Loss of One Copy of Zfp148 Reduces Lesional Macrophage Proliferation and Atherosclerosis in Mice by Activating p53

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Rationale: Cell proliferation and cell cycle control mechanisms are thought to play central roles in the pathogenesis of atherosclerosis. The transcription factor zinc finger protein 148 (Zfp148) was shown recently to maintain cell proliferation under oxidative conditions by suppressing p53, a checkpoint protein that arrests proliferation in response to various stressors. It is established that inactivation of p53 accelerates atherosclerosis, but whether increased p53 activation confers protection against the disease remains to be determined.

Objective: We aimed to test the hypothesis that Zfp148 deficiency reduces atherosclerosis by unleashing p53 activity.

Methods and Results: Mice harboring a gene-trap mutation in the Zfp148 locus (Zfp148<sup>gt/+</sup>) were bred onto the apolipoprotein E (Apoe)<sup>–/–</sup> genetic background and fed a high-fat or chow diet. Loss of 1 copy of Zfp148 markedly reduced atherosclerosis without affecting lipid metabolism. Bone marrow transplantation experiments revealed that the effector cell is of hematopoietic origin. Peritoneal macrophages and atherosclerotic lesions from Zfp148<sup>gt/+</sup>Apoe<sup>–/–</sup> mice showed increased levels of phosphorylated p53 compared with controls, and atherosclerotic lesions contained fewer proliferating macrophages. Zfp148<sup>gt/+</sup>Apoe<sup>–/–</sup> mice were further crossed with p53-null mice (T<sub>a</sub>53<sup>–/–</sup>) [the gene encoding p53]. There was no difference in atherosclerosis between Zfp148<sup>gt/+</sup>Apoe<sup>–/–</sup> mice and controls on a T<sub>a</sub>53<sup>–/–</sup> genetic background, and there was no difference in levels of phosphorylated p53 or cell proliferation.

Conclusions: Zfp148 deficiency increases p53 activity and protects against atherosclerosis by causing proliferation arrest of lesional macrophages, suggesting that drugs targeting macrophage proliferation may be useful in the treatment of atherosclerosis. (Circ Res. 2014;115:781-789.)

Key Words: atherosclerosis • cell proliferation • genes, p53 • macrophages • mice, transgenic • Zfp148 protein, mouse

Atherosclerosis is a chronic inflammatory disease driven by hyperlipidemia. Consequently, drug therapies in clinical use or in late-stage clinical trials target inflammation or lipid metabolism. However, despite the success of cholesterol-lowering drugs, disease progression is seen in many patients, emphasizing the need for alternative therapies that target other pathways.1

Recent findings implicate cell cycle control mechanisms in the pathogenesis of atherosclerosis. Genetic risk variants for atherosclerosis cluster at chromosome 9p21 in proximity of the cyclin-dependent kinase inhibitor 2a (Cdkn2a) gene, which encodes p16<sup>ink4a</sup> and p14<sup>ARF</sup>.2-4 Studies in mice confirm that inactivation of Cdkn2a in bone marrow–derived cells accelerates atherosclerosis in the low-density lipoprotein receptor (L<sub>d</sub>lr)<sup>–/–</sup> model, and that selective targeting of p14<sup>ARF</sup>, the mouse homolog of p19<sup>ARF</sup>, accelerates atherosclerosis in the apolipoprotein E (Apoe)<sup>–/–</sup> model.5,6 The main function of p19<sup>ARF</sup> is to activate p53, a checkpoint protein that induces cell cycle arrest or apoptosis in response to various stressors, suggesting that p53 protects against atherosclerosis. Consistent with this argument, knockout of p53 in the Apoe<sup>–/–</sup> model,7,8 or knockout of p53 in bone marrow–derived cells in the Apoe<sup>–/–</sup>, Apoe<sup>–/–</sup>:Leiden, or L<sub>d</sub>lr<sup>–/–</sup> models,9-10 leads to increased levels of atherosclerosis. However, whether increased p53 activation would reduce the levels of atherosclerosis remains to be determined.

Zinc finger protein 148 (Zfp148, ZBP-89, BFCOL, BERF1, h<sub>lf</sub>) is a Krüppel type transcription factor that binds to GC-rich DNA sequences and thereby activates or represses transcription of target genes.11-18 Zfp148 interacts physically with p53<sup>19</sup> and the significance of this interaction was recently discovered.10,18 Mice lacking both copies of Zfp148 exhibit...
respiratory distress because of arrested cell proliferation in the prenatal lung and half of the mice die shortly after birth. Deletion of 1 or 2 copies of Trp53 (the gene encoding p53) restored cell proliferation and rescued Zfp148-deficient mice from neonatal lethality. In cultured cells, Zfp148 deficiency lowered the threshold for p53 activation under oxidative conditions. Mice lacking 1 copy of Zfp148 have no apparent phenotypes. The role of Zfp148 and its impact on p53 activity in the pathogenesis of atherosclerosis are unknown.

In this study, we hypothesized that Zfp148 deficiency would increase p53 activation and reduce atherosclerosis development in mice. To test this we bred Zfp148<sup>−/−</sup> mice with Apoe<sup>−/−</sup> mice and studied atherosclerosis development in mice fed high-fat or normal diets.

### Methods

#### Mice

Zfp148<sup>−/−</sup> mice<sup>20</sup> and wild-type littermates were backcrossed >10 generations and maintained on a pure Apoe<sup>−/−</sup> C57Bl/6 N genetic background (Taconic, Denmark) and subsequently crossed with Trp53<sup>+/+</sup> mice (JAX Mice). Polymerase chain reaction primers used for genotyping are described in Online Table I. The mice were housed in individually ventilated cages with 12 hours light cycle and access to food and water ad libitum. All animal experiments were approved by the animal research ethics committee in Gothenburg, Sweden.

#### Diet

Mice were fed a high-fat (Harlan TD-88137 42% fat 0.2% cholesterol) or a normal chow diet (Harlan 2016 Teklad Global 16% Protein Rodent Diet).

#### Analysis of Aortas

Whole aortas were dissected, fixed in 4% formaldehyde, pinned out, and stained with Sudan IV as described.<sup>21</sup> Aortic roots were frozen in optimal cutting temperature freezing medium, sectioned and stained with Oil Red O. Total amount of triglyceride-rich particles, 50 μg/mL minimally oxidized LDL, or 50 μg/mL acetylated LDL and stained with Oil Red O. Total amount of Oil Red O-stained lesions were quantified from images using the photomerge function (Adobe) and image analyses were done with BioPix iQ software (version 2.1.8., BioPix).

