SUPPLEMENTAL INFORMATION

Blockade of VEGF-C and VEGF-D modulates adipose tissue inflammation and improves metabolic parameters under high-fat diet

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SUPPLEMENTAL EXPERIMENTAL METHODS

Whole-mount immunofluorescence stains

Approximately 10 mm long pieces of small intestine were dissected from mice. The intestines were cut longitudinally, rinsed with PBS and fixed in 4% PFA for 2 h at 4ºC. Samples were washed with PBS several times for 30 min, followed by blocking with a mixture of 5% non-immune donkey serum, 0.1% Triton-X, 1% BSA and 0.05% NaN_3 in PBS for 2 h, and by incubation with the respective primary antibodies overnight. The samples were then incubated with Alexa 488- and 594- conjugated secondary antibodies (Invitrogen; 1:200 dilution) for 2 h, washed for 2 h with PBS and mounted on glass slides in VectaShield (Vector). Z-stack images were acquired with confocal imaging, which was performed on a Zeiss LSM 710-FCS confocal microscope equipped with a 10× 0.3NA EC Plan-Neofluar objective and a 20× 0.8 NA Plan-Apochromat objective (Carl Zeiss). Images were acquired using the Zeiss ZEN 2009 software and processed using Imaris software (version 7.1.1, Bitplane) or ImageJ (NIH).

Computed tomography analyses of adipose tissue

After 19 weeks of HFD, computed tomography (CT) scans were performed as described with slight modifications [1]. Briefly, mice were kept under isoflurane anesthesia and were imaged with a La Theta LCT-100 (Aloka) Micro-CT scanner. A pitch size of 1 mm was used and the tube voltage of the X-ray source was adjusted to 50 kV with 1 mA constant current. The volumes of SWAT and EWAT were corrected and analyzed with the LaTheta software (V 2.10, Aloka), and the corresponding weights were calculated using a density factor of 0.92 g/cm^3.

Fasting insulin and glucose measurements

Mice that were on chow or HFD for 10, 20 or 38 weeks were fasted for 12 h during the light cycle (6 a.m. to 6 p.m.). Blood glucose was measured with a Contour glucometer (Bayer HealthCare). Serum insulin levels were measured with an ultra sensitive mouse Insulin ELISA kit (Crystal Chem Inc.). The homeostatic model assessment of insulin resistance (HOMA-IR) index was calculated using fasting glucose and insulin concentrations with the following formula: fasting blood glucose (mmol/L) × fasting insulin (μU/mL)/22.5.

Fasting - refeeding studies

Mice that were on chow or HFD for 38 weeks were fasted for 12 h during the light cycle (6 a.m. to 6 p.m.). Mice were given access to food after collection of fasting blood. Three hours after re-feeding, blood samples were collected again. Free-fatty acid levels in the serum were measured with NEFA-HR kit (Wako) following the manufacturer's instructions.

Cytokine analysis

For the conditioned media measurements, $CD11b^+$ cells from the SVF were isolated by MACS (Miltenyi Biotech) from 24-week-old mice on chow diet (WT and sR3, *n*=4 each). Cells were cultured in DMEM (Gibco), supplemented with 1% mouse serum (obtained from the corresponding macrophage donor). After 24 h, conditioned media were collected, centrifuged and the supernatants were stored at –20 ºC until used. The mouse Th1/Th2 10plex FlowCytomix kit (eBioscience) was used to measure the levels of cytokines in macrophage conditioned media, following the manufacturer's instructions.

ELISA

The levels of sR3 protein were detected as described previously with slight modifications [2]. ELISA plates (Nunc) were coated with anti-human IgG (Sigma, 0.5 µg/mL), washed 6 times and blocked with 1% non-fat milk powder, 1% gelatin (Hänseler AG) in PBS for 1 h. Lysates of SWAT, EWAT and liver were incubated for 1 h. After washing 4 times, biotinylated anti-human VEGFR-3 antibody (R&D Systems, 1:4,500) was added for 2 h. After washing 4 times, streptavidin conjugated horseradish peroxidase (GE Healthcare, 1:5,000) was added for 30 min, followed by addition of BM Blue POD substrate (Roche). The reaction was stopped with 2 M H_2SO_4 and absorbance was determined at 450 nm using a Sunrise microplate reader (Tecan).

