps19</i>	<i>Rps19</i>	<i>Hbb-bs</i>
<i>Vim</i>	<i>S100a6</i>	<i>Hbb-bs</i>	<i>Rpl18a</i>	<i>Ubc</i>	<i>Rps27</i>	<i>Cxcl1</i>	<i>Rpl32</i>	<i>Rpl35</i>	<i>Rpl37a</i>	<i>Col3a1</i>	<i>Rps4x</i>	<i>Ftl1</i>
<i>S100a10</i>	<i>Hbb-bs</i>	<i>Rps27</i>	<i>Rpl10</i>	<i>Vim</i>	<i>Pcolce</i>	<i>Rpl37a</i>	<i>Hbb-bs</i>	<i>Rps11</i>	<i>Bgn</i>	<i>Mt2</i>	<i>Rps5</i>	<i>Rpl35</i>
<i>Ftl1</i>	<i>Rpl10</i>	<i>Rpl37a</i>	<i>Rps8</i>	<i>Rps27</i>	<i>Col1a2</i>	<i>S100a9</i>	<i>Ifitm2</i>	<i>Wif1</i>	<i>Jund</i>	<i>Rpl37a</i>	<i>Mfap5</i>	<i>S100a8</i>
<i>Serf2</i>	<i>Serping1</i>	<i>Vim</i>	<i>Igfbp5</i>	<i>Rpl18a</i>	<i>Lum</i>	<i>Rps19</i>	<i>Rps27</i>	<i>Rplp0</i>	<i>Junb</i>	<i>Rps27</i>	<i>Cst3</i>	<i>S100a11</i>
<i>Tppp3</i>	<i>Rps8</i>	<i>Rps5</i>	<i>Rps15a</i>	<i>Rps5</i>	<i>Plac8</i>	<i>Tmsb10</i>	<i>Ifitm3</i>	<i>Rpl18a</i>	<i>Mt2</i>	<i>S100a11</i>	<i>Rpl18a</i>	<i>Rpl18a</i>
<i>Cxcl2</i>	<i>Mt2</i>	<i>Rplp0</i>	<i>Vim</i>	<i>Dpt</i>	<i>Tmsb10</i>	<i>Rpl35</i>	<i>Rpl35</i>	<i>Vim</i>	<i>Rps27</i>	<i>Rpl18a</i>	<i>Rpl10</i>	<i>Tmsb10</i>
<i>Cd63</i>	<i>Rps9</i>	<i>Rpl13a</i>	<i>Rpl32</i>	<i>Rpl32</i>	<i>S100a9</i>	<i>Hbb-bs</i>	<i>Rpl10</i>	<i>Rps27</i>	<i>Rps19</i>	<i>Rpl10</i>	<i>Vim</i>	<i>Rpl37a</i>
<i>Hbb-bs</i>	<i>Rpl18a</i>	<i>S100a8</i>	<i>Rpl9</i>	<i>Pcolce</i>	<i>Rps4x</i>	<i>Rpl13a</i>	<i>Rps4x</i>	<i>Rpl13a</i>	<i>Cst3</i>	<i>Igfbp5</i>	<i>Rps8</i>	<i>Rps19</i>
<i>Cxcl1</i>	<i>B2m</i>	<i>Cyt11</i>	<i>Rps3</i>	<i>Tppp3</i>	<i>Gstm1</i>	<i>Serpinh1</i>	<i>Col6a2</i>	<i>Rpl32</i>	<i>S100a6</i>	<i>Hbb-bs</i>	<i>Hbb-bs</i>	<i>Dpt</i>

Top 20 transcripts for each cell with a *Fap* sequencing count > 2. Original data from <sup>1</sup>.

