SUPPLEMENTARY DATA

Immunoprecipitation. Each plasma sample (500μl) was pre-cleared with protein G-Sepharose beads (Invitrogen) in lysis buffer (10 mM Tris, 10 mM NaCl, 0.1mM EDTA, 1% NP-40, 0.5% deoxycholate, 1mM $MgCl_2$) containing a protease inhibitor mixture (Sigma) for 1 hour at 4°C in order to remove nonspecific proteins. Supernatants were obtained by centrifugation for 30 seconds at 14,000 g, and 2 μg of affinity-purified polyclonal anti-4-1BBL antibody was added and the mixtures were incubated overnight at 4°C with shaking. They were then incubated at 4°C for 2 hours with 20 μl of protein G-Sepharose beads. The beads were sedimented by 30 seconds centrifugation at 14,000 g, and washed three times with 500 μl of lysis buffer. After the final wash, the buffer was replaced with 30 μl of sample buffer and the samples were boiled for 5 minutes. The immunoprecipitated proteins were fractionated by 11% SDS-PAGE and transferred to nitrocellulose membranes.

Immunohistochemistry. For immunohistochemistry of F4/80 or CD3, the paraffin-embedded sections were dewaxed and rehydrated. Endogenous peroxidase was quenched with 3% H_2O_2 for 10 min, and the sections were blocked with Protein Block Serum-Free (DakoCytomation, Carpinteria, CA). They were incubated with anti-F4/80 antibody (1:100 eBioscience) or anti-CD3 antibody (1:100 BD Pharmingen, San Diego, CA) overnight at 4° C, then washed and incubated with HRP-conjugated secondary antibody. Immunoperoxidase staining was carried out with a Vector VIP substrate kit (Vector Labs, Burlingame, CA). Slides were counterstained with hematoxylin.

Quantitative real time PCR. Tissues were collected and stored at -20°C in RNAlater (Ambion, Austin, TX). Total RNA was extracted from tissues using TRIzol (Invitrogen, Carlsbad, CA), and cDNA was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI). Real-time PCR amplification of the cDNA was performed in duplicate with a SYBR premix Ex Taq kit (TaKaRa bio inc, Forster, CA) using a Thermal Cycler Dice (TaKaRa bio inc, Japan). All reactions were performed in the same way: 95°C for 10s, 45cycles of (95°C for 5s and 60°C for 30s). Results were analyzed with real time system TP800 software and all values were normalized to the levels of house-keeping genes 36B4 for adipose tissue and GAPDH for liver. Mouse primer sequences used were as follows: 4-1BB, 5'-CTCTGTGCTCAAATGGATCAGGAA -3' (forward), 5'-TGTGGACATCGGCAGCTACAA -3' (reverse); 4-1BBL, 5'-CCTGTGTTCGCCAAGCTACTG -3' (forward), 5'-CGGGACTGTCTACCACCAACTC -3' (reverse); IRS-1, 5'-GGTAAGCTCTTGCCTTGCAC -3' (forward), 5'-CAGACTGTCGCTGCTGGTAG -3' (reverse); Glut4, 5'-CTGTAACTTCATTGTCGGCATGG -3' (forward), 5'-AGGCAGCTGAGATCTGGTCAAAC -3' (reverse); Sterol regulatory element binding protein (SREBP)-1c, 5'-GGAGACATCGCAAACAAGCTGA -3' (forward), 5'-CAGACTGCAGGCCAGATCCA -3' (reverse); Acetyl CoA carboxylase (ACC)1, 5'-GAAGTCAGAGCCACGGCACA -3' (forward), 5'-GGCAATCTCAGTTCAAGCCAGTC-3' (reverse); Fatty acid synthase (FAS), 5'-AGCACTGCCTTCGGTTCAGTC -3' (forward), 5'-AAGAGCTGTGGAGGCCACTTG -3' (reverse); 36B4, 5'-TTC CAG GCT TTG GGC ATC A-3' (forward), 5'-ATG TTC AGC ATG TTC AGC AGT GTG-3' (reverse); GAPDH, 5'-GGCTATCACGGAGGCTGTGAA -3' (forward), 5'-CCAGCCTTAGCATCAAAGATGGA -3' (reverse).

