Supporting Information

Notch Ligand Delta-like 4 Blockade Attenuates

Atherosclerosis and Metabolic Disorders

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Methods and Materials

Mice and anti-Dll4 Ab treatment

Eight-week-old male $Ldlr^{-/-}$ mice, female Lep^{ob}/Lep^{ob} mice, and male Notch reporter transgenic mice

(1) were purchased from The Jackson Laboratory. $Ldlr^{-/-}$ mice were fed a high-fat, high-cholesterol diet

(D12108, Research Diets, Inc.) from 8 weeks of age through the completion of the study. Lep^{ob}/Lep^{ob}

mice and Notch reporter transgenic mice were fed normal chow. All mice were housed on a 12-hour

light/dark cycle, with food and water ad libitum. Animal care and experimentation were approved by

the Harvard Medical School Institutional Animal Care and Use Committee. Mice were treated with

well-characterized hamster derived anti-mouse Dll4 antibody (2-8). $Ldlr^{-/-}$ mice were injected with 250

μg of anti-mouse Dll4 antibody or isotype control IgG (BioXcell) intraperitoneally twice a week from 8

weeks of age (early-phase) or from 20 weeks of age (late-phase) for 12 weeks. For Lep^{ob}/Lep^{ob} mice,

the amount of Dll4 Ab and IgG was adjusted according to body weight (10 μg/g) and administered for

10 weeks. Mice were weighed twice a week. For bolus injection, we administered 250 μg of Dll4 Ab or

IgG to fat-fed 10-week-old $Ldlr^{-/-}$ mice, or Notch reporter transgenic mice, and harvested them at 6

hours after injection.

Histology and immunohistochemical analysis

For tissue harvesting, we perfused 0.9% sodium chloride solution at a constant pressure via the left

ventricle and then removed tissues immediately. Thoracic aorta, aortic root, liver, and thymus were

embedded in OCT compound (TissueTek). Epididymal fat pads and small intestines were fixed with

formalin or 4% paraformaldehyde, respectively, and embedded in paraffin. Tissues were sectioned

serially (6 μm) and stained with hematoxylin and eosin (H&E) for general morphology.

Immunohistochemistry by the avidin-biotin complex method employed anti-Mac3, anti-MCP-1 (both

from BD Biosciences), and anti-Dll4 (Rockland Immunochemicals) antibodies. Collagen was detected

by picrosirius red staining and viewed under a polarized microscope, as described previously (9).

Alkaline phosphatase (ALP) activity was detected, according to the manufacturer's instructions

(alkaline phosphatase substrate kit, Vector Laboratories). Calcium deposition in plaques was detected

by von Kossa staining, using a commercially available kit (Polysciences, Inc.). To analyze adipocyte

size, we measured the two-dimensional size of 100 adipocytes in five fields and obtained the average

value of each field. PAS staining on the small intestine used a commercially available kit

(Sigma-Aldrich). Macrophage accumulation in fat tissue (Mac3-positive cells, %) was analyzed by the

ratio of the number of Mac3-positive cells to the number of total cells (total number of nucleus). Images

were captured and processed with OPTIPHOT (Nikon) with a digital camera (DXM1200F, Nikon).

Morphometric analysis was performed using image analysis software (Image-Pro Plus 6.0, Media

Cybernetics).

Ex vivo fluorescence reflectance imaging of the aorta and aortic valve

Ldlr^{-/-} mice that received late-phase treatment were used for fluorescence reflectance imaging. Two

spectrally distinct near-infrared fluorescent agents were administered to each mouse 24 hours before

imaging — cross-linked iron oxide fluorescent iron nanoparticles for detection of macrophage

accumulation (CLIO750, 750 nm), and OsteoSense680 for detection of osteogenic activity (680 nm,

VisEn). After perfusion with 0.9% sodium chloride solution at a constant pressure via the left ventricle, we removed the heart and aorta. Signals from macrophages and osteogenic activity were detected on aortas using the Kodak Imaging Station 4000MM Pro (Kodak). Laser scanning fluorescence microscopy (FLUOVIEW1000, Olympus) was used on the opened aortic root to detect signals for osteogenic activity in the aortic valves. The target-to-background ratio was calculated as described previously (10).

Analysis of metabolic parameters

Serum insulin (Alpco), total cholesterol, and triglyceride levels (Wako) were measured with commercially available kits after 4-hour fasting. Serum MCP-1 levels (Signosis) were measured with samples obtained at the harvest. Glucose and insulin tolerance tests were performed after 16-hour and 4-hour fasting, respectively. Glucose and insulin solutions were injected into the peritoneal cavity at doses of 1.0 g/kg and 0.5 unit/kg, respectively. Blood was collected via tail vein at different time points, and glucose levels were measured with a glucometer.

Metabolic studies

The details of indirect experiments were described previously (11). Mouse physical activity was monitored with OPTO-M3 Activity Application Device (Columbus Instruments). Each animal's movements (other than scratching, grooming, digging, etc.) were determined using infrared beams in x, y, and z axes and recorded for 48 consecutive hours. Animals were placed in an indirect open circuit calorimeter (Oxymax System, Columbus Instruments). Oxygen and carbon dioxide

concentrations by volume were monitored at the inlet and outlet parts of a partially sealed chamber, through which a known flow of ambient air was forcibly ventilated. The concentration difference measured between the parts was used to compute oxygen consumption (VO_2) and carbon dioxide production (VCO_2). The consumption and production information were presented in units of ml/kg/min and normalized to 0°C and 760 mmHg. Food intake was investigated with the Oxymax Feed Scale Device (Columbus Instruments) and manual measurements.

Blood pressure measurement

Blood pressure of each conscious mouse was measured with a non-invasive blood pressure system (CODA, Kent Scientific Corporation). In each animal, the mean value of three measurements was used for comparison.

Fractionation of adipose tissue

Previous papers describe fractionation of adipose tissue (12). We minced epididymal fat pads in PBS containing 2% BSA, and then incubated it on a shaking platform for 60 min at 37 °C with the same medium containing collagenase (1250 U/ml). We then passed the mixture through a nylon filter (pore size, 250 μm) to remove undigested material, and centrifuged the filtrate for 5 min at 200g at 4 °C. We recovered floating cells and the pellet as the mature adipocyte fraction and the stromal vascular fraction (SVF), respectively.