**Table S3.** Pathway analyses of the selected transcripts from table S2.

<b>Top 10 GOTERM_BP Term</b>	<b>P Value</b>	<b>Fold Enrichment</b>	<b>Bonferroni</b>	<b>Benjamini</b>	<b>FDR</b>
GO:0006412~translation	3.99E-15	12.599311	1.90E-12	1.90E-12	5.72E-12
GO:0032496~response to lipopolysaccharide	8.19E-06	10.798447	0.003885	0.001944	0.011715
GO:0030199~collagen fibril organization	1.28E-05	34.091252	0.006081	0.002031	0.018358
GO:0007568~aging	4.33E-05	10.759436	0.020352	0.005127	0.061869
GO:0009612~response to mechanical stimulus	8.70E-05	21.104108	0.040482	0.008231	0.124299
GO:0009314~response to radiation	1.84E-04	35.454902	0.083596	0.014444	0.262405
GO:0042060~wound healing	4.09E-04	14.144243	0.176457	0.027353	0.582618
GO:0043206~extracellular fibril organization	7.28E-04	72.521390	0.292442	0.042320	1.035793
GO:0001649~osteoblast differentiation	8.19E-04	11.766007	0.322242	0.042298	1.163880
GO:0042989~sequestering of actin monomers	8.72E-04	66.477941	0.339104	0.040570	1.238798
<b>Top 10 GOTERM_CC Term</b>	<b>P Value</b>	<b>Fold Enrichment</b>	<b>Bonferroni</b>	<b>Benjamini</b>	<b>FDR</b>
GO:0005840~ribosome	2.15E-21	28.38738602	2.57E-19	2.57E-19	2.46E-18
GO:0031012~extracellular matrix	9.45E-21	20.06326531	1.13E-18	5.67E-19	1.08E-17
GO:0070062~extracellular exosome	5.39E-16	4.09668768	6.66E-14	2.22E-14	6.33E-13
GO:0022627~cytosolic small ribosomal subunit	2.45E-15	58.29703504	2.93E-13	7.33E-14	2.81E-12
GO:0005576~extracellular region	1.99E-13	4.806943199	2.38E-11	4.77E-12	2.28E-10
GO:0030529~intracellular ribonucleoprotein complex	4.76E-12	13.16651786	5.71E-10	9.52E-11	5.47E-09
GO:0005925~focal adhesion	6.88E-11	10.77566679	8.25E-09	1.18E-09	7.90E-08
GO:0005615~extracellular space	1.07E-10	4.668977964	1.29E-08	1.61E-09	1.23E-07
GO:0022625~cytosolic large ribosomal subunit	3.07E-08	24.69324961	3.69E-06	4.10E-07	3.53E-05
GO:0005578~proteinaceous extracellular matrix	1.55E-06	8.888788427	1.86E-04	1.86E-05	0.001782
GO:0015935~small ribosomal subunit	3.09E-06	48.42857143	3.70E-04	3.37E-05	0.003544
<b>Top 10 GOTERM_MF Term</b>	<b>P Value</b>	<b>Fold Enrichment</b>	<b>Bonferroni</b>	<b>Benjamini</b>	<b>FDR</b>



GO:0003735~structural constituent of ribosome	4.48E-20	20.651042	5.82E-18	5.82E-18	5.21E-17
GO:0044822~poly(A) RNA binding	6.98E-09	4.898360	9.08E-07	4.54E-07	8.13E-06
GO:0019843~rRNA binding	2.67E-05	28.395182	0.003461	0.001155	0.031050
GO:0003729~mRNA binding	1.60E-04	11.518046	0.020602	0.005191	0.186307
GO:0005518~collagen binding	0.001263	18.480932	0.151523	0.032328	1.461140
GO:0005201~extracellular matrix structural constituent	0.009613	19.945884	0.715115	0.188831	10.638815
GO:0008201~heparin binding	0.017288	7.221026	0.896388	0.276657	18.379381
GO:0035662~Toll-like receptor 4 binding	0.017928	109.037500	0.904799	0.254701	18.996059
GO:0048306~calcium-dependent protein binding	0.025784	11.851902	0.966489	0.314303	26.228997
GO:0050786~RAGE receptor binding	0.028532	68.148438	0.976789	0.313613	28.616601

<b>Top 10 UP_KEYWORDS Term</b>	<b>P Value</b>	<b>Fold Enrichment</b>	<b>Bonferroni</b>	<b>Benjamini</b>	<b>FDR</b>
Ribosomal protein	2.15E-23	31.471588	2.50E-21	2.50E-21	2.46E-20
Ribonucleoprotein	6.32E-20	20.742638	7.33E-18	3.66E-18	7.21E-17
Secreted	2.12E-13	5.308146	2.46E-11	8.21E-12	2.42E-10
Extracellular matrix	4.44E-08	13.593048	5.15E-06	1.29E-06	5.06E-05
Acetylation	8.75E-07	2.759932	1.01E-04	2.03E-05	9.98E-04
Disulfide bond	3.23E-06	2.658563	3.74E-04	6.24E-05	0.003683
Signal	9.87E-05	2.039107	0.011380	0.001634	0.112503
Chemotaxis	2.06E-04	16.991310	0.023592	0.002980	0.234539
Pyrrolidone carboxylic acid	8.09E-04	21.656720	0.089582	0.010374	0.918799
Innate immunity	8.97E-04	7.952779	0.098838	0.010353	1.018331

Top 10 terms as indicated in the column titles: BP, biological process; CC, cellular component; MF, molecular function; UP, UniProtKB keywords.