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Glucose uptake. Basal and insulin-stimulated glucose uptake was measured in epididymal fat pads isolated from 4-1BB-deficient and WT mice, and cut into 1-2mm pieces as previously reported (Cooney GJ, 2004). These were placed in 12-well plates (50 mg per well) in 0.5ml KRB buffer (pH 7.4) supplemented with 0.2% BSA and 2mM sodium pyruvate, and incubated at 37°C for 30 minutes to establish basal conditions. Following this preincubation, the pieces were incubated in the presence or absence of insulin (100 nM) for 30 minutes, and transport was measured for 10 min in KRB buffer containing 1 mM 2-deoxy-D-[1,2-3H]-glucose (1.5 μCi/ml). To terminate transport, the fat pad fragments were dipped in KRB buffer containing 50 μM cytochalasin B at 4°C. They were blotted on filter paper, incubated in 200 μl 1N NaOH at 80°C for 10 minutes and neutralized with 200 μl 1N HCl, and particulates were sedimented by centrifugation at 13,000 g for 2minutes. Radioactivity in aliquots of the digested adipose tissue was determined by scintillation counting.

Preparation of adipocytes. Adipose tissue-derived SVFs from WT and 4-1BB-deficient mice were cultured in a basal medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 200 μM ascorbic acid, 10% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. Two days after reaching confluence (day 0), the cells were incubated in differentiation medium containing the inducing mixture (0.25 μM dexamethasone, 10 μg/ml insulin, and 0.5 mM 1-methyl-3-isobutylxanthine) as in the basal medium. After 40 hours, the culture medium was changed to maturation medium consisting of 5 μg/ml insulin in the basal medium, and the maturation medium was changed every 2 days for 4 days.

Preparation of hepatocyte and kupffer cell. WT and 4-1BB-deficient mice were anesthetized with Nembutal (Dainippon Sumitomo Pharma) intraperitonealy and the livers were perfused with 40 ml of Liver Perfusion Medium (Invitrogen) followed by 30 ml of Liver Digestion Medium (Invitrogen), both at a flow rate of 5 ml/minutes. Hepatocytes were dispersed in Hepatocyte Wash Medium (Invitrogen) supplemented with 1% P/S by dissection and gentle shaking. After filtration through a 100 μm nylon mesh filter, hepatocytes were isolated by repeated centrifugation (3–5 times) at 50×*g* for 3 minutes. The isolated hepatocytes were resuspended in DMEM (Invitrogen) supplemented with 10% FBS and 1% P/S, and cultured in type-1 collagen-coated 12-well plates at a cell density of 2×10^5 cells/well. After 2 hours incubation, hepatocytes were used for mRNA quantification assay.

The non-parenchymal cell-enriched supernatant was centrifuged at 800 *g* for 10 min, the pellet resuspended in 40 ml of PBS, and portions of 10 ml were layered on top of preformed two-step Percoll gradient (the bottom cushion with a density of 1.066 g/mL; the overlying cushion with a density of 1037 g/mL), and centrifuged at 400 *g* for 15 minutes at 4℃. Purified non-parenchymal cells enriched in Kupffer cells extended throughout the lower Percoll cushion. The Kupffer cells-enriched fraction was diluted in PBS and centrifuged at 800 *g* for 10 min. The resulting pellet was resuspended in culture medium (RPMI 1640 with 10% fetal calf serum). Kupffer cells were selected by allowing them to adhere for 2 h at 37℃. After nonadherent cells were removed by gentle washing, adherent cells were used for mRNA quantification assay.

REFERENCE

Cooney GJ, Lyons RJ, Crew AJ, Jensen TE, Molero JC, Mitchell CJ, Biden TJ, Ormandy CJ, James DE, Daly RJ. Improved glucose homeostasis and enhanced insulin signalling in Grb14-deficient mice. 2004 EMBO J.;23(3):582-93.