Flow cytometry analysis

To investigate the effects of Dll4 blockade on tissue monocytes/macrophages and circulating leukocytes, we performed flow cytometry analysis (13). Samples were collected from $Ldlr^{-/-}$ mice treated with Dll4 Ab or IgG for 12 weeks. Anti-Ly-6C-FITC, anti-B220-PE, anti-CD49b-PE, anti-CD90-PE, anti-TER119-PE, anti-Ly-6G-PE, anti-NK1.1-PE, biotinylated-anti-MHC class II, anti-F4/80-PE-Cy7, anti-CD11b-APC-Cy7, and anti-CD11c-Alexa700 (BD Biosciences) were used. Strep-PerCP (BD Biosciences) was used to label biotinylated Ab. Monocytes were identified as CD11b^{high} (CD90/B220/CD49b/NK1.1/TER119/Ly-6G)^{low} (F4/80/I-Ab/CD11c)^{low} Ly-6C$^{high/low}$. Neutrophils were identified as CD11b^{high} (CD90/B220/CD49b/NK1.1/Ly-6G)^{high} (F4/80/I-Ab/CD11c)^{low} Ly-6C^{int}. Data were acquired on an LSRII (BD Biosciences) and analyzed with FlowJo v.8.5.2 (Tree Star, Inc.).

Isolation of macrophages from adipose tissue

From SVF obtained from adipose tissue, we isolated F4/80-positive macrophages using magnetic sorting (EasySep system, StemCell Technologies). SVF was obtained from $Ldlr^{-/-}$ mice treated with Dll4 Ab or IgG for 12 weeks. We used a PE-conjugated anti-F4/80 antibody as a primary Ab (Biolegend).

Cell culture

RAW264.7 cells and 3T3-L1 preadipocytes (both from American Type Culture Collection) were maintained in DMEM (Gibco) containing 10% FBS. Day 10 3T3-L1 adipocytes were used as differentiated 3T3-L1 adipocytes. To activate Dll4-mediated Notch signaling, we seeded RAW264.7

cells and 3T3-L1 adipocytes to plates coated with recombinant mouse Dll4 (immobilized rDll4) (R&D Systems, Inc.) (14) at the same cell density, and then harvested cells after 24-hour or 48-hour incubation, respectively. Culture supernatant was collected for further investigation. We used SN50 (25 μg/ml, Calbiochem) NF-κB inhibitor to determine the interaction between Dll4-mediated Notch signaling and the NF-κB pathway. As a control, we used SN50M (Calbiochem), an inactive control for SN50.

Human saphenous vein endothelial cells were maintained in M-199 (LONZA) supplemented with 10% FBS, and endothelial cell growth factor. Cells up to the seventh passage were used for this study. Cells were stimulated with human rDll4 (R&D Systems, Inc.) (1 μg/ml), and effects of anti-human Dll4 Ab or IgG (10 μg/ml) and DAPT (10 μM) or DMSO were investigated after 24-hour incubation.

Peritoneal macrophages collected by peritoneal lavage were seeded to plates at the same cell density and cultured with RPMI containing 5% FBS. At 16 hours after seeding, lipids were stained with Oil red O and percentage of positive area in macrophage was obtained.

Transient Transfection and Luciferase Assay

We transfected siRNA against mouse Dll4 (Dharmacon) and plasmid encoding mouse Dll4 (GeneCopoeia) to RAW264.7 cells and 3T3-L1 adipocytes, and RBP-Jκ reporter (SABiosciences) to 3T3-L1 adipocytes by electroporation (Nucleofector system, Amaxa), according to the manufacturer's instructions. We transfected 200 ng of siRNA or 2 μg of plasmid to cells and harvested them 48 hours or 24 hours after transfection, respectively. We used 5 μl of RBP-Jκ reporter solution for each

transfection. After incubation for 24 hours, cells were seeded to rDll4-coated plates and used for the luciferase assay after 48-hour incubation. Luciferase activity was determined using a Dual-Luciferase Reporter Assay System (Promega).

Quantitative RT-PCR

We synthesized cDNA from total RNA extracted from tissues and cells. Real-time quantitative RT-PCR was done with a MyiQ single-color real-time PCR detection system (BioRad) and PerfeCTa SYBR Green SuperMix for iQ (Quanta Biosciences). Data are expressed in arbitrary units that were normalized by β-actin.

Immunoblotting

Lysates prepared from tissues and cells in lysis buffer containing protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail (Roche) were separated with SDS-PAGE gels. We used the following antibodies: Dll4 (Rockland Immunologics), IκBα (Cell Signaling), GFP (Invitrogen), and β-actin (Sigma).

Statistics

Data are expressed as mean ± SEM for continuous variables. Comparisons between two groups were performed with the unpaired Student's t-test. Comparisons of multiple groups were made with one-way ANOVA, followed by the Student–Newman–Keuls multiple comparison test. P values of <0.05 were considered statistically significant.

References

1. Duncan AW, et al. (2005) Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. *Nat Immunol* **6**:314-322.

2. Fukushima A, et al. (2008) Notch ligand Delta-like4 inhibits the development of murine experimental allergic conjunctivitis. *Immunol Lett* **121**:140-147.

3. Kassner N, et al. (2010) Cutting Edge: Plasmacytoid Dendritic Cells Induce IL-10 Production in T Cells via the Delta-Like-4/Notch Axis. *J Immunol* **108**:550-554.

4. Koyanagi A, Sekine C, Yagita H. (2012) Expression of Notch receptors and ligands on immature and mature T cells. *Biochem Biophys Res Commun* **418**:799-805.

5. Moriyama Y, et al. (2008) Delta-like 1 is essential for the maintenance of marginal zone B cells in normal mice but not in autoimmune mice. *Int Immunol* **20**:763-773.

6. Oishi H, et al. (2010) Blockade of delta-like ligand 4 signaling inhibits both growth and angiogenesis of pancreatic cancer. *Pancreas* **39**:897-903.

7. Sekine C, et al. (2009) Differential regulation of splenic CD8- dendritic cells and marginal zone B cells by Notch ligands. *Int Immunol* **21**:295-301.

8. Yamanda S, et al. (2009) Role of ephrinB2 in nonproductive angiogenesis induced by Delta-like 4 blockade. *Blood* **113**:3631-3639.