#### Blood Analysis

Cholesterol and triglycerides were measured on a Konelab 20 autoanalyzer (Thermo Scientific; Vantaa, Finland). Lipoprotein fractions were determined by size exclusion chromatography using 1 Superdex 200 column (24 mL) and 1 Superose 6HR 10/30 column (24 mL) coupled in series on an ÄKTA Explorer chromatography system (Amersham Pharmacia Biotech, Uppsala, Sweden). Plasma cytokines were analyzed with a SECTOR imager 2400 reader (Meso Scale Discovery, Gaithersburg, MD). Peripheral blood was transferred to EDTA-coated Microvette tubes (Sarstedt) for total white blood cell counts and analyzed on a Hemato analyzer KX21N (Sysmex).

#### Macrophage Isolation

Peritoneal macrophages were isolated by flushing the peritoneum with PBS and were then cultured overnight as described.<sup>23</sup> Lesional macrophages were isolated as described.<sup>23,24</sup> Briefly, aortas were perfused with PBS, cut up longitudinally, pinned with intimal area facing upward. A thin layer of digestion mixture containing 450 U/mL collagenase I, 125 U/mL collagenase XI, 60 U/mL DNase I and 60 U/mL hyaluronidase (Sigma-Aldrich) was applied for 15 minutes in room temperature on top of lesions. After the incubation, lesions were scraped off under media with a glass cover slip and further digested for 40 minutes at 37°C to a single-cell suspension with the enzymatic digestion mixture described above.

#### Real-Time Reverse Transcription Polymerase Chain Reaction

TaqMan assays were performed as described<sup>25</sup> using TaqMan universal polymerase chain reaction mastermix (Applied Biosystems) and the predesigned TaqMan assays (Applied Biosystems) listed in Online Table I.

#### Isolation and Modification of Triglycerides and Low-Density Lipoprotein Particles

Triglyceride-rich particles were isolated from plasma obtained from Apoe<sup>−/−</sup> mice fed a high-fat diet for 12 weeks as described. Low-density lipoprotein (LDL) and acetylated LDL were purchased from Kalen biomedicals.

Minimally oxidized LDL was prepared by incubating LDL at 4°C for 3 months, after which thiobarbituric acid–reactive substances were determined (<3.7 nmol thiobarbituric acid–reactive substance/mg protein) as described.<sup>27</sup>

#### Accumulation of Lipids in Macrophages

Peritoneal macrophages were cultured for 24 hours in the presence of triglyceride-rich particles, 50 μg/mL minimally oxidized LDL, or 50 μg/mL acetylated LDL and stained with Oil Red O. Total amount of Oil Red O–stained lipids were quantified with white blood cell counts 3 days after irradiation. Two weeks after transplantation the mice were switched to a high-fat diet for 12 weeks and analyzed for atherosclerosis at 22 weeks of age.

#### Bone Marrow Transplantation

Bone marrow cells were harvested from femur and tibia of donor mice. One week before and 2 weeks after transplantation, recipient mice were given acidified water with 100 mg/L neomycin and 10 mg/L polymyxin B sulfate. Eight-week-old recipient mice were irradiated with 9.25GY and reconstituted with 10<sup>5</sup> unfractionated donor cells in 200 μL RPMI-1640 as described.<sup>28</sup> Irradiation efficacy was verified with white blood cell counts 3 days after irradiation. Two weeks after transplantation the mice were switched to a high-fat diet for 12 weeks and analyzed for atherosclerosis at 22 weeks of age.

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>Apoe</td>
<td>apolipoprotein E</td>
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<tr>
<td>Cdkn2a</td>
<td>cyclin-dependent kinase inhibitor 2a</td>
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<td>IL</td>
<td>interleukin</td>
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<td>Ldr</td>
<td>low-density lipoprotein receptor</td>
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<td>p53</td>
<td>tumor protein p53</td>
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<td>Trp53</td>
<td>gene encoding mouse p53</td>
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<td>Zfp148</td>
<td>zinc finger protein 148</td>
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**Blood Analysis**

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Fluorescence-Activated Cell Sorter Analysis
Peripheral blood, spleen, and bone marrow were isolated from bone marrow–transplanted mice on a high-fat diet at 22 weeks of age or from nontransplanted mice on a Chow diet at 46 weeks of age, and single-cell suspensions were prepared in PBS with 2 mmol/L EDTA. Samples were treated with Red Cell Lysis (Sigma) according to the manufacturer’s recommendation, to reduce the erythrocyte population. Atherosclerotic lesions were isolated from mice put on high-fat diet for 12 weeks. Isolated cells (5×10⁶ cells/sample) were washed once in PBS with 0.1% bovine serum albumin, blocked with Fc-block (CD16/CD32; Becton Dickinson Biosciences) at a 1:100 dilution and incubated on ice in the presence of specific monoclonal antibodies for 20 minutes in the dark. Labeled antibodies against the following cell surface markers were purchased from Becton Dickinson Biosciences; CD4, CD8α, B220, Gr1, CD45, CD11b/Mac1, c-kit, Sca-1, Thy1/CD90, CD115, Ly6c, F4/80, lineage marker cocktail for bone marrow cells (Lin including B220, CD4, CD8α, Ter119, Gr1, CD11b/Mac1) and lineage marker cocktail for lesional macrophages (Lin including Thy1/CD90, B220, CD49b, NK1.1, Gr1, Ter119, CD11c). For detection of 5-bromo-2-deoxyuridine (BrDU)-positive cells, BrdU Flow Kit (BD Biosciences) was used according to the manufacturer’s instructions in mice pulsed 2 hours with BrdU. 7-Aminoactinomycin D was used as a viability stain. Expression of cell surface markers was detected on a FACS Aria or an Accuri C6 flow cytometer (Becton Dickinson Biosciences) and data were analyzed using BD FACS Diva or CFlow Plus Analysis (Becton Dickinson Biosciences) software.