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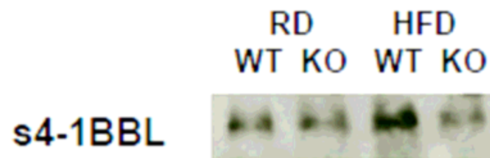
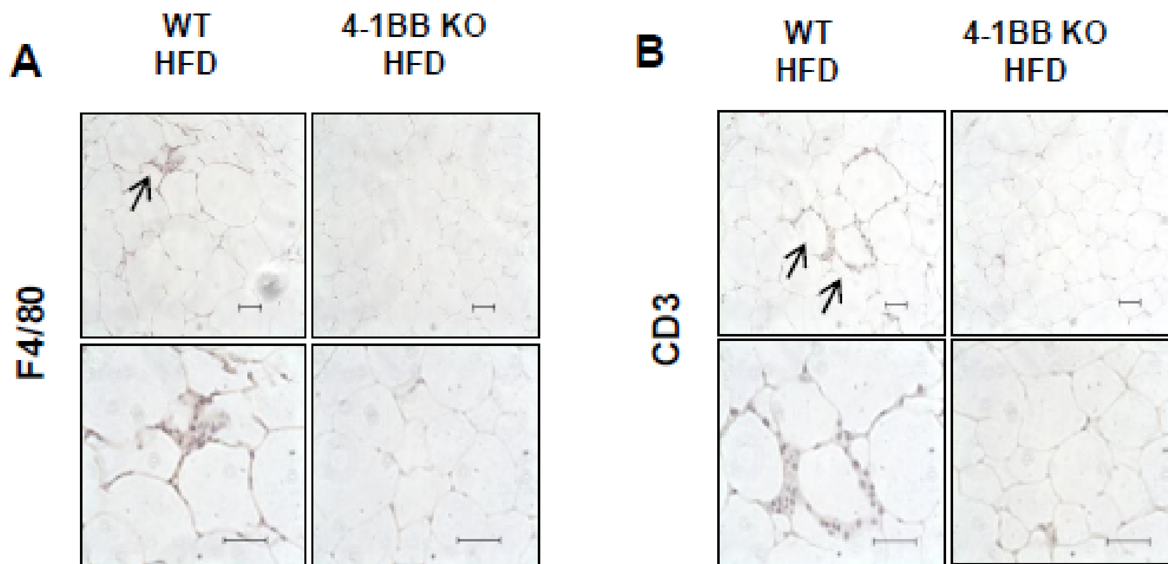
SUPPLEMENTARY DATA

Supplementary Figure 1. Levels of s4-1BBL in plasma**.** s4-1BBL protein was immunoprecipitated from plasma samples, separated by SDS-PAGE, and analyzed by Western blotting using a polyclonal anti-4-1BBL.



Supplementary Figure 2. Adipose tissue macrophages and T cells in HFD-fed 4-1BB-deficient mice. Immunohistological analysis of epididymal adipose tissue from WT and 4-1BB-deficient mice fed a HFD. Sections were stained with (A) Anti-F4/80, and (*B*) anti-CD3, in epididymal adipose tissue from WT and 4-1BB-deficient mice fed an HFD. Stained cells are indicated by arrows. Original magnification are x 200 (upper) and x 400 (lower) (Scale bar=50μm).



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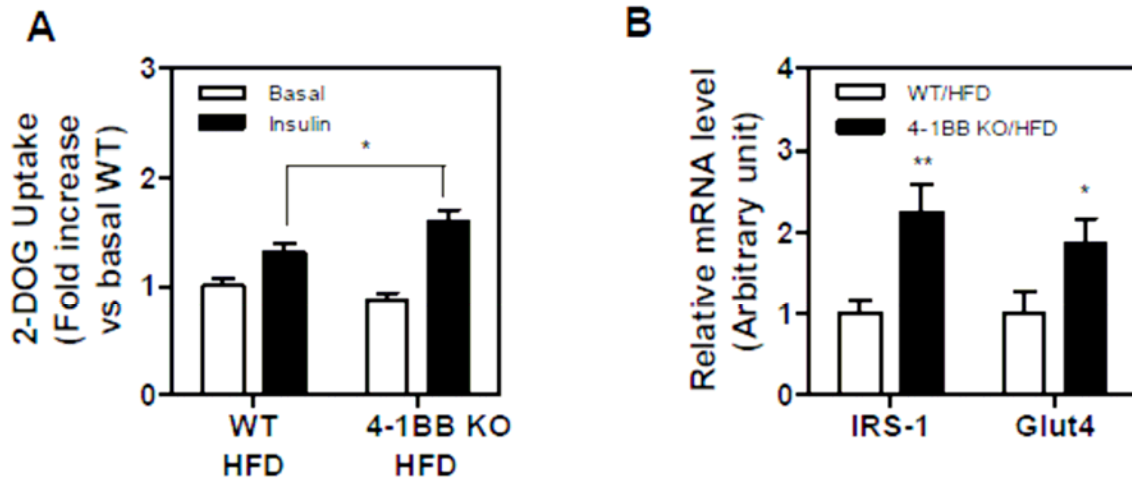
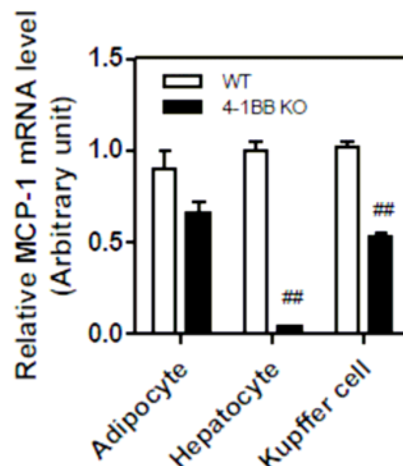
Supplementary Figure 3. Glucose uptake and IRS-1/Glut4 gene expression in adipose tissue. (*A*) Basal and insulin-stimulated glucose uptake in isolated adipose tissue from WT and 4-1BB-deficient mice fed an HFD. Results are means ± SEM. **P* < 0.05 compared with WT mice fed an HFD. (*B*) Expression of IRS-1 and Glut4 mRNA in adipose tissue from WT and 4-1BB-deficient mice fed an HFD. Levels of mRNA were estimated by qPCR. Results are means ± SEM. **P* < 0.05 compared to WT mice fed an HFD.