9. Deguchi J, et al. (2005) Matrix metalloproteinase-13/collagenase-3 deletion promotes collagen accumulation and organization in mouse atherosclerotic plaques. *Circulation* **112**:2708-2715.

10. Aikawa E, et al. (2007) Multimodality molecular imaging identifies proteolytic and osteogenic activities in early aortic valve disease. *Circulation* **115**:377-386.

11. Maeda K, et al. (2005) Adipocyte/macrophage fatty acid binding proteins control integrated

metabolic responses in obesity and diabetes. *Cell Metab* **1**:107-119.

12. Hosooka T, et al. (2008) Dok1 mediates high-fat diet-induced adipocyte hypertrophy and obesity through modulation of PPAR-gamma phosphorylation. *Nat Med* **14**:188-193.

13. Swirski FK, et al. (2009) Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* **325**:612-616.

14. Williams CK, Li JL, Murga M, Harris AL, Tosato G. (2006) Up-regulation of the Notch ligand Delta-like 4 inhibits VEGF-induced endothelial cell function. *Blood* **107**:931-939.
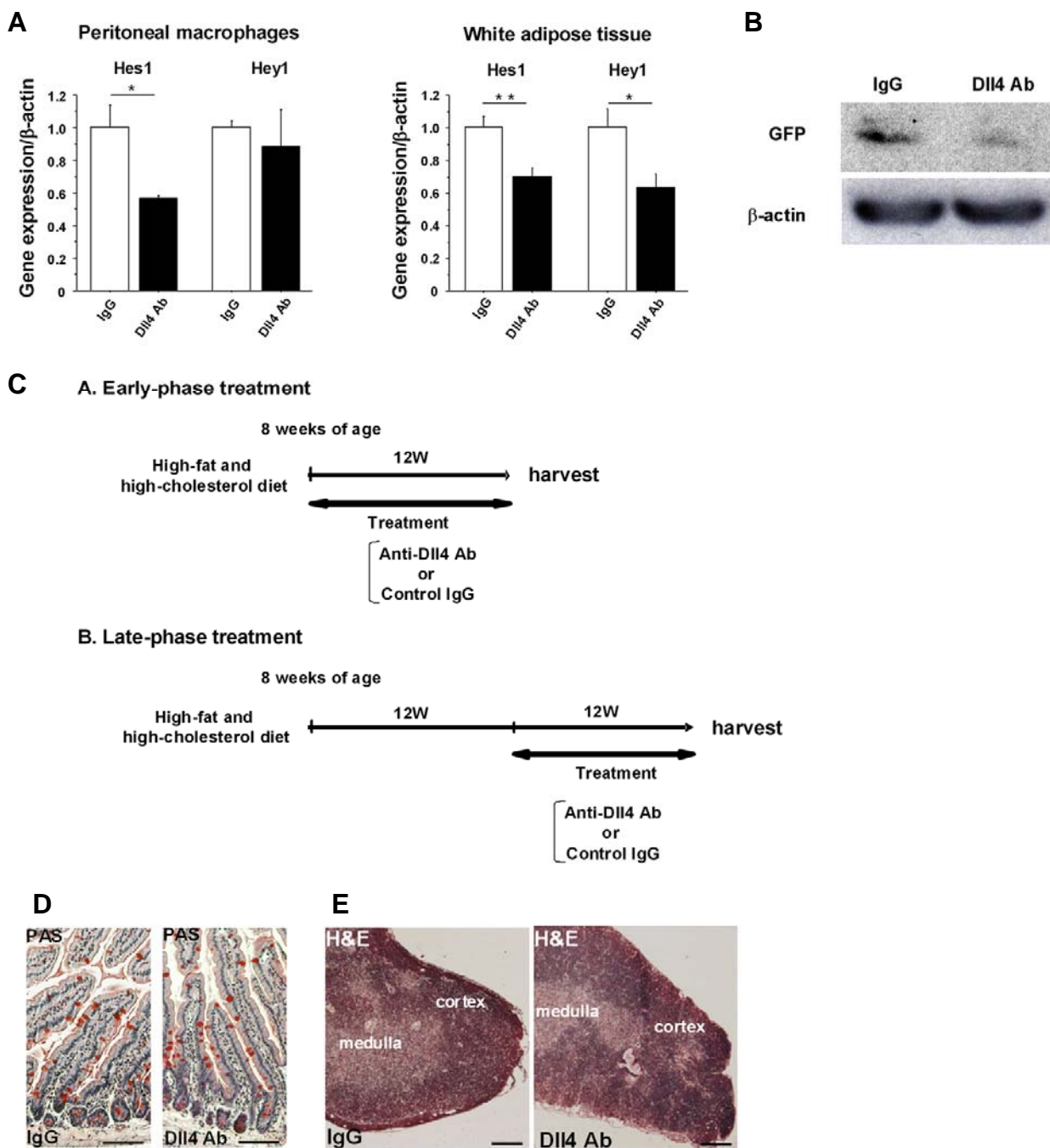
Fig. S1. Effects of anti-Dll4 Ab and study protocol.