Western Blotting
Total protein extracts from macrophages were analyzed as before⁵⁰ using antibodies recognizing total p53 (fl393, Santa Cruz Biotechnology), phospho-p53 (ser15, 9284, Cell Signaling Technology), γH2AX (05–636, Millipore), and β-actin (20672–1-IR, Cell signaling Technology). Secondary antibodies were anti-mouse IRDye 680RD (926–68072, Li-Cor) and antirabbit 680RD (926–68071, Li-Cor). Protein bands were detected on a Li-Cor Odyssey Imager with Odyssey Software (version 3.0, Li-Cor).

Apoptosis
Apoptosis was evaluated with terminal deoxynucleotidyl transferase dUTP nick end labeling using the ApopTag Fluorescence In Situ Apoptosis Detection Kit (Millipore) or with antibodies recognizing cleaved caspase-3 as described before.⁵¹

Carotid Atherosclerotic Plaques
Samples of symptomatic carotid atherosclerotic plaques were obtained from the Göteborg Atheroma Study Group biobank of patients undergoing carotid endarterectomy at the Sahlgrenska University Hospital (Gothenburg, Sweden)⁵² The study was approved by the regional ethics committee, and all subjects gave informed consent to participate.

Statistics
Values are means±SEM. Statistics were performed with 2-tailed Student t test for comparisons between 2 groups: 1-way ANOVA with Tukey’s post hoc test for multiple groups and 2-way ANOVA with Bonferroni correction for multiple groups and genotypes. Statistics for plasma cytokines were performed with nonparametric Mann–Whitney test. Differences between groups were considered significant when P<0.05.

Results
To define the importance of Zfp148 in atherogenesis, Zfp148gt/+ mice were bred onto an Apoec– genetic background (from here on designated Zfp148gt/+). Attempts to generate Zfp148gt/gt mice failed, suggesting that the Zfp148gt/+ genotype is lethal on this background (data not shown). The study was therefore done on Zfp148gt/+ mice. Zfp148gt/+ and Zfp148gt/+ littermate controls were fed a high-fat diet from 8 weeks of age and euthanized at 20 weeks of age. En face analysis of Sudan IV–stained aortas showed markedly smaller lesion areas in Zfp148gt/+ mice compared with Zfp148gt/+ controls (Figure 1A). Similarly, lesion areas were smaller on sections from the aortic root of Zfp148gt/+ mice (Figure 1B; Online Figure I). There was no difference in cellular composition of lesions from Zfp148gt/+ mice or controls, as judged by staining for biomarkers of smooth muscle cells (α-smooth muscle actin), macrophages (Mac2), or T cells (CD3; Figure 1C–1E). And there was no difference in necrotic area per lesion area (Figure 1F). To test whether the extent and length of hyperlipidemia influence the effect of Zfp148 deficiency, a second cohort of mice was fed a Chow diet for 50 weeks. In line with our first experiment, lesion areas were significantly smaller in Zfp148gt/+ mice compared with Zfp148gt/+ controls (Figure 2A and 2B). We conclude that loss of a single copy of Zfp148 reduces atherogenesis without affecting the cellular composition of the plaques and irrespective of the type of diet. All experiments from hereon were performed on mice fed a high-fat diet, unless otherwise indicated.

We next investigated the effect of Zfp148 deficiency on systemic inflammation. Quantification of inflammatory markers in plasma at 20 weeks of age showed lower levels of the proinflammatory cytokines interferon-γ and interleukin (IL)-12 and lower levels of the anti-inflammatory cytokine IL-10 in Zfp148gt/+ mice compared with controls (Online Figure II). To investigate the possibility that Zfp148 is directly involved in the regulation of inflammatory cytokines, peritoneal macrophages from Zfp148gt/+ mice and controls were investigated for cytokine expression after stimulation with triglyceride-rich lipoproteins. There were no differences in mRNA levels for chemokine (C-X-C motif) ligand 1, chemokine (C-X-C motif) ligand 2, IL-10, IL-1β, IL-6, or tumor necrosis factor-α between Zfp148gt/+ macrophages and Zfp148gt/+ controls, whereas the levels of chemokine (C-C motif) ligand 10 were increased (P<0.05) and the levels of chemokine (C-C motif) ligand 2 were decreased (P<0.05) (Figure 3B). Although decreased chemokine (C-C motif) ligand 2 expression could reduce atherosclerosis, there was no difference in protein concentration of chemokine (C-C motif) ligand 2, or any of the other tested cytokines, between media conditioned by Zfp148gt/+ macrophages and controls after stimulation with triglyceride-rich lipoproteins, minimally modified LDL, or acetylated LDL (Online Figure II). Thus, smaller lesion areas in Zfp148gt/+ mice are not likely caused by mitigated inflammation.

Hyperlipidemia and the accumulation of cholesterol in macrophages play a central role in the pathogenesis of atherosclerosis. We therefore investigated our mice for signs of disturbed lipid metabolism. Body weight, plasma triglycerides, and plasma cholesterol levels were similar in Zfp148gt/+ mice compared with controls (Online Figure IIIA and IIIB). Furthermore, there was no difference in plasma lipoprotein fractions of very low-density lipoprotein, LDL, intermediate-density lipoprotein, or high-density lipoprotein between Zfp148gt/+ mice and Zfp148gt/+ controls, as judged by size exclusion chromatography (Online Figure IIIB). To determine whether the smaller lesion area in Zfp148gt/+ mice could be explained by reduced accumulation of lipids in macrophages, we...
measured Oil red O–stained lipids in peritoneal macrophages after 24-hour incubation with triglyceride-rich lipoproteins, minimally modified LDL, or acetylated LDL. There was no difference in the amount of stained lipids between cells from Zfp148gt/+ mice and controls (Online Figure IID–IIIF). There was further no difference in mRNA levels for genes coupled to lipid uptake or export (Online Figure IIIG). Thus, Zfp148gt/+ mice exhibited reduced atherosclerosis without evidence for effects on lipid metabolism.

To define the effector cell that suppresses atherosclerosis in Zfp148gt/+ mice, we performed bone marrow transplants to lethally irradiated recipients at 8 weeks of age. After 2-week recovery, the mice were put on a high-fat diet for 12 weeks. Zfp148+/+ mice and Zfp148gt/+ mice that received Zfp148gt/+ bone marrow exhibited markedly smaller lesion areas compared with mice transplanted with Zfp148+/+ bone marrow (Figure 4A). In contrast, there was no difference between Zfp148gt/+ mice and Zfp148+/+ mice that were both transplanted with Zfp148gt/+ bone marrow. Thus, the effector cell is of hematopoietic origin.

