Supplementary Information

Material and Methods

Animals

Generation of *Bbs2*, *Bbs4*, and *Bbs6* null mice and genotyping was described previously[6-8]. Animals were housed in a room with constant temperature (23 $^{\circ}$C) and 12 hr light/dark cycle with free access to standard mouse chow (Harlan Teklad) and water. The University of Iowa Animal Research Committee approved all protocols.

Leptin normalization/leptin resistance study

Sex- and age-matched control and BBS mice were housed in individual cages at 6-8 weeks of age. Control animals were fed *ad libitum* and daily food consumption of control mice was measured. BBS animals were given 70-80% of food consumed by wild-type controls everyday until the day before leptin treatment. Animals were equipped with ICV cannulae as previously described[1] and allowed to recover for at least one week. During ICV cannulation blood was collected from each mouse and serum leptin concentrations were measured using Mouse Leptin ELISA Kit (Crystal Chem Inc.) to ascertain that circulating leptin levels were comparable between BBS animals and wild-type controls. For food intake and body weight measurement, recombinant mouse leptin (2 μg in 1 μl, R&D Systems) or vehicle was injected ICV. One hour after ICV injections (onset of dark cycle), mice were given free access to food. Mice were sacrificed 24 hrs after ICV leptin administration, and food intake and body weight were measured. Blood was collected to confirm that circulating leptin levels were normalized in BBS animals. For protein extraction and STAT3 phosphorylation analysis, food was removed 18 hrs before ICV

injection. Leptin (2 μg in 1 μl) or vehicle was injected and animals sacrificed 2 hrs later

by CO_2 asphyxiation. Hypothalami were quickly dissected and homogenized in the lysis

buffer (50 mM HEPES pH7.5, 137 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 2 mM

EDTA, 2 mM Na_3VO_4, 10 mM NaF, 10% glycerol, protease inhibitor cocktail). After

clarification by centrifugation, total protein concentrations were measured using DC

Protein Assay kit (Bio-Rad) and equal amounts of proteins were loaded onto 4-12%

NuPAGE Bis-Tris gels. Western blotting was performed using standard protocol. Blood

was collected and plasma leptin concentrations were measured as above. Blots were

scanned and band intensities were quantitated using Image J program.

MTII study

Sex- and body weight-matched control and BBS animals were housed in individual

cages at 9-13 weeks of age with free access to food and water. Two weeks later, mice

were implanted with ICV cannulae as described above and given at least a week for

recovery. Body weight and food intake were measured daily for 4 consecutive days

before ICV injection and used as baseline values. Animals were sacrificed 24 hrs after

MTII (1 μg in 1 μl, Phoenix Pharmaceuticals Inc) or vehicle injection by CO_2

asphyxiation, and body weight, food consumption and the weights of brown adipose

tissue and reproductive fat were measured.

Antibodies

Antibodies were purchased from the following sources: rabbit anti-phospho-STAT3

(Tyr705; Cell Signaling), rabbit anti-STAT3 (C-20: SantaCruz), mouse anti-β-catenin

(BD Transduction Laboratories), mouse anti-Synaptophysin (MAB368; Millipore), rabbit

anti-Syntaxin-1 (Sigma), mouse anti-SOCS3 (1B2; Millipore), mouse anti-Myc antibody (9E10; SantaCruz), rat anti-HA (3F10; Roche Applied Science), mouse anti-FLAG (M2; Sigma), rabbit anti-POMC (Phoenix Pharmaceuticals Inc), rabbit anti-TGN46 (Sigma), rabbit anti-EEA1 (Cell Signaling), biotinylated goat anti-rabbit IgG (Vector Laboratories).

RNA extraction and qRT-PCR

Mice were sacrificed by CO_2 asphyxiation and hypothalami were excised, homogenized in TRIzol Reagent (Invitrogen) and total RNA extracted following manufacture's instruction. One μg of total RNA was used for cDNA synthesis with random primers and SuperScript II reverse transcriptase (Invitrogen). Quantitative reverse-transcription PCR was performed with iQ SYBR Green Supermix (Bio-Rad) and Mx3000P QPCR System (Stratagene). PCR was carried out in duplicate. Relative gene expression was calculated following normalization with RPL19. The PCR products were confirmed by the melt-curve analysis and sequencing. Primer sequences are shown in Table 1.