(A) Bolus injection of Dll4 Ab decreased expression of Notch target genes in peritoneal macrophages and adipose tissues (n =4, per group). **(B)** Bolus injection of Dll4 Ab reduced GFP signal in small intestines in Notch reporter transgenic mice determined by Western blotting (representative figures, n = 3). **(C)** Study protocols (*in vivo*). We administered 250 μg of Dll4 Ab or isotype control hamster IgG by intraperitoneal injection twice a week to *Ldlr*^{-/-} mice. We initiated Ab or IgG administration at 8 weeks (early phase) or 20 weeks (late phase) of age, to examine the effects on the initiation and progression of the cardiometabolic syndrome, respectively. All mice consumed a high-fat, high-cholesterol diet from 8 weeks of age to the harvest. **(D)** PAS staining of small intestine. Dll4 Ab treatment did not increase goblet cells (red). Scale bar, 100 μm. **(E)** H&E staining of thymus. Dll4 Ab treatment did not cause atrophy of thymus. Scale bar, 200 μm. D and E, *Ldlr*^{-/-} mice received Dll4 Ab treatment for 12 weeks (n = 5). * *P*<0.05, ** *P*<0.01. Values are mean ± SEM.
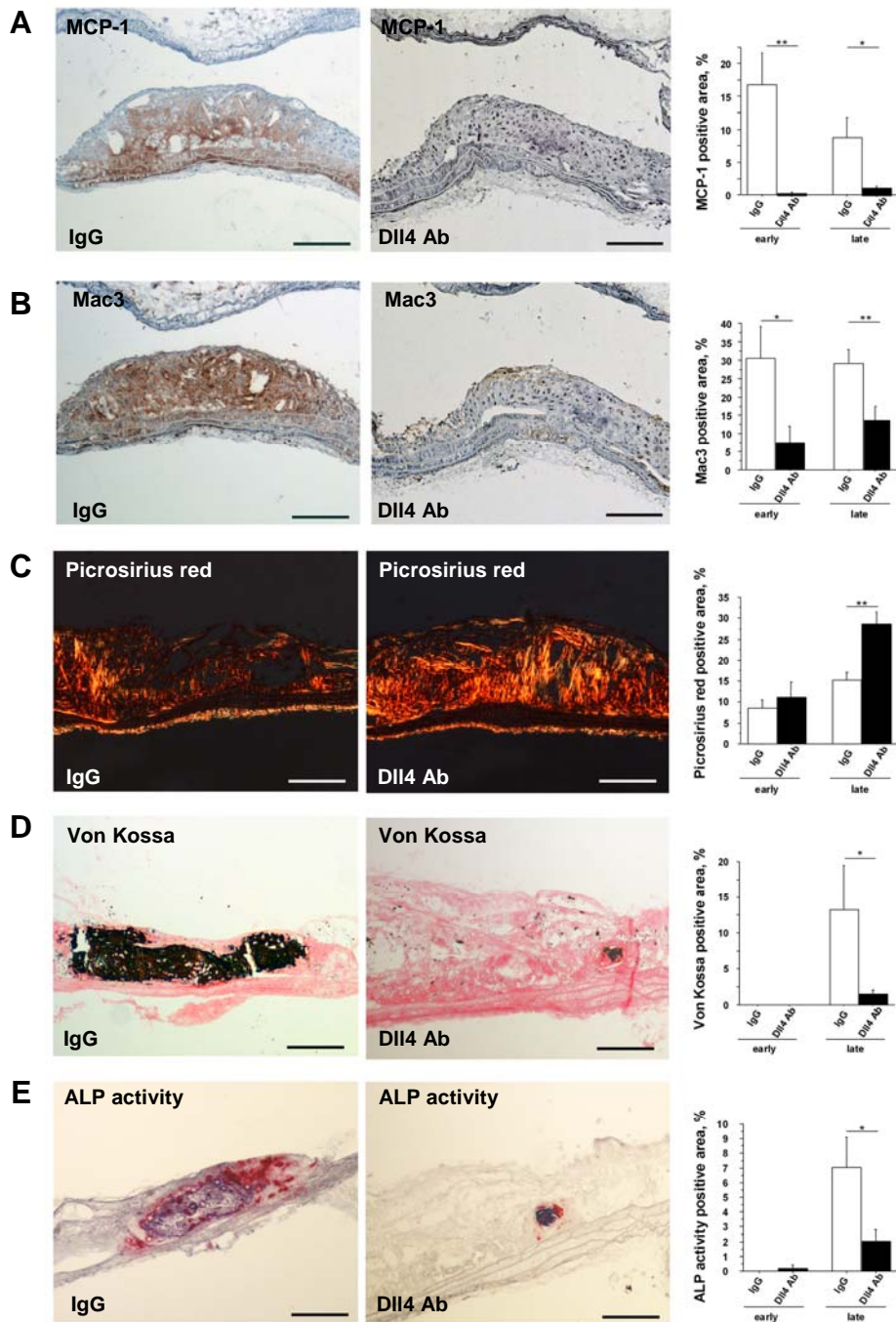
Fig. S2. Effects of Dll4 blockade on atherosclerotic lesions in the brachiocephalic artery.
(A) Immunostaining of MCP-1, **(B)** Immunostaining of Mac3, **(C)** Picrosirius red staining, **(D)** von Kossa staining, and **(E)** ALP activity. Scale bars: 100 μm. A and B; early-phase treatment (n = 7-8). C-E; late-phase treatment (n = 14-15). * $P < 0.05$, ** $P < 0.01$. All values are mean ± SEM.
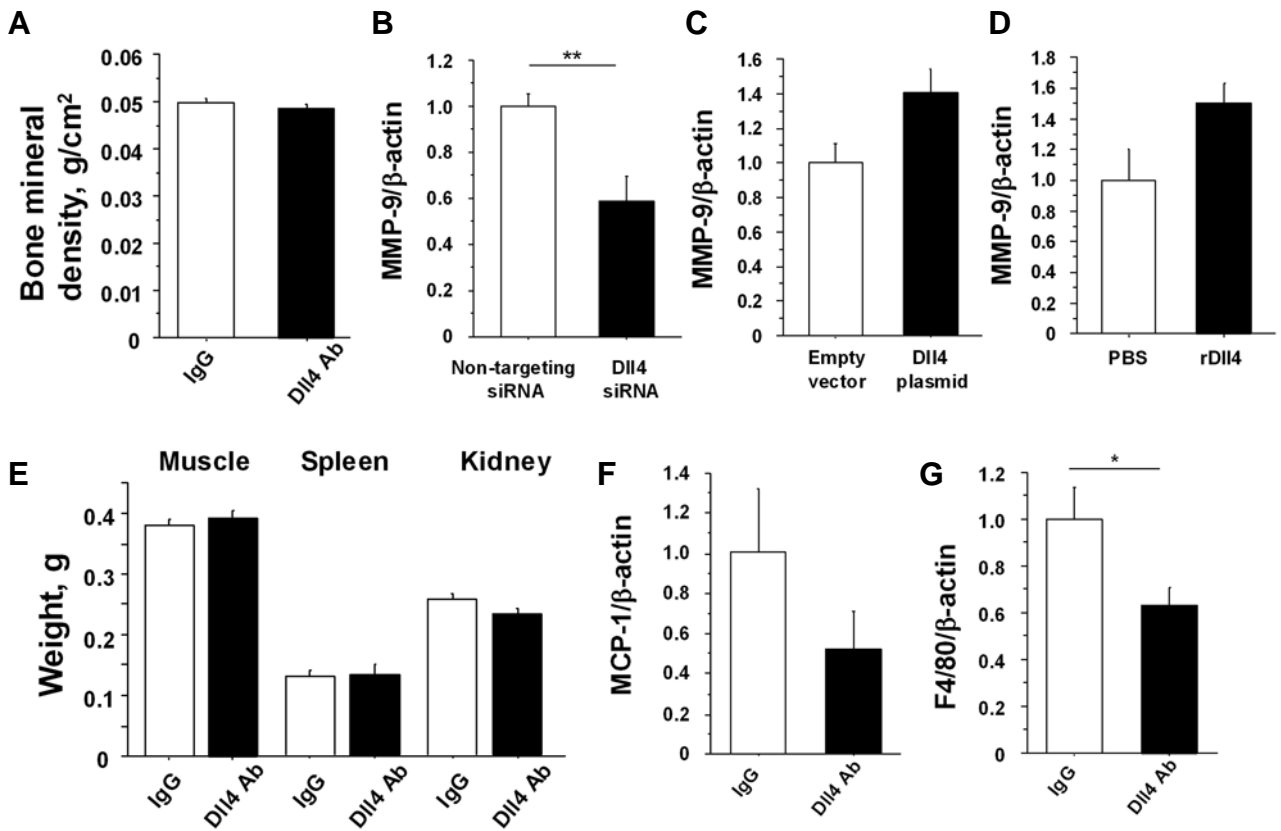
**Fig. S3. Effects of Dll4 blockade on bone mineral density and liver, and the role of Dll4 in MMP9 expression in RAW264.7 cells.**
(A) Dll4 blockade by 12 weeks of Dll4 Ab administration did not decrease bone mineral density in Ab-treated animals (n = 6). **(B)** Effect of RNAi silencing of Dll4 on MMP9 expression in RAW264.7 cells. **(C and D)** Effect of transfection of the plasmid encoding mouse Dll4 **(C)** and stimulation with immobilized rDll4 **(D)** on MMP9 expression in RAW264.7 cells. G-I, (n = 6). **(E)** There were no differences in weight of muscle, spleen, and kidney after 12 weeks of Dll4 Ab administration (n = 19–20). **(F and G)** Quantitative RT-PCR analysis of expression of MCP-1 **(F)** and F4/80 **(G)** in the liver, (n = 7). * $P < 0.05$. Values are mean $\pm$ SEM.