Tamoxifen administration and X-gal staining

Flt4-Cre-ERT2;ROSA26-LSL-LacZ double knock-in mice on mixed (129/C57BL/6) background [3] were used to assess the Flt4 (VEGFR-3) expression in tissues. Tamoxifen injections (50 µL of 1 mg/mL) were done on postnatal days P1, P2 and P3 intragastrically. The mice were sacrificed on day P11 and tissues were harvested for X-gal staining. The tissues were fixed in 2% formaldehyde, 0.2% glutaraldehyde and 0.02% NP-40 in PBS at 4 ºC for 1 h, washed with PBS, and then stained overnight with 1 mg/mL (1:500) 5- bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) in the preheated (37 ºC) X-gal staining buffer (5mM potassium ferrocyanide ($K_4[Fe(CN)_6]$), 5mM potassium ferricyanide ($K_3[Fe(CN)_6]$), 2mM $MgCl_2$, 0.02% Np-40 in PBS) at 37 ^oC. The following day the tissues were post-fixed with 4% PFA in PBS for 1 h, washed with PBS for 1 h and imaged.

SUPPLEMENTAL TABLES

Table S1: Serum parameters of 12-h fasted wildtype and sR3 mice on HFD or chow for 38 weeks. Data are expressed as mean ± SEM. Parentheses indicate number of mice per group. *P<0.05, one-tailed Student's t-test, sR3 mice compared to WT under same diet conditions. † P<0.05, one-tailed Student's t-test, fasting level compared to post-prandial level.

	Chow		**HFD**	
	Wildtype	**sR3**	**Wildtype**	**sR3**
Fasting glucose (mmol/L)	7.1 ± 0.6 (5)	6.5 ± 0.4 (5)	8.0 ± 0.6 (5)	7.8 ± 0.4 (6)
Fasting insulin (ng/mL)	6.8 ± 2.2 (5)	1.6 ± 0.4 (6) *	12.7 ± 1.7 (5)	7.8 ± 1.5 (6) *
HOMA-IR	59.5 ± 23.2 (5)	13.0 ± 3.0 (6) *	119.5 ± 21.5 (5)	68.1 ± 11.2 (6) *
FFA (mmol/L) (fasting)	1.1 ± 0.1 (5)	1.1 ± 0.1 (6)	1.0 ± 0.1 (5)	0.7 ± 0.1 (6)
FFA (mmol/L) (post-prandial)	0.8 ± 0.1 (5)	0.6 ± 0.2 (6)†	1.1 ± 0.2 (5)	0.9 ± 0.1 (6)

Table S2: Primers used for the quantitative real-time PCR analyses.

