SUPPLEMENTAL MATERIAL

Methods

Animals

Animal experiments were performed in accordance with the guidelines of the Canada Council on Animal Care, and with the approval of the University of Toronto Faculty of Medicine Animal Care Committee. *Ddr1*$^{+/+}$*; Ldlr*$^{-/-}$ (SKO) and *Ddr1*$^{-/-}$*; Ldlr*$^{-/-}$ (DKO) mice were generated as described previously.[1] At 6 weeks of age, male mice were fed Western-type high-fat diet (40% fat, 43% carbohydrate, 0.5% cholesterol; D05011404; Research Diets, New Brunswick, NJ) for 12 weeks to induce insulin resistance and atherosclerosis. Animals were fasted over-night (16 hours) prior to assessment of blood parameters. Blood glucose was measured by tail-vein bleed using the OneTouch® Ultra® 2 glucometer. Plasma samples were collected using K2 EDTA-coated Microvette® capillary tubes (16.444.100; Sarstedt, Numbrecht, DE). Plasma was separated by centrifugation at 5,000 rpm for 5 minutes. Plasma parameters were assayed using the Beckman AU480 Biochemistry Analyzer at the Toronto Centre for Phenogenomics. Animals were euthanized and perfusion-fixed with 4% paraformaldehyde (PFA). Aortic arch tissue was isolated and longitudinal sections were mounted onto slides by the Toronto Centre for Phenogenomics.

Immunohistochemistry

Paraffin-embedded aortic arch sections were rehydrated and stained using standard procedures. Antibodies were used at a concentration of 1:100 unless otherwise indicated. Tissues were immunolabelled with antibodies for RUNX2 (12556; Cell Signaling Technology, Danvers, MA), Mac-2 (87985; Cell Signaling Technology), Sox-9 (82630; Cell Signaling Technology), BMP-2 (ab14933; Abcam, Cambridge, UK), Osteocalcin (ab93876; Abcam), 1:200 secondary goat anti-rabbit Alexa-Fluor™ 488 IgG antibody (A11008; Thermo Fischer Scientific, Waltham, MA),and counterstained with Hoechst-33342 (4082; Cell Signaling Technology). Staining was visualized by epifluorescence microscopy using the Nikon Eclipse Ci microscope (Nikon, Tokyo, Japan). Image analysis was performed using Nikon Elements Software. To measure overall staining in plaque, a region of interest was drawn around the plaque by tracing between the internal elastic lamina (IEL) and plaque fibrous cap, followed by measuring stain intensity by applying a threshold on the green channel.

Cell Culture

Primary VSMCs were isolated from *Ddr1*$^{+/+}$ and *Ddr1*$^{-/-}$ C57BL/6 mouse carotid arteries as described in Hou *et. al.*[2] Cells were propagated in DMEM (11885084; Thermo Fischer Scientific) supplemented with 10% FBS (12483020; Thermo Fischer Scientific) and 1% penicillin-streptomycin (15140122; Thermo Fischer Scientific). Experiments were performed using cells at passage 5 – 10 For experiments relating to calcification assays and immunoblot, VSMCs were cultured for 2 or 12 days (as indicated) in normal media (DMEM with 5mM glucose (118850684; Thermo Fischer Scientific), 3% HyClone™ FBS (SH3007003HI; Thermo Fischer Scientific), 1% penicillin-streptomycin (15140122; Thermo Fischer Scientific)), or calcifying media (DMEM with 25mM glucose (11995065; Thermo Fischer Scientific), 3% HyClone™ FBS (SH3007003HI; Thermo Fischer Scientific), 1% penicillin-streptomycin (15140122; Thermo Fischer Scientific), 2.4 mM Pi).