Supplementary Figure 4. MCP-1 gene expression in adipocytes, hepatocytes and kuppfer cell. Adipose tissue stromal vascular fractions (SVFs) were isolated from WT and 4-1BB-deficient mice. 48 hours after reaching confluence, the SVF were incubated in differentiation medium containing 0.25 µM dexamethasone, 10 µg/ml insulin, and 0.5 mM 1-methyl-3-isobutylxanthine. After 40 hours, the culture medium was changed to maturation medium containing 5µg/ml insulin, and the maturation medium was replaced with fresh medium every 2 days for 4 days. Hepatocytes and kupffer cells were isolated from WT and 4-1BB-deficient mice as described in *Supplementary Methods*. Levels of MCP-1 mRNA were estimated by qPCR. Results are means ± SEM. ##P < 0.001, versus the WT control.



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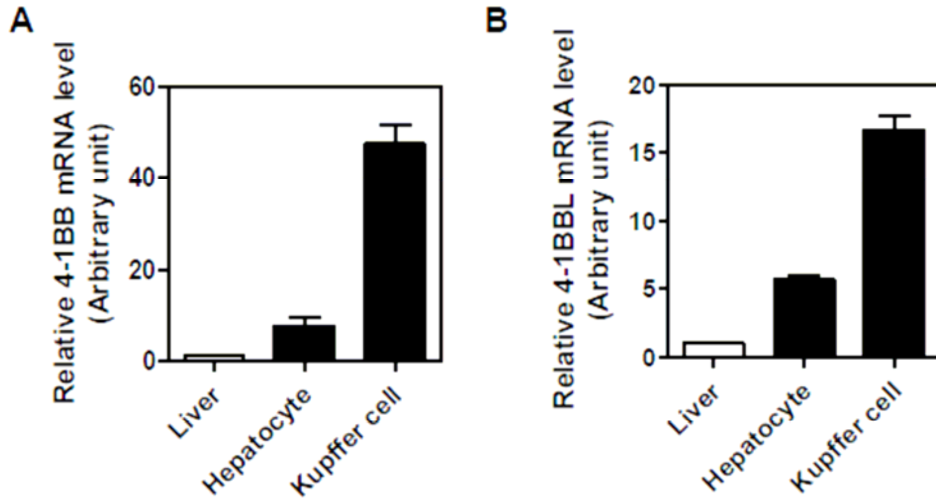
Supplementary Figure 5. 4-1BB and 4-1BBL mRNA levels in liver, hepatocytes, and kupffer cells fractions. Hepatocytes and kupffer cells were isolated from mice. Expression of (*A*) 4-1BB and (*B*) 4-1BBL gene in liver, hepatocytes, and the kupffer cells. Levels of mRNA were estimated by qPCR. Results are expressed relative to liver and are the meam±SEM.



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