Primer	Sequence	Reference
36b4 Forward	5'-AGATTCGGGATATGCTGTTGGC-3'	Primer bank ID: 6671569a1
36b4 Reverse	5'-TCGGGTCCTAGACCAGTGTTC-3'	
Inos Forward	5'-CCTGGTACGGGCATTGCT-3'	[4]
Inos Reverse	5'-GCTCATGCGGCCTCCTT-3'	
Mrc1 Forward	5'-GCTTCCGTCACCCTGTATGC-3'	Primer bank ID: 224967061c2
Mrc1 Reverse	5'-TCATCCGTGGTTCCATAGACC-3'	
Tnf Forward	5'-CAGGCGGTGCCTATGTCTC-3'	[5]
Tnf Reverse	5'-CGATCACCCCGAAGTTCAGTAG-3'	
CD11b Forward	5'-CCTTGTTCTCTTTGATGCAG-3'	[6]
CD11b Reverse	5'-GTGATGACAACTAGGATCTT-3'	
CD11c Forward	5'-CCAAGACATCGTGTTCCTGATT -3'	Primer bank ID:
CD11c Reverse	5'- ACAGCTTTAACAAAGTCCAGCA-3'	118130485c3
CD68 Forward	5'- CCAATTCAGGGTGGAAGAAA -3'	[7]
CD68 Reverse	5'- CTCGGGCTCTGATGTAGGTC-3'	
Il6 Forward	5'-TCTATACCACTTCACAAGTCGGA-3'	[8]
Il6 Reverse	5'- GAATTGCCATTGCACAACTCTTT-3'	
Arg1 Forward	5'-CTCCAAGCCAAAGTCCTTAGAG-3'	Primer bank ID: 7106255a1
Arg1 Reverse	5'-AGGAGCTGTCATTAGGGACATC-3'	
Ym1 Forward	5'-GGGCATACCTTTATCCTGAG-3'	[9]
Ym1 Reverse	5'-CCACTGAAGTCATCCATGTC-3'	
Vegfc Forward	5'-GGGGGCGAGGTCAAGGCTTTT-3'	Self-designed
Vegfc Reverse	5'-CCTGGTATTGAGGGTGGGCTGC-3'	
Vegfd Forward	5'-AGCGAACATGGACCAGTGAAGGATT-3'	Self-designed
Vegfd Reverse	5'-CCTCCAAACTAGAAGCTGCTCGGA-3'	
Vegfr2 Forward	5'-TTTGGCAAATACAACCCTTCAGA-3'	Primer bank ID: 27777648a1
Vegfr2 Reverse	5'-GCAGAAGATACTGTCACCACC-3'	
Vegfr3 Forward	5'-CTGGCAAATGGTTACTCCATGA-3'	Primer bank ID: 6679813a1
Vegfr3 Reverse	5'-ACAACCCGTGTGTCTTCACTG-3'	
Nrp1 Forward	5'-GACAAATGTGGCGGGACCATA-3'	Primer bank ID: 6679134a1
Nrp1 Reverse	5'-TGGATTAGCCATTCACACTTCTC-3'	
Nrp2 Forward	5'-CCAGAACTGTGAGTGGATTGTC-3'	Self-designed
Nrp2 Reverse	5'-CCATCCCGAATCTCAATGAAGTC-3'	

SUPPLEMENTAL FIGURES and FIGURE LEGENDS

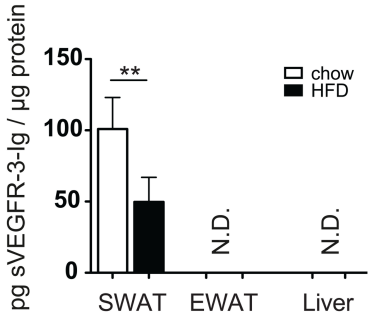

Figure S1: sR3 protein is detectable in SWAT, but not in EWAT or liver. ELISA analysis of adipose tissue and liver lysates showing detectable amounts of sR3 protein only in SWAT (*n*=3-4 each group). N.D., not-detected. ***P*<0.01, two-tailed Student's *t*-test, WT under HFD compared to WT under chow diet. Data are mean ± SD.