A

B
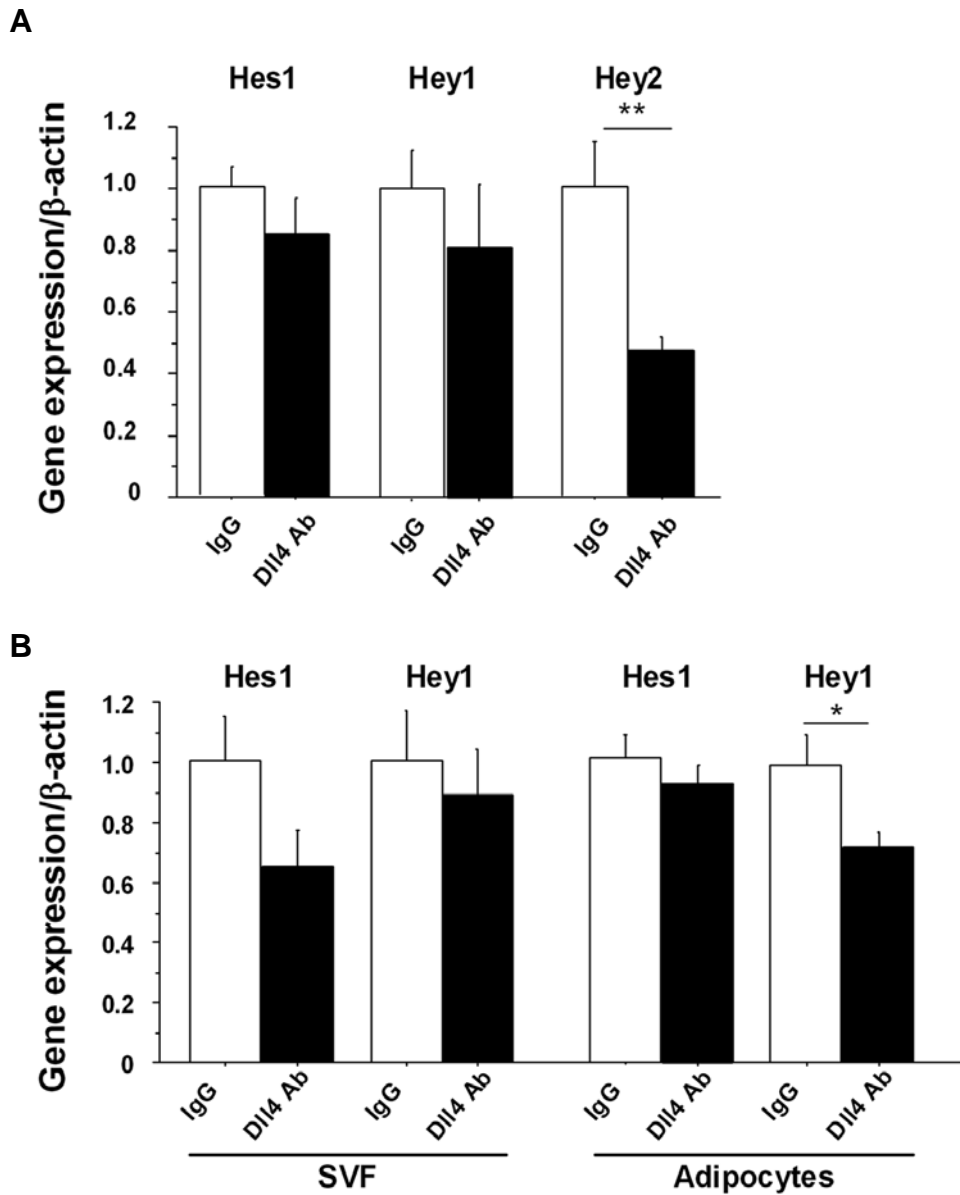
Fig. S4. Expression of Notch target genes.
Quantitative RT-PCR analysis of Notch target genes. Dll4 blockade using neutralizing Dll4 Ab decreased expression of Notch target genes in aortas (n = 7-8) **(A)** and SVF and adipocytes (n = 9-10) **(B).** ** P < 0.01. All values are mean ± SEM.
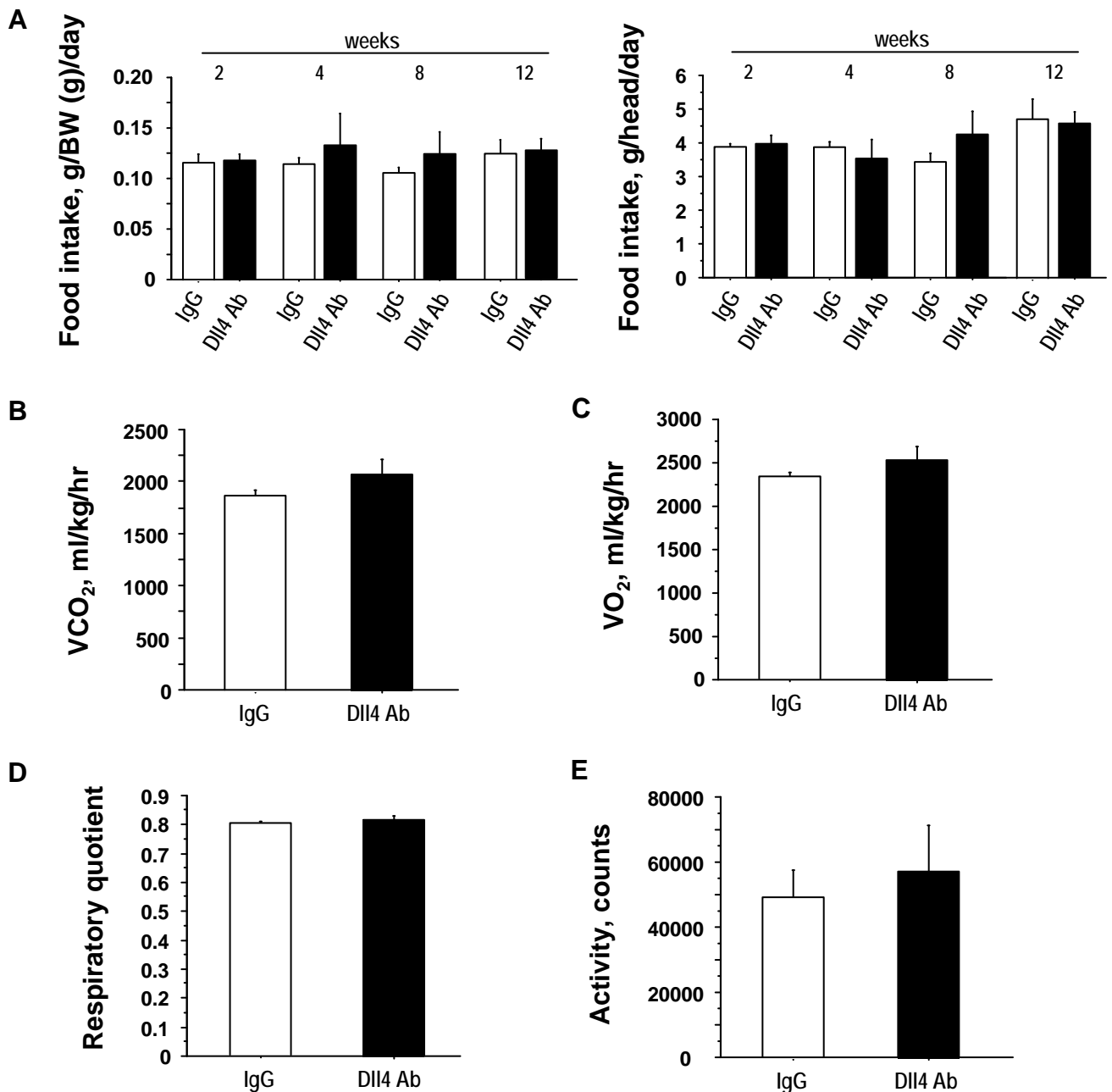
**Fig. S5. Food consumption and metabolic studies of mice after late-phase treatment.**
(A) We measured food intake over 7 days at 2, 4, 8, and 12 weeks of treatment. Data are expressed as grams of food per gram of body weight per day (left) and grams of food per day (n = 8 per group). Dll4 Ab treatment did not affect food consumption. Indirect calorimetry was performed at the end of late-phase treatment (n = 6). **(B-D)** There were no differences in carbon