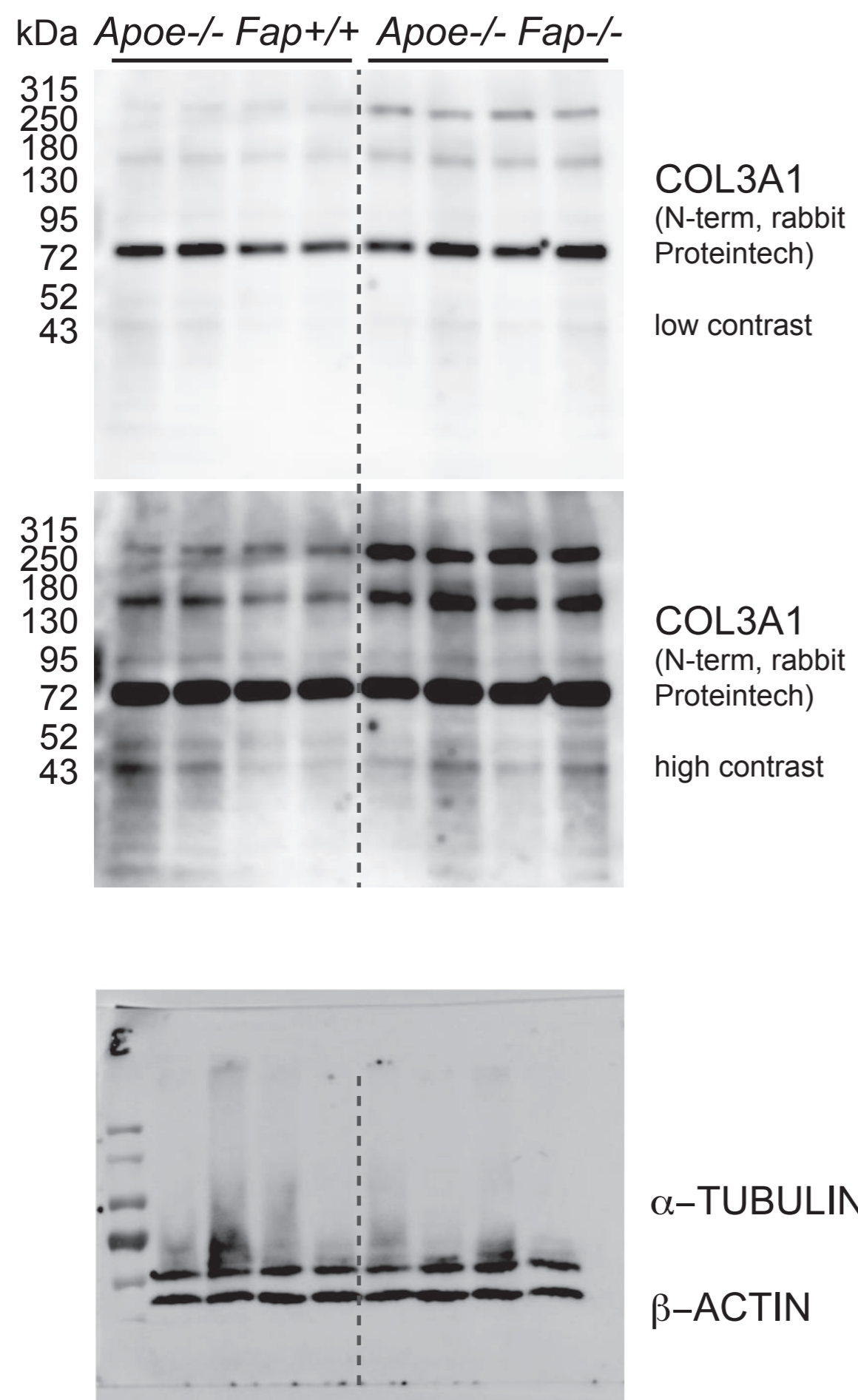
**Table S4.** Primers used for qPCR.