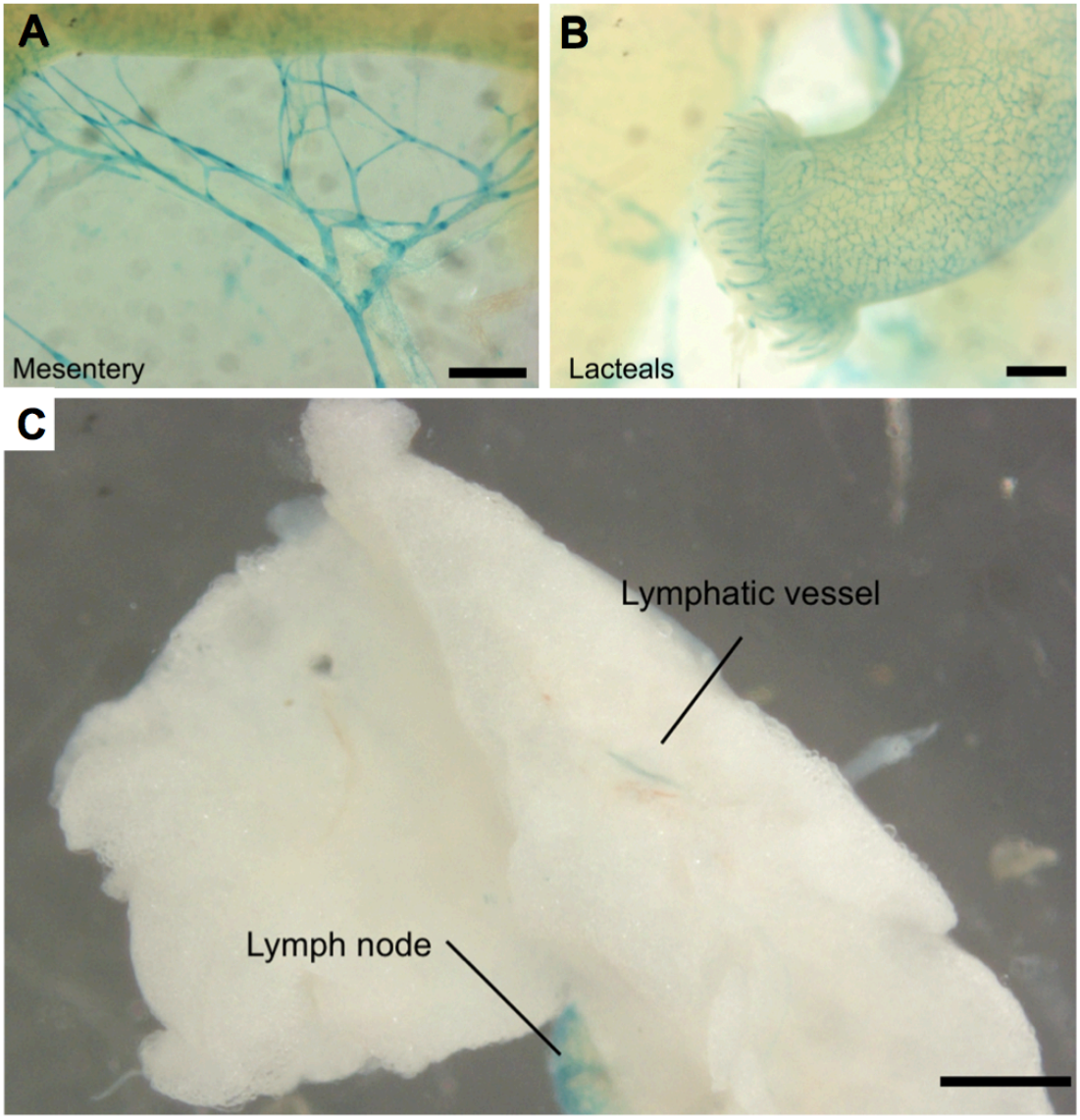

Figure S2: Whole mount X-gal staining in Flt4CreErt2 Rosa26LacZ double knock-in mice reveals lack of VEGFR-3 in adipose tissue. VEGFR-3 positive lymphatic vessels are visible in different tissues such as the mesentery (A) and the lacteals (B). (C) Lack of VEGFR-3 expressing cells in the adipose tissue except for the collecting lymphatic vessels and lymph nodes. Scale bars: A, B = 500 µm; and C = 200 µm.