dioxide production **(B)**, oxygen consumption **(C)**, and respiratory quotients **(D)** between Ab-treated mice and control mice. **(E)** There was no difference in total 24-hour physical activity as determined by automatic sensors on x-axis. All values are mean ± SEM.
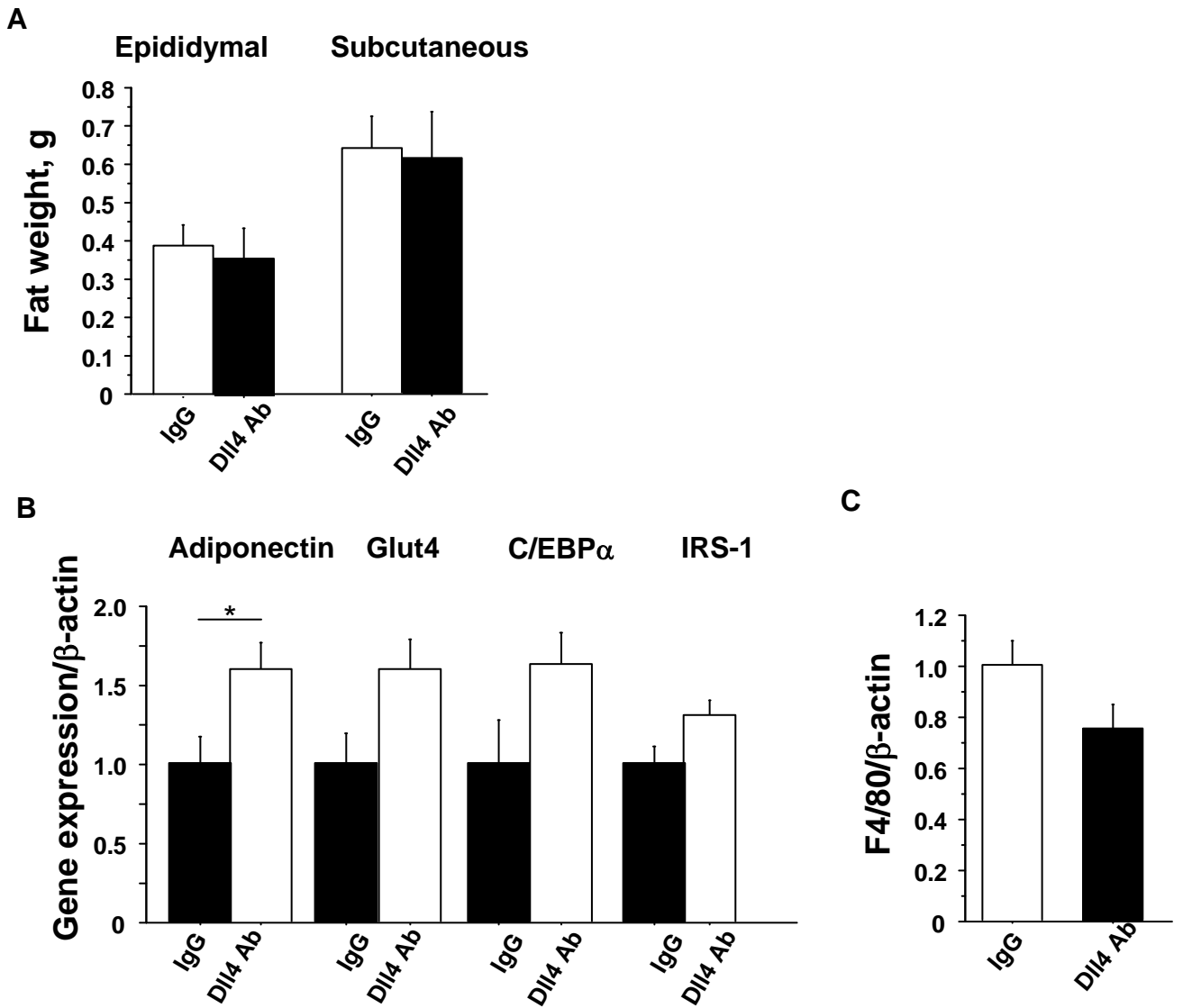
**Fig. S6. Analyses of epididymal fat after early-phase treatment.**
(A) Dll4 blockade did not affect fat weight after early-phase treatment (n = 7-8). **(B)** Results of quantitative RT-PCR analyses of genes related to insulin sensitivity showed that Dll4 Ab administration significantly increased adiponectin and tended to increase GLUT4, C/EBPα (both P = 0.06) and IRS-1 (P = 0.09) expression in epididymal fat, regardless of the similarity in fat weight (n = 6). **(C)** Dll4 blockade also tended to decrease macrophage accumulation in fat (P = 0.09) (n = 6). * P < 0.05. All values are mean ± SEM.