T cells, B cells, and monocytes are important for the formation of atherosclerosis. Because Zfp148 has been implicated in hematopoiesis, development of those lineages could be negatively affected in Zfp148gt/+ mice. To test this possibility, we performed fluorescence-activated cell sorter analysis of peripheral blood, bone marrow, and spleens from Zfp148+/+ mice transplanted with Zfp148gt/+ or Zfp148+/+ bone marrow and from nontransplanted Zfp148gt/+ mice and controls. There was no difference in circulating T cells, B cells, neutrophils, monocytes, or total white blood cells between mice transplanted with Zfp148gt/+ or Zfp148+/+ bone marrow and from nontransplanted Zfp148gt/+ mice and controls. There was no difference in circulating T cells, B cells, neutrophils, monocytes, or total white blood cells between mice transplanted with Zfp148+/+ bone marrow and from nontransplanted Zfp148+/+ mice and controls (Figure 4B and 4F; Online Figures IV–VI). Notably, levels of Ly6C low monocytes were lower in spleens from Zfp148+/+ mice transplanted with Zfp148+/+ or Zfp148gt/+ bone marrow compared with controls (Online Figure IVB). However, this difference was not recapitulated.

Figure 1. Reduced atherosclerotic lesions in aortas in Zfp148+/+ Apoe–/– mice fed a high-fat diet. A, Quantification of subendothelial lipid accumulation in aorta as percentage of Sudan IV–stained area per total area (n=23 Zfp148+/+Apoe–/–, 19 Zfp148gt/+Apoe–/–). Photos show representative en face preparations of Sudan IV–stained aortas. B, Quantification of subendothelial lipid accumulation in aortic roots stained with Oil Red O (n=23 Zfp148+/+Apoe–/–, 16 Zfp148gt/+Apoe–/–). Photomicrographs show representative Oil Red O staining. C to E, Analysis of plaque composition: sections from aortic sinuses were stained with antibodies against α-smooth muscle actin (αSMA; C, smooth muscle cells), Mac2 (D, macrophages), or CD3 (E, T cells; n=16). Photomicrographs show representative stainings. F, Quantification of necrotic area in lesions (n=16). Photomicrographs show hematoxylin–eosin stainings. *Necrotic area. Scale bars, 100 μm; ****P<0.0001, **P<0.01. a.u. indicates arbitrary unit.

Figure 2. Reduced atherosclerotic lesions in 50-week-old Zfp148gt/+–Apoe–/– mice fed a chow diet. A, Quantification of subendothelial lipid accumulation in aorta (n=15). Photos show representative en face preparations of Sudan IV–stained aortas. B, Quantification of subendothelial lipid accumulation in aortic roots (n=15). Photomicrographs show representative Oil Red O stainings. Scale bars, 100 μm; **P<0.01.
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in spleens from nontransplanted Zfp148<sup>gt/+</sup> mice and controls (Online Figure V). And there was no difference in bone marrow progenitor cells (lineage-negative or Lin<sup>-</sup>Sca1<sup>-</sup>cKit<sup>+</sup>; Online Figures IV and V). The results are consistent with our earlier data showing that Zfp148 is dispensable for hematopoietic development in mice. In conclusion, reduced atherosclerosis in Zfp148<sup>gt/+</sup> mice is not caused by intrinsic defects in hematopoietic development.

Zfp148 interacts physically with the check point protein p53, and inactivation of both copies of Zfp148 leads to p53-dependent cell proliferation arrest and lethality in new-born mice. We therefore hypothesized that Zfp148 haploinsufficiency could reduce atherosclerosis by unleashing p53 activity. Consistent with this idea, peritoneal macrophages from Zfp148<sup>gt/+</sup> mice contained more phosphorylated p53 after stimulation with minimally modified LDL, compared with controls (Figure 5A). There was, however, no difference in total amount of p53, suggesting that Zfp148 deficiency accentuates p53 activation without affecting p53 stabilization. Moreover, levels of γH2AX were similar in Zfp148<sup>gt/+</sup> macrophages and controls arguing against differences in DNA damage. To assess whether Zfp148 deficiency affects p53 activity in vivo, we measured phosphorylated p53 at the level of the aortic root. Atherosclerotic plaques from Zfp148<sup>gt/+</sup> mice contained more cells expressing phosphorylated p53 compared with controls, in line with the in vitro data (Figure 5B).

We next investigated whether cell proliferation or apoptosis was affected. Aortic roots from Zfp148<sup>gt/+</sup> mice contained fewer Ki67-positive cells per lesion area compared with controls (Figure 5C). There was no difference in Ki67-positive cells in the aortic media/adventitia (Online Figure VIIA), indicating that cell proliferation is reduced in lesions, but not in normal arterial walls. Moreover, the number of apoptotic cells per lesion area was markedly increased in Zfp148<sup>gt/+</sup> mice compared with controls (Figure 5D; Online Figure VIIB). To define the identity of the proliferating cells, atherosclerotic lesions from the whole aorta were isolated after a 2-hour BrdU pulse and analyzed by fluorescence-activated cell sorter. Lesions from Zfp148<sup>gt/+</sup> mice showed a 50% reduction in BrdU-positive Lin<sup>-</sup>CD11b<sup>+</sup>F4/80<sup>+</sup> macrophages compared with controls (Figure 5E). Because monocytes that proliferate in the bone marrow do not enter the circulation during a 2-hour BrdU pulse, the data indicate proliferation of tissue macrophages in lesions. There was no difference in the percentage of BrdU-positive CD45<sup>+</sup>Thy1<sup>+</sup> cells, which mainly

Figure 3. Lower systemic inflammation in Zfp148<sup>gt/+</sup>–Apoe<sup>−/−</sup> mice. A, Plasma concentrations of the indicated cytokines in 20-week-old mice fed a high-fat diet (n=23 Zfp148<sup>−/−</sup>–Apoe<sup>−/−</sup>, 19 Zfp148<sup>gt/+</sup>–Apoe<sup>−/−</sup>). B, Real-time reverse transcription polymerase chain reaction of mRNA normalized to 18S for the indicated genes in intraperitoneal macrophages after incubation with or without triglyceride-rich lipoproteins (TRL; n=4). *P<0.05.