Transcript	Forward (5' to 3')	Reverse(5' to 3')
<i>Acta</i>	CCAGAGCAAGAGAGGGATCCT	TGTCGTCCCAGTTGGTGATG
<i>Actb</i>	TGTTACCAACTGGGACGACA	GGGGTGTGAAGGTCTCAA
<i>B2m</i>	TTCTGGTGCTTGTCTCACTG	TATGTTCCGGCTTCCCATTCT
<i>Col1a1</i>	GCACGAGTCACACCGGAACT	AAGGGAGCCACATCGATGAT
<i>Col1a2</i>	CTACTGGTGAAACCTGCATCCA	GGGCGGGCTGTATGAG
<i>Col3a1</i>	TCCTGAAGATGTCGTTGATGTG	TTTTTGCAGTGGTATGTAATGTTCTG
<i>Ctgf</i>	CACTCTGCCAGTGGAGTTCA	AAGATGTCATTGTCCCCAGG
<i>Cthrc1</i>	CGGGATGGATTCAAAGGGGA	CGTGAATGTACACTCCGCAAT
<i>Des</i>	CTCGGAAGTTGAGAGCAGAGA	GTGAAGATGGCCTTGGATGT
<i>Fn1</i>	ACTGGATGGGGTGGGAAT	GGAGTGGCACTGTCAACCTC
<i>Fn1</i>	ACTGGATGGGGTGGGAAT	GGAGTGGCACTGTCAACCTC
<i>Gfap</i>	CCTTCTGACACGGATTGGT	ACATCGAGATCGCCACCTAC
<i>Htra1</i>	GATCTTCCCTGCCCTTGGC	CTACACCAACCTGTGCCAGC
<i>Mgp</i>	AGGACTCCATGCTTTCGTGA	ACCCGAGACACCATGAAGAG
<i>Mgp</i>	AGGACTCCATGCTTTCGTGA	ACCCGAGACACCATGAAGAG
<i>Mmp13</i>	TTGCCCTGGGAAGGAGAGA	AGTCCAGCTCAACAAGAAGAAGGT
<i>Mmp14</i>	AGTCAGGGTCACCCACAAAGA	TTTGGGCTTATCTGGGACAGA
<i>Mmp2</i>	ACCACCTTAACTGTTGCTTTTG	AGGAAATGCAGTGGAGTGGA
<i>Mmp3</i>	GGAGCTAGCAGGTTATCCTAAAAGC	TAGAAATGGCAGCATCGATCTTC
<i>Mmp7</i>	GGTGAGGACGCAGGAGTGAA	GAAGAGTGACTIONCAGACCCAGA
<i>Mmp8</i>	AAAAGGGAAGCTCAGTCTGTATACTC	AGAGGGCTGCAGAGTTAGTTACCA
<i>Mmp9</i>	GGACGACGTGGGCTACGT	CACGGTTGAAGCAAAGAAGGA
<i>Npc2</i>	TTGTGCAGCTGACAGGGAT	CCCCTGCACTTCAAGGACT
<i>Npc2</i>	TTGTGCAGCTGACAGGGAT	CCCCTGCACTTCAAGGACT
<i>Ppib</i>	CAGGGGAGATGGCACAGGAG	CGGCTGTCTGTCTTGGTGCTCTCC
<i>Reln</i>	ACATGAGAGGCCACCACACT	CTTCTCAGAGCATTGGAGGC
<i>Serpine1</i>	TTGTCCAGCGGGACCTAGAG	AAGTCCACCTGTTTCACCATAGTCT
<i>Tgfb1</i>	CAACCCAGGTCTTCCCTAAA	GGAGAGCCCTGGATACCAAC
<i>Tgfb1</i>	CAACCCAGGTCTTCCCTAAA	GGAGAGCCCTGGATACCAAC
<i>Thbs2</i>	AGTGCACAGCTACAGCCTGA	ACAGAGTACTGGCGTCGGTC
<i>Thbs2</i>	AGTGCACAGCTACAGCCTGA	ACAGAGTACTGGCGTCGGTC
<i>Timp1</i>	CCTTCGCATGGACATTATTCTC	TCTCTAGGAGCCCCGATCTG
<i>Vim</i>	GGATTCCACTTCCGTTCAA	GAAATTCAGGAGGAGATGC