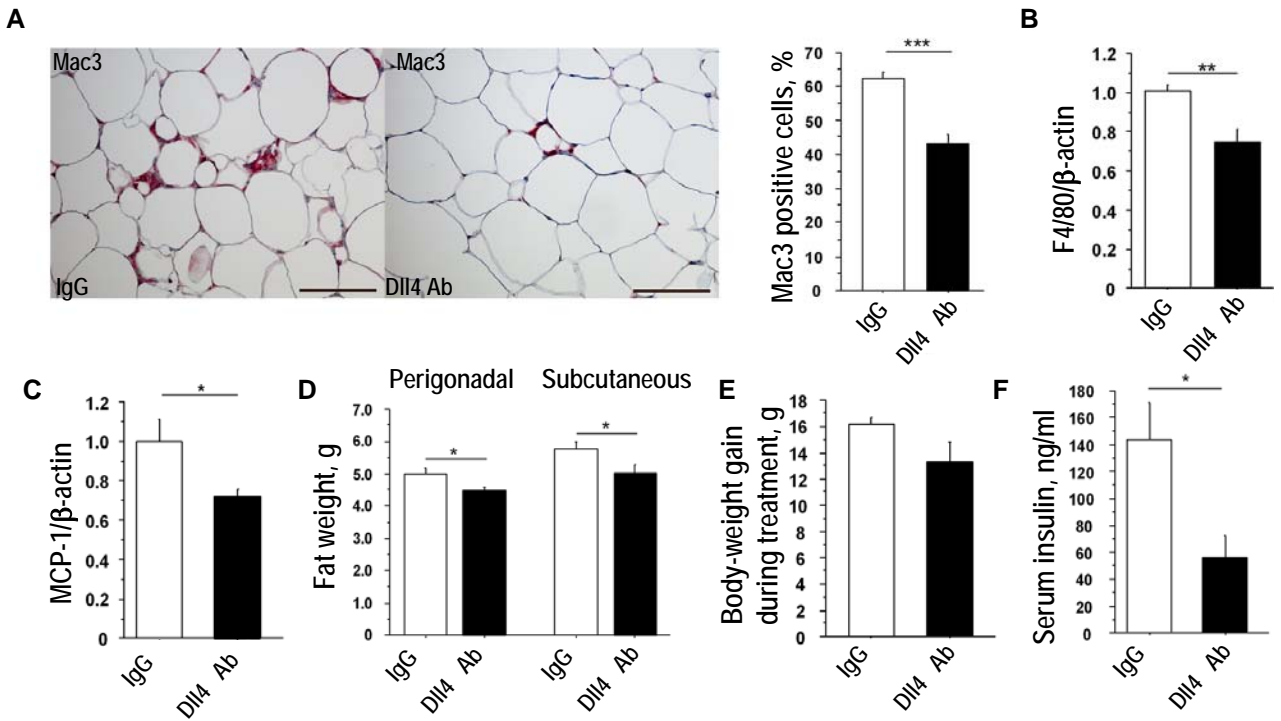
Fig. S7. Effects of Dll4 blockade on white adipose tissue in *Lep^{ob}/Lep^{ob}* mice
(A) Mac3 staining and population of Mac3-positive cells in perigonadal fat obtained from
Lep^{ob}/Lep^{ob} mice after 10-week treatment. Scale bar: 100 μm. **(B** and **C)** Quantitative RT-PCR
analysis of F4/80 **(B)** and MCP-1 **(C)** expression in fat. **(D** and **E)** Effects of Dll4 Ab treatment on
fat weight **(D)** and body weight gain **(E)**. **(F)** Serum insulin levels. (n = 9). * $P < 0.05$, **$P < 0.001$,
*** $P < 0.05$. All values are mean ± SEM.
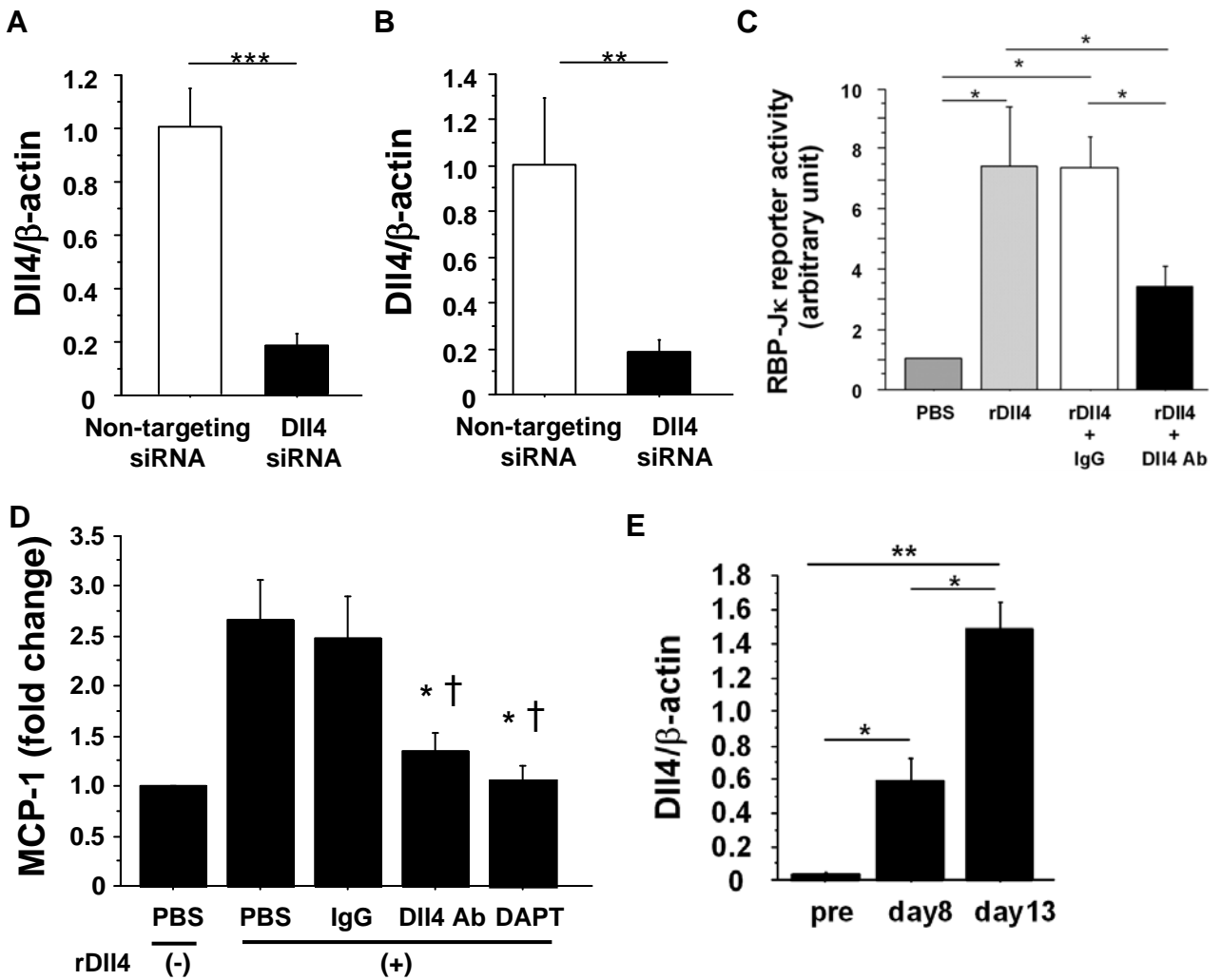
Fig. S8. Results of *in vitro* experiments using RAW264.7 cells, differentiated 3T3-L1 adipocytes, and endothelial cells.