Figure 4. Reduced atherosclerosis in Zfp148<sup>gt/+</sup>–Apoe<sup>−/−</sup> mice is mediated by cells of hematopoietic origin. A, Quantification of subendothelial lipid accumulation in aorta after bone marrow transplantation: Zfp148<sup>−/−</sup>–Apoe<sup>−/−</sup> mice were transplanted with Zfp148<sup>−/−</sup>–Apoe<sup>−/−</sup> bone marrow (n=12) or Zfp148<sup>gt/+</sup>–Apoe<sup>−/−</sup> bone marrow (n=11) and Zfp148<sup>−/−</sup>–Apoe<sup>−/−</sup> mice were transplanted with Zfp148<sup>−/−</sup>–Apoe<sup>−/−</sup> bone marrow (n=9) or Zfp148<sup>gt/+</sup>–Apoe<sup>−/−</sup> bone marrow (n=6). Photos show representative en face preparations of Sudan IV–stained aortas. B to E, Fluorescence-activated cell sorter analysis of circulating immune cells in Zfp148<sup>−/−</sup>–Apoe<sup>−/−</sup> mice transplanted with Zfp148<sup>−/−</sup>–Apoe<sup>−/−</sup> or Zfp148<sup>gt/+</sup>–Apoe<sup>−/−</sup> bone marrow (n=5). Graphs show T cells (B), B cells (C), Ly6C<sup>low</sup> (left) and Ly6C<sup>high</sup> (right) monocytes (D), and neutrophils (E) in peripheral blood (PB). F, Total white blood cells (WBCs) in PB. *P<0.05, **P<0.01.
consists of T cells, suggesting that the effect is selective for macrophages (Online Figure VIIIC).

To assess whether p53 is required for attenuating atherosclerosis in Zfp148+/- mice, we crossed the Zfp148+/- mice onto a Trp53+/– genetic background. Zfp148+/- mice developed less atherosclerosis compared with Zfp148+/+ controls on Trp53+/– background (Figure 6A and 6B), thus reproducing earlier data. Strikingly, there was no difference in lesion area between Zfp148+/- and Zfp148+/- mice on Trp53+/– background. Similarly, there was no difference in expression of phosphorylated p53 or the proliferation marker Ki67 on Trp53+/– background (Figure 6A and 6B), thus reproducing earlier data. Notably, and for reasons we do not know, the effect was greater in the whole aorta compared with the aortic root. The data did not reveal any differences in lipid metabolism or inflammation. We further show that the effector cell is of hematopoietic origin and that Zfp148 deficiency affects atherosclerosis independently of lipid metabolism. We conclude that Zfp148 deficiency confers protection against atherosclerosis by increasing p53 activation, thus reducing proliferation of lesional macrophages.

This is the first report that associates Zfp148 with atherosclerosis or cardiovascular disease. Importantly, we recorded reduced plaque burden in Zfp148+/- mice compared with controls in 4 independent experiments making this a robust observation. Notably, and for reasons we do not know, the effect was greater in the whole aorta compared with the aortic root. This proposal is supported by 4 lines of evidence. First, bone marrow transplantation showed that the effector cell is of hematopoietic origin. Second, peritoneal macrophages and atherosclerotic lesions from Zfp148+/- mice showed increased levels of phosphorylated p53 compared with controls. Third, there were fewer proliferating macrophages in atherosclerotic plaques opening up for the possibility that the mechanism we outline is clinically relevant.

### Discussion

Our results demonstrate that Zfp148 deficiency reduces atherosclerosis in the Apoe-/- model without affecting lipid metabolism. We further show that the effector cell is of hematopoietic origin and that Zfp148 deficiency confers protection against atherosclerosis by increasing p53 activation, thus reducing proliferation of lesional macrophages.

Our results demonstrate that Zfp148 deficiency reduces atherosclerosis in the Apoe-/- model without affecting lipid metabolism. We further show that the effector cell is of hematopoietic origin and that Zfp148 deficiency confers protection against atherosclerosis by increasing p53 activation, thus reducing proliferation of lesional macrophages.

One plausible mechanism behind the reduced atherosclerosis in Zfp148-deficient mice is that p53 activation attenuates proliferation of macrophages in atherosclerotic plaques. This proposal is supported by 4 lines of evidence. First, the bone marrow transplantation showed that the effector cell is of hematopoietic origin. Second, peritoneal macrophages and atherosclerotic lesions from Zfp148+/- mice showed increased levels of phosphorylated p53 compared with controls. Third, there were fewer proliferating macrophages in atherosclerotic plaques.
lesions from Zfp148gt/+ mice compared with controls. And finally, deletion of 1 copy of Trp53 abolished the effects of Zfp148 deficiency on (1) atherosclerosis development, (2) p53 activation, and (3) cell proliferation. The lack of effect in Trp53+/– mice is not proof that Zfp148 works through this mechanism, as the atheroma is complex and multiple pathways influence its progression in hierarchical but not necessarily interdependent systems. However, the genetic interaction between Zfp148 and Trp53 strongly supports a causal relationship.

Compelling evidence shows that inactivation of p53 accelerates atherosclerosis development in mice, but the mechanism downstream of p53 is not well understood. Although there is a consensus on the hematopoietic origin of the effector cell, conflicting data exist on whether inactivation of p53 increases lesional cell proliferation, decreases apoptosis, or both.4 Our results show that proliferation of lesional macrophages is critical for atherosclerosis development in Apoe–/– mice, and that p53 controls the proliferation rate.

Recent findings suggest that local proliferation of macrophages during inflammation is more important than previously thought.38,39 In the Apoe–/– model, macrophage proliferation, but not recruitment of monocytes from the blood, is critical for the accumulation of lesional macrophages.24 Proliferating macrophages/monocytes are consistently observed within human primary atherosclerotic plaques at proliferation rates that are similar to those observed in Apoe–/– mice.40–43 Thus, drugs targeting cell proliferation may confer protection against atherosclerosis.

Apoptosis have pleiotropic effects on atherosclerosis. In early stages, it limits accumulation of macrophages and slows progression of the disease.44,45 But it may also cause endothelial dysfunction that accelerates the disease.56–58 In advanced lesions, apoptosis of macrophages reduces the capacity for effecytosis, the removal of dead cells, and thus promotes necrotic core formation and plaque instability.44,45 Zfp148 deficiency increased apoptosis in lesions on the Trp53+/– genetic background without affecting atherosclerosis development, in potential conflict with the literature. However, without determining the identity of the apoptotic cells in the Zfp148gt/+ lesions, data cannot be readily compared. And we did not investigate the mice for effects on effecytosis or plaque instability.