Immunohistochemistry

Wild-type and BBS mice anesthetized with ketamine (200 mg/kg) and xylazine (10 mg/kg) were perfused with PBS (5 ml/min; 50 ml) followed by 4% paraformaldehyde/0.5% glutaraldehyde in PBS (2.5 ml/min; 50 ml) using Harvard PHD 22/2000 Syringe Pump. Entire brain was excised and incubated in the same fixative overnight at 4 °C. Fixed brains were washed 3 times with PBS and incubated in 30% sucrose/PBS overnight with one change of solution after 4-6 hrs of initial incubation. Brains were vibratome-sectioned with 45 μm thickness. Sections were washed with

PBS, permeabilized in PBST (0.25% Triton X-100), and blocked by 3% normal goat serum. POMC neurons were decorated with rabbit anti-POMC antibody (1:4000), biotinylated goat anti-rabbit IgG (1:200), and Vectastain Elite ABC kit (Vector Laboratories) following manufacturer's instruction. Images were taken using Olympus IX71 inverted microscope.

Transfection and co-immunoprecipitation

HEK293T cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen). Cells were transfected in 6-well plates with 1 μg of CS2FLAG-LepR and 1 μg of indicated HA-tagged BBS proteins using FuGENE HD (Roche Applied Science). After 30 hrs of incubation, cells were lysed in the lysis buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 0.3% NP-40) supplemented with Complete Protease Inhibitor Cocktail (Roche Applied Science). Lysates were immunoprecipitated with anti-Myc (9E10; SantaCruz) or anti-HA (F-7; SantaCruz) antibodies conjugated to agarose beads for 2 hrs at 4°C. Beads were washed in the lysis buffer 4 times, and precipitated proteins were analyzed by SDS-PAGE and western blotting following standard protocol.

Immunofluorescence

ARPE-19 cells were used for immunofluorescence study. Cells were maintained in DMEM/F12 medium (Invitrogen) supplemented with 10% FBS (Invitrogen). Cells were seeded on glass cover slips in 24-well plates. ARPE-19 cells were transfected with total 1 μg of DNA using FuGENE HD (Roche). Cells were shifted to serum-free medium 24hr after transfection and further incubated for 48 hr. Cells were fixed with methanol for 6

minutes at -20°C, blocked with 5% BSA and 3% normal goat serum, and decorated with indicated primary antibodies. Alexa Flour 488 goat anti-mouse IgG (Invitrogen) and Alexa Flour 568 goat anti-rabbit IgG (Invitrogen) were used to detect primary antibodies. Cover slips were mounted on VectaShield mounting medium with DAPI (Vector lab) and images were taken with Olympus IX71 inverted microscope. RNAi constructs for human *BBS1* and *BBS2* genes were purchased from Open Biosystems.

Statistical analysis

Results were expressed as mean ± s.e.m. Comparisons between groups were made by one-way or two-way analysis of variance (ANOVA) with Fisher LSD method post-test analysis for pairwise multiple comparisons, as appropriate. $P < 0.05$ was considered to be statistically significant.

Table 1. Primer sequences used for qRT-PCR

Name	Sequence	Product size (bp)
F-mRPL19 R-mRPL19	GCAAGCCTGTGACTGTCCATT GCATTGGCAGTACCCTTCCTC	106
F-mAgRP R-mAgRP	CAGAAGCTTTGGCGGAGGT AGGACTCGTGCAGCCTTACAC	80
F-mNPY R-mNPY	TCAGACCTCTTAATGAAGGAAAGCA GAGAACAAGTTTCATTTCCCATCA	92
F-mPOMC R-mPOMC	CTGCTTCAGACCTCCATAGATGTG CAGCGAGAGGTCGAGTTTGC	120
F-mSOCS-3 R-mSOCS-3	CAGGACCTGGAATTCGTCTGAG CCGCTTGTCAAAGGTATTGTCC	125
F-mLepRb-qRT1 R-mLepRb-qRT1	TGTTTTGGGACGATGTTCCA GCTTGGTAAAAAGATGCTCAAATG	95
F-mMC3R R-mMC3R	CCTGCCTGCTTATTGGCTTTG TCTCCCGGTAGTGGTCAGGAG	140
F-mMC4R R-mMC4R	TGAGCCGAACCCAGAAGAGAC TCAGGGAAGCTCCGAGTGATT	138

References

1. Rahmouni, K., Haynes, W. G., Morgan, D. A. and Mark, A. L. (2003) Role of melanocortin-4 receptors in mediating renal sympathoactivation to leptin and insulin. *J. Neurosci.* **23**, 5998-6004.
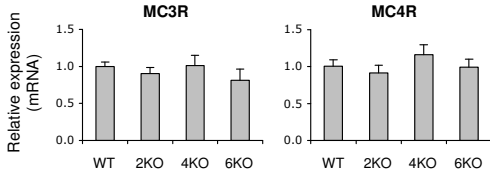
Fig. S1