(A and B) Silencing efficacy of Dll4 by transfection of siRNA in RAW264.7 cells **(A)** and 3T3-L1 adipocytes **(B)**, (n = 6). **(C)** To examine Notch activation by Dll4 and effect of Dll4 Ab in 3T3-L1 adipocytes, we performed RBP-Jκ reporter activity assay. Stimulation with immobilized rDll4 promotes RBP-Jκ reporter activity in this cell type, which was abrogated by anti-Dll4 Ab, (n = 4). A-C, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. **(D)** We examined the role of Dll4-Notch signaling in MCP-1 expression in endothelial cells. Immobilized rDll4 promoted expression of MCP-1 in human saphenous vein endothelial cells *in vitro*, which were abrogated by anti-human Dll4 Ab or DAPT, a g-secretase inhibitor (n = 6 different donors). * $P < 0.05$ vs. rDll4 + PBS, † $P < 0.05$ vs. rDll4 + IgG. **(E)** Quantitative RT-PCR analysis of Dll4 in differentiated 3T3-L1 adipocytes. Dll4 expression increased during differentiation (n = 4). * $P < 0.05$, ** $P < 0.01$. All values are mean ± SEM.

Supporting Table. Blood pressure and serum lipid levels

	IgG	Dll4 Ab	*P-value*
Early-phase Treatment			
Systolic blood pressure, mmHg	102.3±5.3	110.1±10.2	NS
Diastolic blood pressure, mmHg	82.5±3.5	88.0±6.8	NS
Total cholesterol, mg/dl	782.7±57.9	716.1±57.3	NS
Triglyceride, mg/dl	249.9±28.1	218.6±19.2	NS
Blood glucose, mg,dl	170.8±9.2	190.6±7.7	NS
Late-phase Treatment			
Systolic blood pressure, mmHg	98.4±2.4	91.5±4.9	NS
Diastolic blood pressure, mmHg	75.5±2.8	70.1±5.8	NS
Total cholesterol, mg/dl	1051.4±74.7	989.9±50.5	NS
Triglyceride, mg/dl	176.4±13.2	161.9±15.4	NS

All values are mean ± SEM. NS, not significant different.