Immunocytochemistry

VSMCs were grown on glass coverslips (CA48366; VWR, Radnor, PA) in 6-well culture plates for 2 or 12 days in normal or calcifying media. For microtubule stabilization experiments cells were treated with 10μM Taxol (T7402; Sigma-Aldrich) for 6 hours prior to staining. The cells were fixed with 4% PFA for 10 minutes, washed 3x5min in PBS, permeabilized with 0.25% Triton X-100 for 10 minutes, and blocked with 1% BSA for 1 hour. Cells were immunolabelled with antibody for α-tubulin (ab18251; Abcam) at a concentration of 1:100, and 1:200 secondary anti-mouse Alexa-Fluor™ 488 IgG (A11001; Thermo Fischer Scientific). Nuclei were stained using Hoechst-33342. Staining was visualized using the Nikon AR1 laser scanning confocal microscope (Nikon, Tokyo, Japan).

Statistical Analyses

Data were expressed as mean±SEM. Statistical methods are summarized in the Major Resources Table in the Supplemental Material. Data were analyzed using GraphPad Prism Software (La Jolla, California, USA). Normality was assessed (D'Agostino-Pearson omnibus test) followed by appropriate parametric or non-parametric statistical analyses as indicated.

References

1. Franco C, Hou G, Ahmad PJ, Fu EY, Koh L, Vogel WF, Bendeck MP. Discoidin domain receptor 1 (DDR1) deletion decreases atherosclerosis by accelerating matrix accumulation and reducing inflammation in low-density lipoprotein receptor-deficient mice. *Circ Res*. 2008;102:1202-1211
2. Hou G, Vogel WF, Bendeck MP. The discoidin domain receptor tyrosine kinase DDR1 in arterial wound repair. *J Clin Invest*. 2001;107:727-735

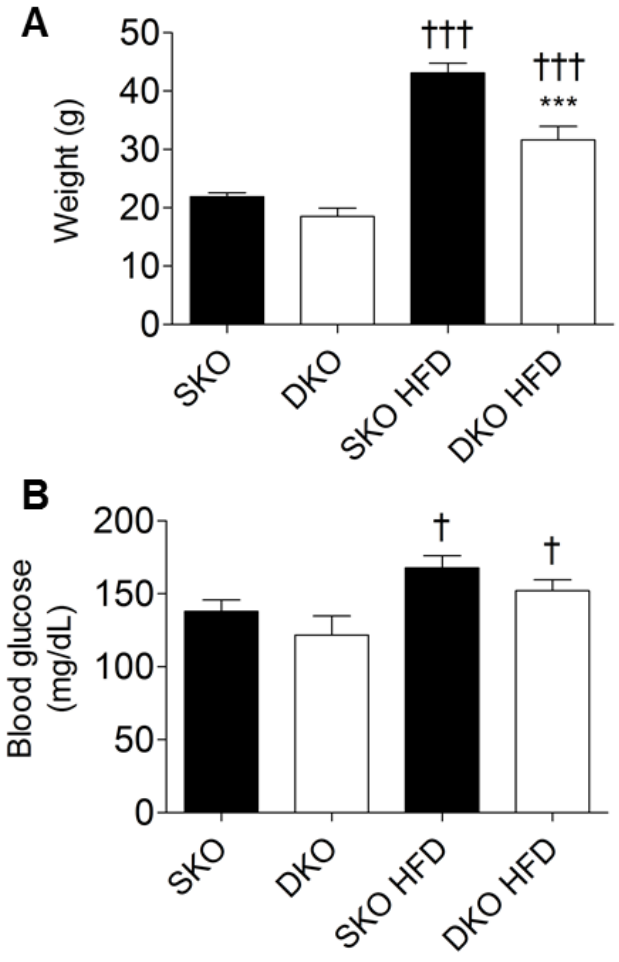

Figure SI.
Effect of high-fat feeding on body weight and plasma glucose. *Ldlr*$^{-/-}$*; Ddr1*$^{+/+}$ (SKO; n=12) and *Ldlr*$^{-/-}$*; Ddr1*$^{-/-}$ (DKO; n=9) mice were fed high-fat diet for 12 weeks. Body weight significantly increased after high fat feeding in both SKO and DKO mice, and DKO mice were smaller after HFD compared to SKO (**A**). Fasting glucose was significantly elevated after HFD-feeding in both SKO and DKO mice (**B**). **A** and **B**, 2-way ANOVA with Bonferroni post hoc test (***p<0.001 comparing SKO to DKO; †p<0.05, †††p<0.001 SKO HFD to SKO and DKO HFD to DKO respectively).