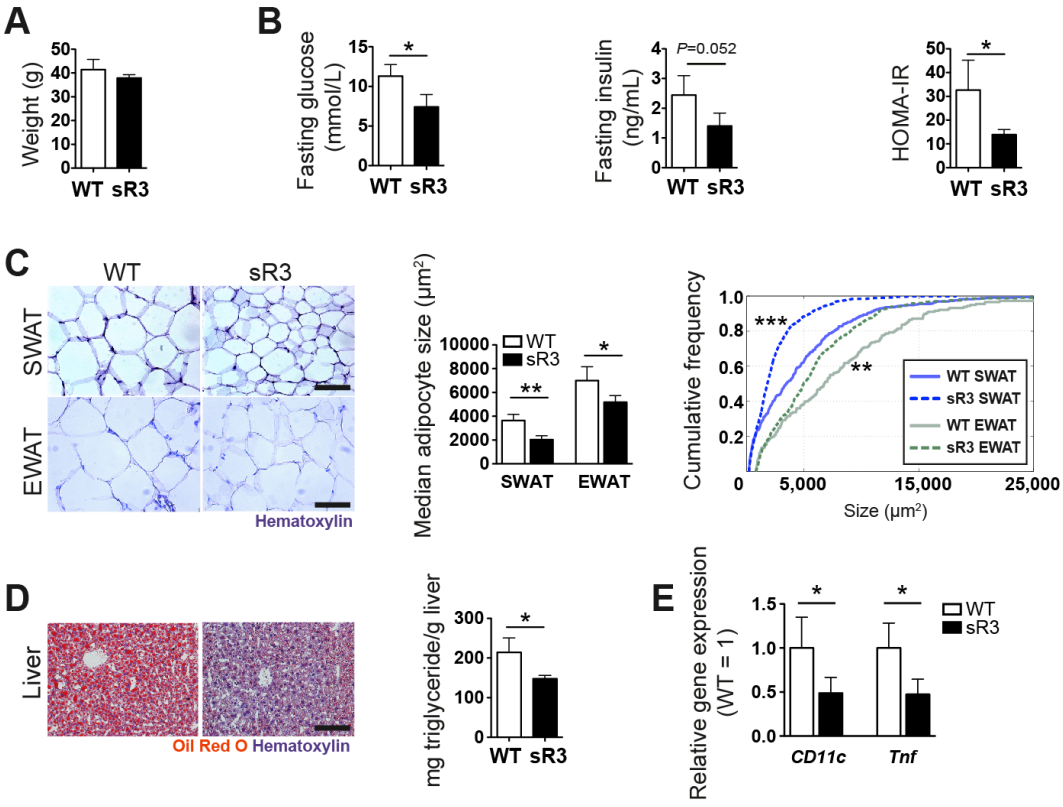

Figure S3: Increased insulin sensitivity of the adipose tissue of sR3 mice on the C57/BL6 background (sR3 B6) on HFD. (**A**) No difference in weight after 10 weeks on HFD (*n*=3-4). (**B**) Lower fasting glucose and insulin levels that result in reduced HOMA-IR indices (*n*=3-4). (**C**) sR3 B6 mice had smaller SWAT and EWAT adipocytes than WT mice, which was confirmed by the median adipocyte size comparison and cumulative frequency plots (*n*=3-4, ***$P<0.001$; two-sample Kolmogorov-Smirnov test). (**D**) sR3 B6 mice also had lower ectopic lipid accumulation in the liver as shown by Oil Red O staining and triglyceride measurements (*n*=3-4). (**E**) Macrophages isolated from SWAT of sR3 B6 mice showed reduced expression of the M1 markers *CD11c* and *Tnf* (*n*=3-4). Scale bars = 100 µm. *$P<0.05$, **$P<0.01$, two-tailed Student's *t*-test compared to WT. All data are mean±SD.