The renewed interest in drugs targeting cell proliferation brings back the question whether increased p53 activation would reduce atherosclerosis development. Our results strongly support this notion. However, earlier attempts to validate the concept have yielded negative or inconclusive results.49,50 Increasing the p53 gene dosage had, for example, no effect on atherosclerosis development.49 Our findings suggest that endogenous Zfp148 may constitute a barrier toward genetic or therapeutic activation of p53 in atherosclerotic plaques. Such interrelation, if verified, raises the possibility that therapeutic targeting of Zfp148 could reduce atherosclerosis by unleashing p53 activity. Moreover, targeting Zfp148 may circumvent some of the anticipated adverse effects of elevated p53 activity. Activation of p53 in the endothelium, for example, leads to endothelial dysfunction and atherosclerosis,46–48 and global activation of p53 is lethal as shown by knockouts of the p53-inhibitor Mdm2.51,52 Deletion of 1 copy of Zfp148 does not seem to trigger these responses.

Figure 6. Activation of p53 reduces atherosclerosis and lesional cell proliferation in Zfp148gt/+ Apoe–/– mice. A, Quantification of subendothelial lipid accumulation in aorta as percentage of Sudan IV–stained area per total area (n=10 Zfp148+/+Apoe–/–Trp53+/+, 8 Zfp148gt/+Apoe–/–Trp53+/+, 24 Zfp148+/+Apoe–/–Trp53+/–, 22 Zfp148gt/+Apoe–/–Trp53+/–). B to E, Quantification at the level of the aortic root for subendothelial lipid accumulation (B), p-p53Ser18 positive area/lesion area (C), proliferating Ki67-positive cells/lesion area (D), and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive cells/lesion area (E; n=6 Zfp148+/+Apoe–/–Trp53+/+, 6 Zfp148gt/+Apoe–/–Trp53+/+, 19 Zfp148+/+Apoe–/–Trp53+/–, 16 Zfp148gt/+Apoe–/–Trp53+/–). Scale bars, 100 μm; *P<0.05, **P<0.01.
Acknowledgments
We thank Josefina Kjelladal and Kristina Skålen for technical assistance.

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Disclosures
None.

References


By inhibiting p53 activation, the transcription factor Zfp148 helps tissue macrophages evade proliferation arrest and accumulate in atherosclerotic plaques.


**Novelty and Significance**

**What Is Known?**

- Deletion of the tumor suppressor p53 accelerates atherosclerosis development in mouse models of atherosclerosis.
- By inhibiting p53 activation, the transcription factor Zinc finger protein 148 (Zfp148) maintains cell proliferation under oxidative conditions in mice and cultured cells.
- Local proliferation of tissue macrophages drives accumulation of inflammatory cells in atherosclerotic lesions in mice.

**What New Information Does This Article Contribute?**

- Increased p53 activation arrests proliferation of lesional macrophages and reduces atherosclerosis in apolipoprotein E-deficient mice.
- By inhibiting p53 activation, the transcription factor Zfp148 helps lesional macrophages evade proliferation arrest and accumulate in atherosclerotic plaques.
Loss of One Copy of Zfp148 Reduces Lesional Macrophage Proliferation and Atherosclerosis in Mice by Activating p53

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Supplemental Material

Loss of one copy of Zfp148 reduces lesional macrophage proliferation and atherosclerosis in mice by activating p53

Includes: Online Figure I-IX, Online Table I and Online Methods

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Online Methods

Mice

Zfp148^{+/-} mice and wildtype littermates were backcrossed more than 10 generations and maintained on a pure ApoE^{-/-} C57Bl/6 N genetic background (Taiconic, Danmark) and subsequently crossed with Trp53^tm1Tyj/^{+/-} (Trp53^{+/-}) mice (JAX Mice, USA). PCR primers used for genotyping are described in Online Table I. The mice were housed in individually ventilated cages with 12 hours light cycle and access to food and water ad libitum. All animal experiments were approved by the animal research ethics committee in Gothenburg, Sweden.

Diet

Mice were fed a high-fat (Harlan TD-88137 42% Fat 0.2% Cholesterol) or a normal chow diet (Harlan 2016 Teklad Global 16% Protein Rodent Diet).

Western blotting

Total protein extracts from macrophages where lysed in 9 M urea (U0631; Sigma-Aldrich) supplemented with complete protease inhibitor cocktail (11836170001; Roche Applied Science, Indianapolis, IN. Samples where then sonicated and cleared by centrifugation (21 000 x g for 10 min). The lysates suspended in 4X LDS sample buffer(NP0008, Life Technologies) were size-fractionated on 4–12% polyacrylamide Bis-Tris gels (Bolt; Life Technologies) and wet transferred to nitrocellulose membranes (0.22 µm; Bio-Rad Laboratories, Hercules, CA). The membranes were blocked in Blocking Buffer for Fluorescent Western Blotting (MB-070, Rockland Immunochemicals) followed by incubation with primary antibodies overnight at 4°C, washed in PBS-T (09-9410-100, Medicago AB) and then probed with secondary antibodies for 1 h at room temperature. All samples were analysed using antibodies recognizing total p53 (fl393, Santa Cruz Biotechnology); phospho-p53Ser15 (9284, Cell Signaling Technology); γH2AX (05-636, Millipore) and β-Tubulin (926-68072, Li-Cor). Secondary antibodies were anti-mouse IRDye 680RD (926-68072) and anti-rabbit 680RD (926-68071, Li-Cor). Protein bands were detected on a Li-Cor Odyssey Imager with Odyssey Software (version 3.0, Li-Cor).

Analysis of Aortas

Whole aortas were dissected, fixed in 4% formaldehyde, pinned out and stained with Sudan IV as described. Aortic roots were frozen in optimal cutting temperature freezing medium, sectioned and stained with Oil Red O as in.