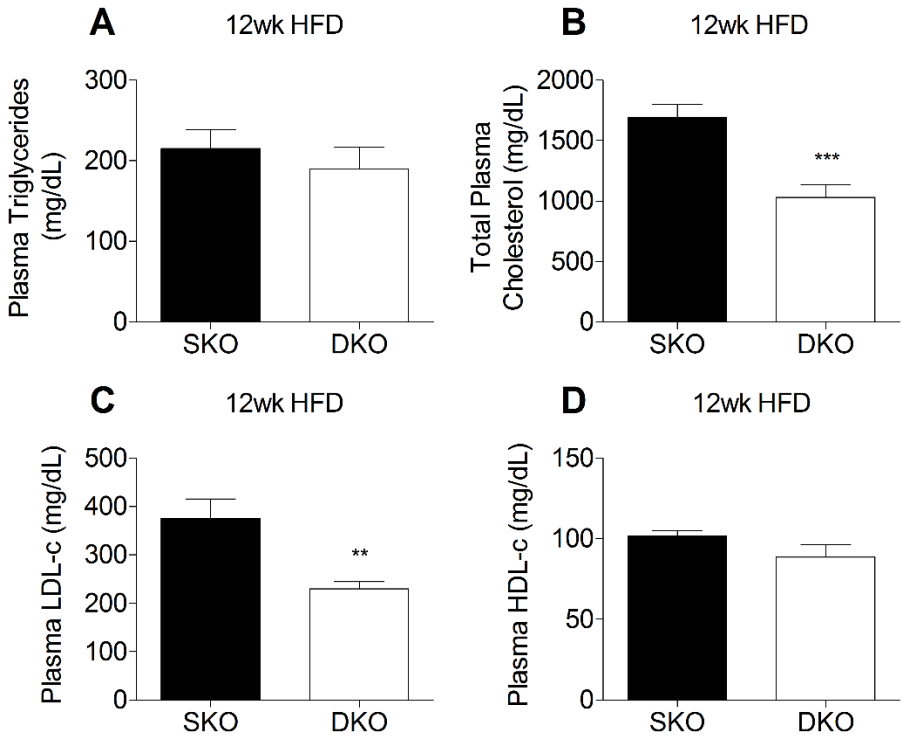

Figure SII.
Blood plasma was collected from *Ddr1*$^{+/+}$*; Ldlr*$^{-/-}$ (SKO; n=8) and *Ddr1*$^{-/-}$*; Ldlr*$^{-/-}$ (DKO; n=6) mice at the end of the 12-week HFD feeding, following an over-night fast. Plasma triglycerides (**A**), total cholesterol (**B**), LDL-cholesterol (**C**), and HDL-cholesterol (**D**) were assessed. **A**, **B**, and **D**, student's t-test; **C**, Mann-Whitney test. (**p<0.01, ***p<0.001). HDL-c denotes High Density Lipoprotein-cholesterol; LDL-c denotes Low Density Lipoprotein-cholesterol.