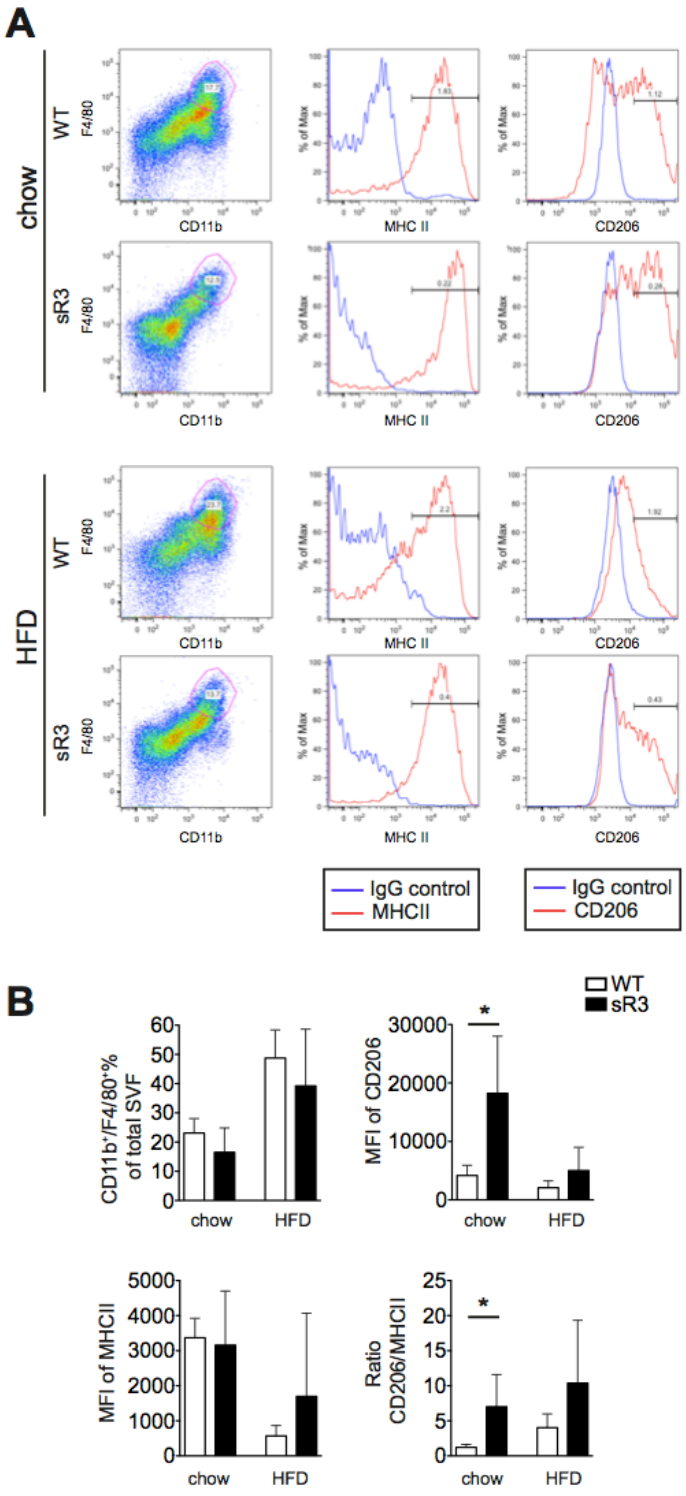

Figure S4: FACS analysis of EWAT macrophages. (**A**) Representative FACS plots. (**B**) FACS analyses showed a higher ratio of CD206/MHC II expression levels in EWAT macrophages of sR3 mice as compared to WT mice (n=3-7). *P<0.05, two-tailed Student's t-test compared to WT under same diet. All data are mean±SD.