Immunohistochemistry

The primary antibodies anti-αSMA (Sigma #F3777; 1:1000) and anti-Mac2 (anti-Mac2-FITC conjugate; Cedarlane; 1:1000) were used for immunohistochemistry and visualized with Vectastain ABS kit alkaline phosphatase (Vector AK-5000) and Fast Red (Vulcan Fast Red Chromogen Kit 2, Biocare Medical). Anti-CD3 (abcam ab5690; 1:1000), anti-cleaved caspase-3 (Cell Signaling Technology, 9961; 1:200) anti-Ki-67 (abcam ab16667; 1:500) and anti-p-p53 (Cell Signaling Technology, 9284; 1:500) were used for immunofluorescence and visualized with the TSA Cyanine 3 System (PerkinElmer) or goat anti-rabbit Cy3. Anti-CD68 (Novocastra Laboratories, Newcastle; 1:500) and anti-Znf148 (HPA001656, atlas antibodies, Sigma Aldrich; 1:25) were used for immunohistochemistry and visualized with Ultra vision LP detection system: HRP polymer /DAB plus Chromogen (TL-015-HD; Thermo scientific). Images were obtained with a Leica Image1 microscope (Leica Microsystems AG), a MIRAX Scan (Carl Zeiss, Germany) or the Metafer Slide Scanning Platform (MetaSystems). Images for quantification were merged with Photoshop using the photomerge function (Adobe) and image analyses were done with BioPix iQ software (version 2.1.8., BioPix).

Blood Analysis

Cholesterol and triglycerides were measured on a Konelab 20 autoanalyzer (Thermo Scientific; Vantaa, Finland). Lipoprotein fractions were determined by size exclusion chromatography using one
Superdex 200 column (24mL) and one Superose 6HR 10/30 column (24mL) coupled in series on an ÄKTA Explorer chromatography system (Amersham Pharmacia Biotech, Uppsala, Sweden). Plasma cytokines were analyzed with a SECTOR imager 2400 reader (Meso Scale Discovery, Gaithersburg, MD). Peripheral blood was transferred to EDTA-coated Microvette tubes (Sarstedt) for total white blood cell counts and analysed on a Hemato analyser KX21N (Sysmex).

**Macrophage Isolation**

Peritoneal macrophages were isolated by flushing the peritoneum with PBS and were then cultured overnight as described. Lesional macrophages were isolated as described. Briefly, aortas were perfused with PBS, cut up longitudinally, pinned with intimal area facing upward. A thin layer of digestion mixture containing 450 U ml \(^{-1}\) collagenase I, 125 U ml \(^{-1}\) collagenase XI, 60 U ml \(^{-1}\) DNase I and 60 U ml \(^{-1}\) hyaluronidase (Sigma-Aldrich) was applied for 15 min in room temperature on top of lesions. After the incubation, lesions were scraped off underlying media with a glass cover slip and further digested for 40 min in 37°C to a single cell suspension with the enzymatic digestion mixture described above.

**Real-time RT-PCR**

TaqMan assays were performed as described using TaqMan universal PCR mastermix (Applied Biosystems) and the pre-designed TaqMan assays (Applied Biosystems) listed in Online Table I.

**Isolation and Modification of triglycerides and LDL Particles**

Triglyceride-rich particles were isolated from plasma obtained from Apoe \(^{-/-}\) mice fed a high fat diet for 12 weeks as described. LDL and Acetylated LDL were purchased from Kalen biomedicals.

Minimally oxidized LDL (mmLDL) was prepared by incubating LDL at 4°C for 3 months, after which Thiobarbituric acid-reactive substances (TBARS) were determined (<3.7 nmol TBARS/mg protein) as described.

**Accumulation of lipids in macrophages**

Peritoneal macrophages were cultured for 24 hours in the presence of Triglyceride-rich particles (TRP), 50 μg/mL mmLDL or 50 μg/mL acLDL and stained with Oil Red O. Total amount of Oil Red O stained lipids were quantified from images using the BioPix iQ software (version 2.3.1., BioPix) as described.

**Bone Marrow Transplantation**

Bone marrow cells were harvested from femur and tibia of donor mice. One week before and two weeks after transplantation, recipient mice were given acidified water with 100 mg/L neomycin and 10 mg/L polymyxin B sulfate. Eight weeks old recipient mice were irradiated with 9.25 GY and reconstituted with 10 \(^{7}\) unfractionated donor cells in 200 μl RPMI-1640 as described. Irradiation efficacy was verified with white blood cell counts 3 days after irradiation. Two weeks after transplantation the mice were switched to a high-fat diet for 12 weeks and analyzed for atherosclerosis at 22 weeks of age.

**Fluorescence-Activated Cell Sorter Analysis**

Peripheral blood, spleen and bone marrow were isolated from bone marrow–transplanted mice on a high-fat diet at 22 weeks of age, or from non-transplanted mice on a chow diet at 46 weeks of age, and single-cell suspensions were prepared in PBS with 2mM EDTA. Samples were treated with Red Cell Lysis (Sigma) according to manufacturer’s recommendation, to reduce the erythrocyte population. Atherosclerotic lesions were isolated from mice put on high-fat diet for 12 weeks. Isolated cells (5 × 10 \(^{6}\) cells/sample) were washed once in PBS with 0.1% bovine serum albumin, blocked with Fc-block (CD16/CD32; Becton Dickinson Biosciences) at a 1:100 dilution and incubated on ice in the presence of specific monoclonal antibodies for 20 min in the dark. Labeled antibodies against the following cell surface markers were purchased from Becton Dickinson Biosciences; CD4, CD8a, B220, Gr1, CD45, CD11b/Mac1, c-kit, Sca-1, Thy1/CD90, CD115, Ly6c, F4/80, lineage marker cocktail for bone marrow cells (“Lin” including B220, CD4, CD8a, Ter119, Gr1, CD11b/Mac1) and lineage marker cocktail for lesional macrophages (“Lin” including Thy1/CD90, B220, CD49b, NK1.1, Gr1, Ter119,
For detection of BrdU positive cells, BrdU Flow Kit (BD Biosciences) was used according to the manufacturer’s instructions in mice pulsed 2h with BrdU. 7AAD was used as a viability stain. Expression of cell surface markers was detected on a FACS Aria or an Accuri C6 flow cytometer (Becton Dickinson Biosciences) and data were analyzed using BD FACS Diva or CFLOW Plus Analysis (Becton Dickinson Biosciences) software.

**Apoptosis**

Apoptosis was evaluated with Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using the ApopTag® Fluorescence In Situ Apoptosis Detection Kit (Millipore) or with antibodies recognizing cleaved caspase-3 as per instructions from manufacturer or described before.11

**Carotid atherosclerotic plaques**

Samples of symptomatic carotid atherosclerotic plaques were obtained from the Göteborg Atheroma Study Group biobank of patients undergoing carotid endarterectomy at the Sahlgrenska University Hospital (Gothenburg, Sweden) as described in.12 The study was approved by the regional ethics committee, and all subjects gave informed consent to participate.