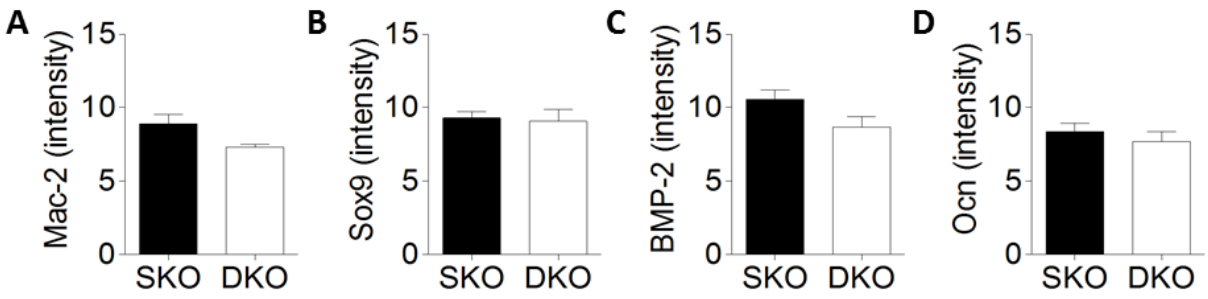

Figure SIII.
DDR1 deletion results in no significant difference in aortic Mac-2, Sox-9, BMP-2, and Ocn staining after 12 weeks on HDF. Aortic arches from *Ddr1*$^{+/+}$*; Ldlr*$^{-/-}$ (SKO, n=8) and *Ddr1*$^{-/-}$*; Ldlr*$^{-/-}$ (DKO, n=5) mice fed high-fat diet for 12 weeks, were immunostained with antibodies against Mac-2 (**A**), Sox9 (**B**), BMP-2 (**C**), and Ocn (**D**). Stain intensity quantified in plaque, revealing no significant differences between SKO and DKO mice. **A**, **C**, and **D**, student's t-test; **B**, Mann-Whitney test.

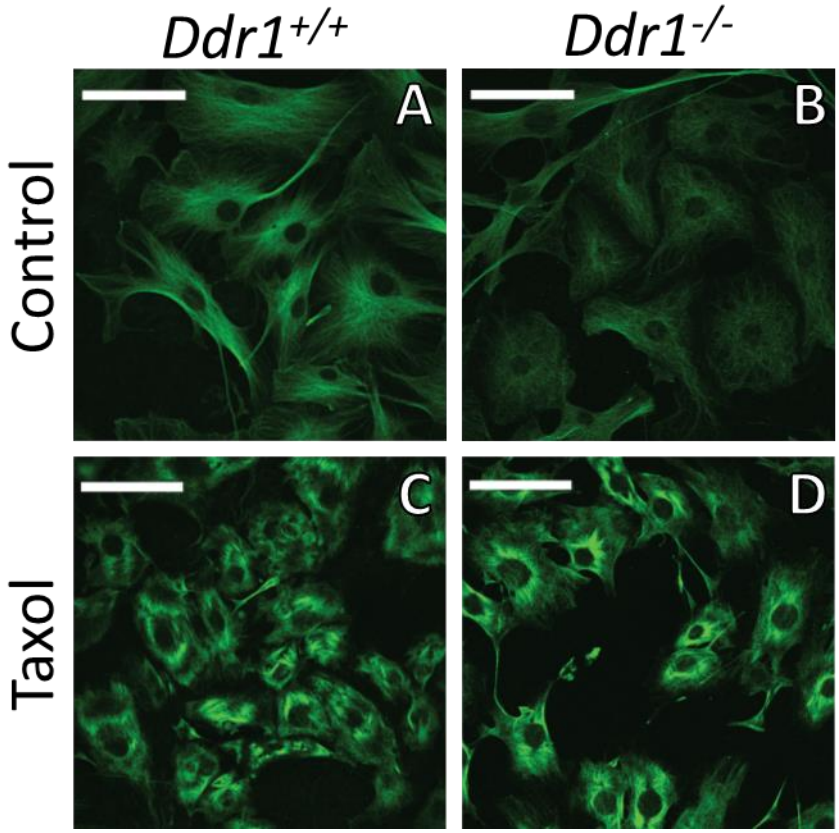

Figure SIV.
Taxol treatment restores microtubule organization in *Ddr1*$^{-/-}$ VSMCs. *Ddr1*$^{+/+}$ and *Ddr1*$^{-/-}$ VSMCs were immunostained for α-tubulin after treatment with control (**A**, **B**) and taxol (**C**, **D**). Microtubules were less organized as demonstrated by weaker, more diffuse staining for tubulin in *Ddr1*$^{-/-}$ VSMCs (**B**). Tubulin staining was enhanced following taxol treatment of *Ddr1*$^{+/+}$ (**C**), and *Ddr1*$^{-/-}$ (**D**) VSMCs. Scale bars = 200μm.