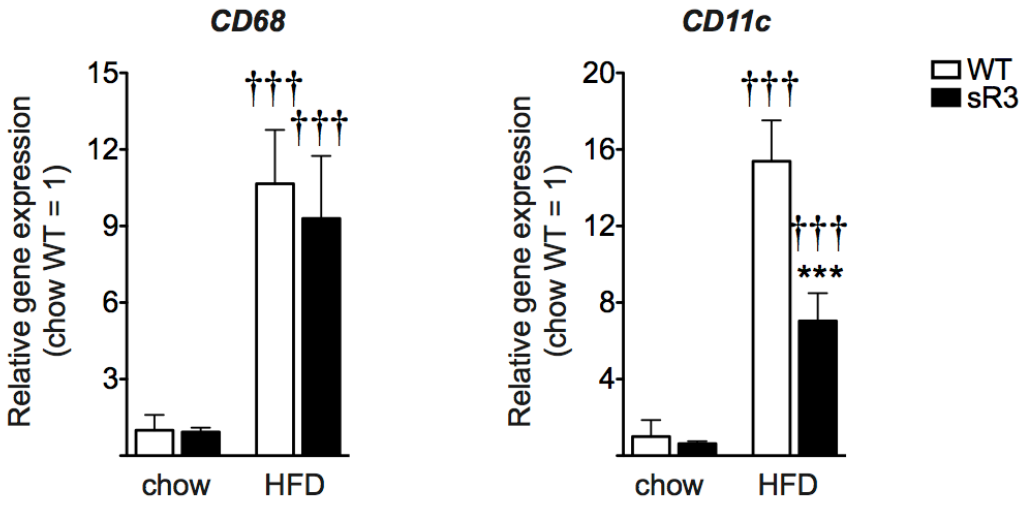

Figure S5: sR3 mice have reduced *CD11c* expression in SWAT under HFD. Comparable expression of *CD68* (common macrophage marker) between sR3 and WT mice under both chow and HFD. *CD68* expression levels were significantly upregulated after HFD. Expression of *CD11c* (an M1 marker) was significantly lower in sR3 mice than in WT mice after HFD (n=5-7 each group). ***P<0.001 two-tailed Student's t-test compared to WT of the same diet, †††P<0.001 two-tailed Student's t-test compared to chow diet of the same genotype. All data are mean±SD.

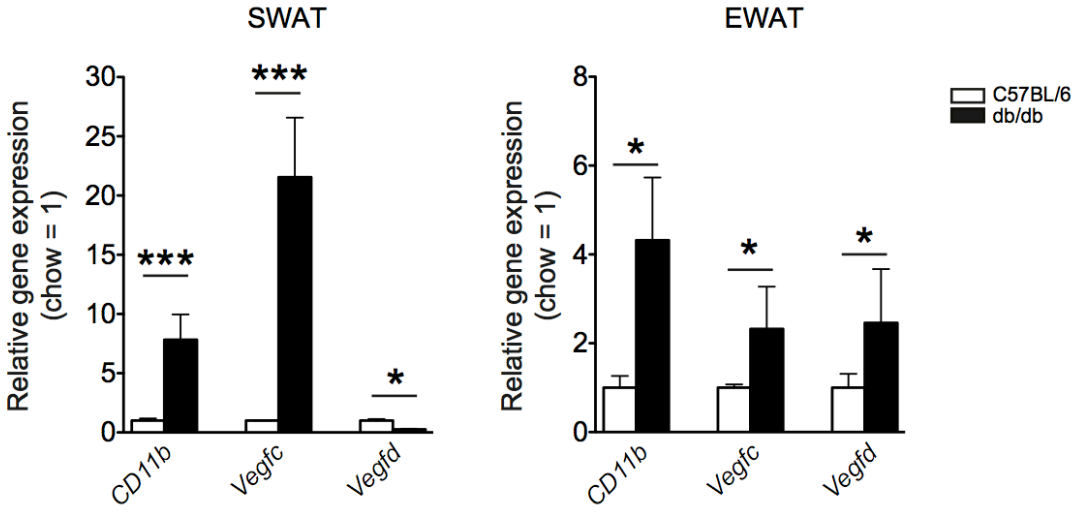

Figure S6: VEGF-C and -D are upregulated in the EWAT of db/db mice. Quantitative real-time PCR analyses of *Vegfc* and *Vegfd* expression together with *CD11b* in total SWAT and EWAT of lean (C57BL/6) and obese (db/db) mice showed that *Vegfc* and *Vegfd* mRNA levels were also upregulated in EWAT in obesity (n=3-5 each group). *P<0.05, ***P<0.001 one-tailed Student's t-test compared to lean (C57BL/6) mice. All data are mean±SD.

SUPPLEMENTAL REFERENCES

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