Fig. S1. Expression levels of the melanocortin-3 and -4 receptor (MC3R and MC4R).
Relative amounts of mRNA were measured by qRT-PCR and normalized to RPL-19. Expression
levels in wild-type mice were arbitrarily set at 1, n = 6-9 mice per group.
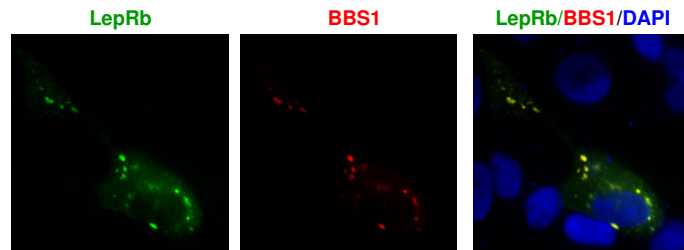
Fig. S2. Co-localization of the LepRb and BBS1. GFP-fusion of the LepRb (left panel) was co-expressed with RFP-fused BBS1 (middle panel) in ARPE-19 cells. Right panel shows the merged image with DAPI staining (blue) for nucleus.
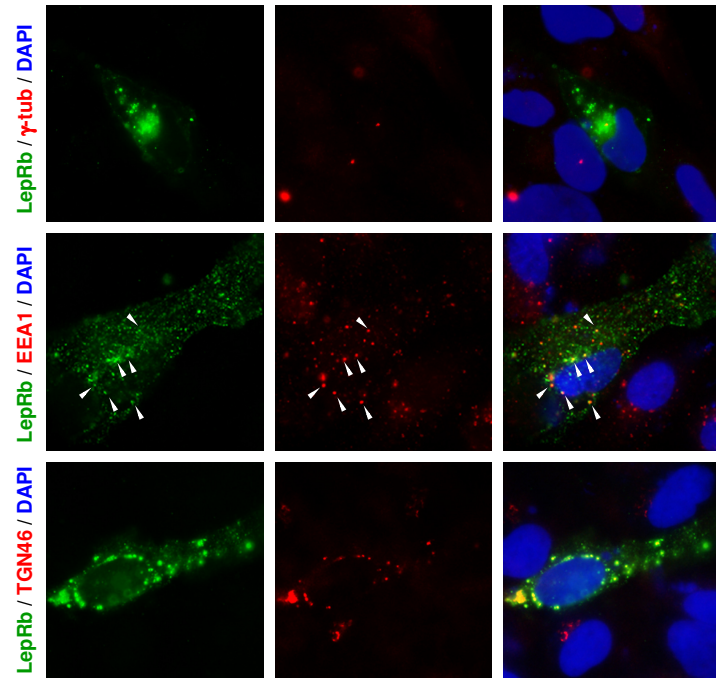
Fig. S3. LepRb localization in the TGN and small vesicles in the secretory and the endocytic pathways.
FLAG-tagged LepRb was transiently transfected into AREP-19 cells and its localization was probed with anti-FLAG antibodies. γ-tubulin (tub) is a marker for centrosomes. TGN46 is a marker for the trans-Golgi network, secretory vesicles, and recycling endosome. EEA1 is a marker for early endosomes.
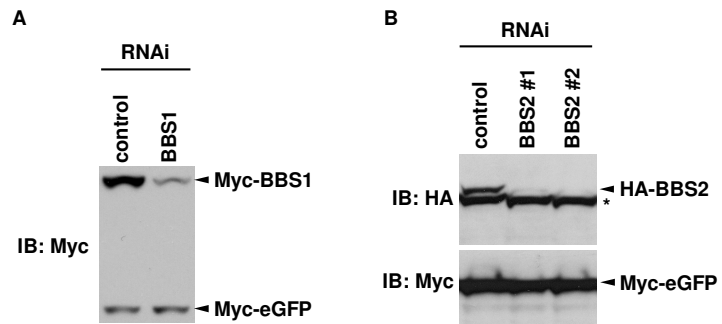
Fig. S4

Fig. S4. Suppression of *BBS1* and *BBS2* gene expressions by RNAi.
Myc-tagged BBS1 (A) and HA-tagged BBS2 (B) expression plasmids were
co-transfected with scrambled (control), BBS1, or BBS2 shRNA constructs.
For BBS2, two shRNA constructs targeting different regions of the *BBS2*
gene showed similar efficiencies. Asterisk denotes a non-specific band
recognized by anti-HA antibody. Equal amounts of GFP expression
construct were co-transfected and used to assess transfection efficiencies
and off-target effect of RNAi. Note that the intensity of the non-specific band
was not reduced by BBS2 RNAi constructs.