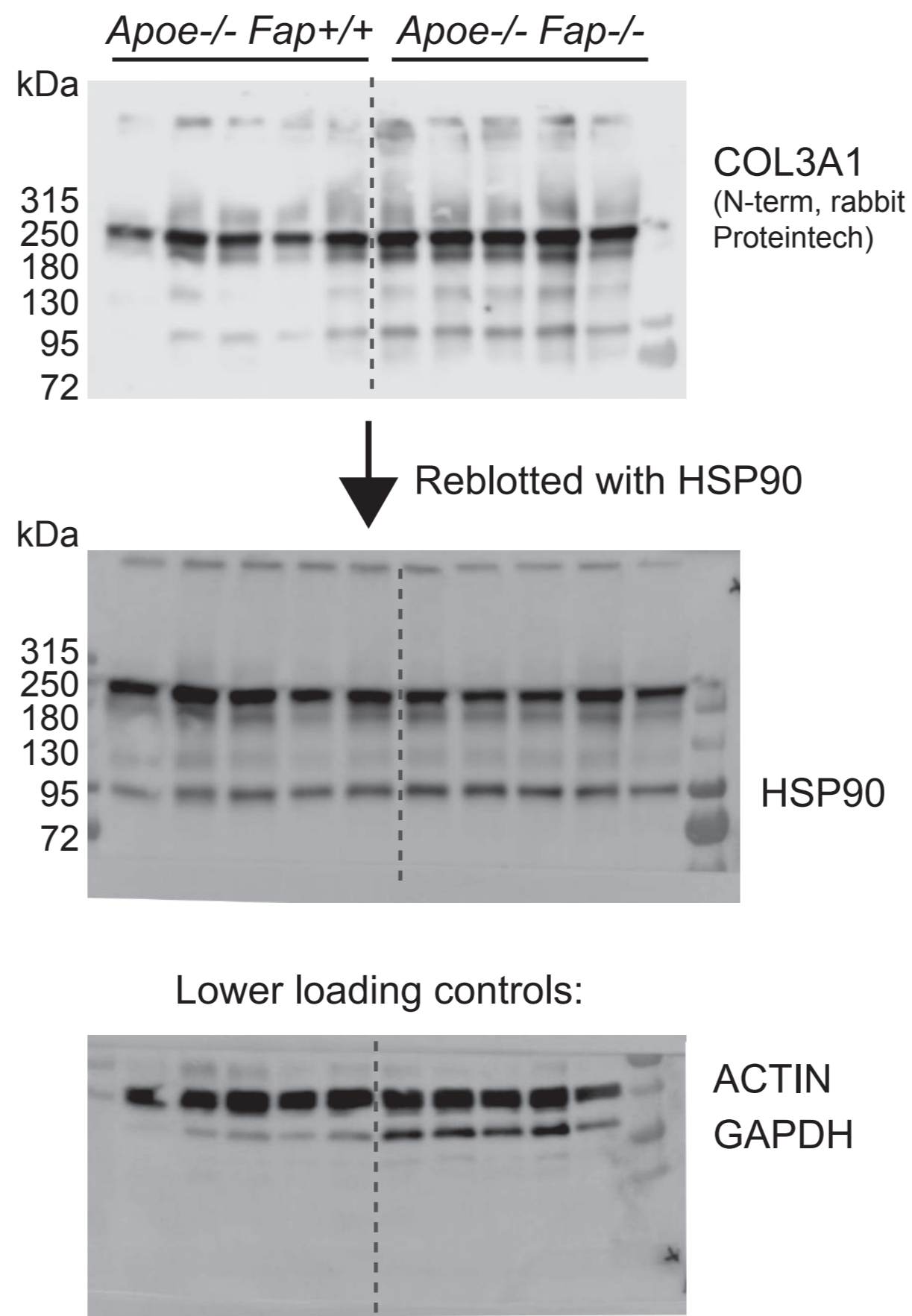
## Supplementary References

1. Gu W, Ni Z, Tan YQ, Deng J, Zhang SJ, Lv ZC, Wang XJ, Chen T, Zhang Z, Hu Y, Jing ZC, Xu Q. Adventitial Cell Atlas of wt (Wild Type) and ApoE (Apolipoprotein E)-Deficient Mice Defined by Single-Cell RNA Sequencing. *Arterioscler Thromb Vasc Biol* 2019;**39**:1055-1071.

**Figure 4E**



**Figure S7A**



**Figure S7C & E**