**Statistics**

Values are mean ± SEM. Statistics were performed with two-tailed Student’s t-test for comparisons between two groups; one-way ANOVA with Tukey’s post-hoc test for multiple groups; two-way ANOVA for multiple groups and genotypes. Statistics for plasma cytokines were performed with non-parametric Mann Whitney test. Differences between groups were considered significant when P < 0.05.


Online Figures and Legends

Online Figure I.

Online Figure I. Reduced atherosclerotic lesions in aortas in Zfp148<sup>gt/+</sup> Apoe<sup>−/−</sup> mice.

Quantification of subendothelial lipid accumulation in aortic roots stained with Oil Red O, in absolute values (n=23 Zfp148<sup>gt/+</sup> Apoe<sup>−/−</sup>, 16 Zfp148<sup>gt/gt</sup> Apoe<sup>−/−</sup>).
Online Figure II. Cytokine concentrations in conditioned media from Zfp148<sup>gt/+</sup> Apoe<sup>−/−</sup> macrophages and controls, after stimulation with lipid particles. Concentrations of the indicated cytokines from macrophage-conditioned media after 24h incubation with triglyceride-rich lipoproteins (TRL), acetylated-LDL (acLDL), or minimally modified LDL (mmLDL) (n=4).
Online Figure III. Zfp148 deficiency reduces atherosclerosis without effects on lipid metabolism. A, Body weight curves for adult Zfp148+/+ Apoe−/− and Zfp148gt/+ Apoe−/− female mice on high-fat diet (n=13 Zfp148+/+ Apoe−/−, 12 Zfp148gt/+ Apoe−/−). B, Graphs show concentration of cholesterol (top panel) and triglycerides (bottom panel) in serum (n=23 Zfp148+/+ Apoe−/−, 19 Zfp148gt/+ Apoe−/−). C, Size exclusion chromatography profiles of triglyceride (top panel) and cholesterol (bottom panel) concentration in serum fractions pooled from 10 mice per genotype. D–F, Oil Red O positive surface area of intraperitoneal macrophages after incubation with triglyceride-rich lipoproteins (TRL) (D), (50 μg/mL) minimally modified LDL (mmLDL) (E) or (50 μg/mL) acetylated LDL (acLDL) (F) (n=5). (G) Real-time RT-PCR showing mRNA levels normalized to 18S for key regulators of lipid metabolism in intraperitoneal macrophages after incubation with TRL (n=4).
Online Figure IV.

Hematopoietic cells in tissues from Zfp148<sup>+++</sup> Apoe<sup>−/−</sup> mice and controls 14 weeks after bone marrow transplantation. A, Gating strategy for FACS data in Figure 5 and Online Figure IV and V. B–C, FACS analysis of immune cells in spleen (B) or bone marrow (D) from Zfp148<sup>+++</sup> Apoe<sup>−/−</sup> mice transplanted with Zfp148<sup>+++</sup> Apoe<sup>−/−</sup> or Zfp148<sup>gt/+</sup> Apoe<sup>−/−</sup> bone marrow (n=5) (LSK, Lin<sup>−</sup>Sca1<sup>−</sup>cKit<sup>−</sup>).
Online Figure V. Hematopoietic cells in tissues from Zfp148gt/+Apoe−/− and control mice. A–C, FACS analysis of immune cells in peripheral blood (A), spleen (B), or bone marrow (C) from Zfp148gt/+Apoe−/− or Zfp148gt/+Apoe−/− mice (n=4) (WBC, total white blood cell counts in peripheral blood; LSK, Lin−Sca1−cKit+).
Online Figure VI. Subpopulations of T-cells and monocytes in peripheral blood from Zfp148gt/+ Apoe–/– mice and controls. A, Gating strategy. B, FACS analysis of subpopulations of T-cells and monocytes in peripheral blood from Zfp148gt/+ Apoe–/– mice and controls (n=6).
Online Figure VII. Analysis of cell proliferation and apoptosis. A, Quantification of cell proliferation in the media and adventitia of the aortic wall, at the level of the aortic root ($n=16$). Photomicrographs show representative staining with antibodies against Ki67 (arrowhead indicate proliferating cell in aortic wall, lesion is within dashed line border). B, Quantification of apoptosis in aortic root lesions ($n=16$). Photomicrograph show representative cleaved caspase-3 (CC3) staining (arrowheads indicate apoptotic cells, dashed line indicates lesion border). C, FACS analysis of lesional cells. Left panels, gating strategy. Right panel, quantification of % BrdU positive CD45^+Thy1^+ cells ($n=6$). Scale bars, 100µm; **$P < 0.01$. 
Online Figure VIII. Lesions from Zfp148\textsuperscript{gt/+} Apoe\textsuperscript{−/−} mice contain more apoptotic cells compared to Zfp148\textsuperscript{gt/+} Apoe\textsuperscript{−/−} controls on Trp53\textsuperscript{+/+} and Trp53\textsuperscript{+/−} genetic backgrounds. Quantification of cleaved caspase-3 (CC3) in aortic root lesions from Zfp148\textsuperscript{+/+} Apoe\textsuperscript{−/−} Trp53\textsuperscript{+/+} (n=6), Zfp148\textsuperscript{gt/+} Apoe\textsuperscript{−/−} Trp53\textsuperscript{+/+} (n=6), Zfp148\textsuperscript{+/+} Apoe\textsuperscript{−/−} Trp53\textsuperscript{+/−} (n=19), and Zfp148\textsuperscript{gt/+} Apoe\textsuperscript{−/−} Trp53\textsuperscript{+/−} (n=16) mice.

*P < 0.05, ***P < 0.001.
Online Figure IX. Znf148 is expressed in macrophage rich areas in human carotid atherosclerotic plaques. Photomicrographs with representative antibody stainings against the macrophage marker CD68 (left panel) and Znf148 (middle and right panels) on adjacent sections from a carotid artery plaque removed by endarterectomy. Boxed area in middle panel is displayed at higher magnification in right panel. Strong Znf148 nuclear staining is indicated with arrows. Scale bars, 200µm.
### Online Table I

#### Genotyping

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#### TaqMan assays (Life Technologies)

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