Extended Results

Figure S1. Identification of Antisense Oligonucleotides Efficiently Targeting Knockdown of ABHD6 Specifically in Peripheral Metabolic Tissues and Not in the Central Nervous System, Related to Figure 2

(A) C57BL/6 mice mice were maintained on chow diet in conjunction with biweekly injections (25 mg/kg) of either saline, a non-targeting control ASO, or four different ASOs targeting knockdown of ABHD6 for 4 weeks. Relative quantification of ABHD6 mRNA levels in the liver was conducted by real-time qPCR, and normalized to cyclophilin. Data represents pooled RNA samples with n=5 mice per group. ABHD6 ASOα and ABHD6 ASOβ were chosen for further characterization in high fat diet fed mice.

(B) ASO-mediated knockdown of ABHD6 is tissue-specific. C57BL/6N mice were maintained on high fat diet in conjunction with biweekly injections of either saline, a non-targeting control ASO (Control), or one of two independent ASOs targeting the knockdown of ABHD6 for 12 weeks. Western blotting was performed as described in materials and methods; 3 representative mice are shown per group.

(C) IHC determination of effective knockdown in mouse liver. C57BL/6 mice were maintained on high fat diet in conjunction with injection of 50 mg/kg per week of either a non-targeting control ASO (Control) or an ASO targeting knockdown of ABHD6 for 21 weeks. Immunohistochemical (IHC) detection using an affinity purified ABHD6 antibody was performed.

(D) ASO treatment does not reduce ABHD6 expression in hippocampal neurons. Hippocampal neurons were subjected to IHC staining from the same experiment shown in panel C.

(E) ASO treatment does not reduce ABHD6 or lipogenic gene expression in the hypothalamus. C57BL/6 Mice were fed a high fat diet and treated with either Control ASO or ABHD6 ASO for a period of 8 weeks. At necropsy, the whole brain was removed and the hypothalamus isolated, homogenized and analyzed for mRNA expression. Data is expressed as fold change from control ASO treated group and no significant differences were detected. Eno2, enolase 2 is a neuron specific marker; Npy, neuropeptide 1 is a hypothalamus marker; SREBP-1c, sterol response element binding protein 1c; SCD1, stearoyl-CoA desaturase 1; FAS, fatty acid synthase; ACC1, acetyl-CoA carboxylase 1.

Figure S2. ABHD6 Knockdown Consistently Reduces Hepatic Lipogenic Gene Expression, Without Altering AMPK Activiation, Related to Figure 3

(A) C57BL/6 mice mice were maintained on chow diet in conjunction with biweekly injections (25 mg/kg) of either saline, a non-targeting control ASO, or four different ASOs targeting knockdown of ABHD6 for 4 weeks. Relative quantification of lipogenic gene expression in the liver was conducted by real-time qPCR, and normalized to cyclophilin. Data represents pooled RNA samples with n=5 mice per group. FAS, fatty acid synthase; ACC1, acetyl-CoA carboxylase 1; SCD1, stearoyl-CoA desaturase 1.

(B) Mice were fed a high fat diet (HFD) and treated with a control non-targeting ASO or two independent ASOs targeting the knockdown of ABHD6 for 12 week. Livers were excised and immediately snap-frozen in liquid nitrogen. Protein extracts from the liver were analyzed by Western blotting for phospho-AMPKα (Thr172), total AMPKα, phospho-AMPKβ1 (Ser108), total AMPKβ, and phospho-ACC (ser79); three representative animals are shown for each group.

Figure S3. Hepatic Triacylglycerol (TAG) and Diacylglycerol (DAG) Levels in ABHD6 Knockdown Mice, Related to Figure 3

Male C57BL/6 Mice were fed a chow or high fat diet (HFD) and treated with a control non-targeting ASO or an ASO targeting the knockdown of ABHD6 for 12 weeks.

(A) Hepatic levels of TAG species were measured by mass spectrometry as described in the methods section.

(B) Hepatic levels of DAG species were measure by mass spectrometry as described in the methods section

Data represent the mean ± SEM (n = 6), * = $P < 0.05$ (vs. control ASO within each diet).

Figure S4. Hepatic Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), and Phosphatidic acid (PA) Levels in ABHD6 Knockdown Mice, Related to Figure 6

Male C57BL/6 Mice were fed a chow or high fat diet (HFD) and treated with a control non-targeting ASO or an ASO targeting the knockdown of ABHD6 for 12 weeks.

(A) Hepatic levels of PC species were measured by mass spectrometry as described in the methods section.

(B) Hepatic levels of PE species were measure by mass spectrometry as described in the methods section.

(C) Hepatic levels of PA species were measured by mass spectrometry as described in the methods section.

Data represent the mean ± SEM (n = 6), * = $P < 0.05$ (vs. control ASO within each diet).

Figure S5. Hepatic Phosphatidylserine (PS), Phosphatidylinositol (PI), and Phosphatidylglycerol (PG) Levels in ABHD6 Knockdown Mice, Related to Figure 6

Male C57BL/6 Mice were fed a chow or high fat diet (HFD) and treated with a control non-targeting ASO or an ASO targeting the knockdown of ABHD6 for 12 weeks.

(A) Hepatic levels of PS species were measured by mass spectrometry as described in the methods section.

(B) Hepatic levels of PI species were measure by mass spectrometry as described in the methods section.

(C) Hepatic levels of PG species were measured by mass spectrometry as described in the methods section.

Data represent the mean ± SEM (n = 6), * = P < 0.05 (vs. control ASO within each diet).

Figure S6. Hepatic Lysophosphatidylglycerol (LPG), Lysophosphatidylcholine (LPC), Lysophosphatidylethanolamine (LPE), Lysophophatidylinositol (LPI), Lysophosphatidylserine (LPS), and Lysophosphatidic Acid (LPA) Levels in ABHD6 Knockdown Mice, Related to Figure 6

Male C57BL/6 Mice were fed a chow or high fat diet (HFD) and treated with a control non-targeting ASO or an ASO targeting the knockdown of ABHD6 for 12 weeks. Hepatic levels of LPG (A), LPC (B), LPI (C), LPE (D), LPS (E), and LPA (F) species were measured by mass spectrometry as described in the methods section. Data represent the mean ± SEM (n = 6), * = P < 0.05 (vs. control ASO within each diet).

Figure S7. ABHD6 Knockdown Does Not Significantly Alter Plasma Lipid or Glucose Homeostasis in Chow-Fed Mice, but Significantly Reduces Certain Species of Hepatic Triacylglycerols (TAG), Related to Figure 3

Male C57BL/6 mice were maintained on a standard chow diet, and starting at 8 weeks of age began receiving biweekly treatments with a control non-targeting ASO or an ASO targeting the knockdown of ABHD6 for 12 weeks.
(A) Plasma TAG levels.
(B) Plasma insulin levels.
(C) Glucose tolerance tests in mice treated with ASOs for 10-11 weeks.
(D) Insulin tolerance tests in mice treated with ASOs for 10-11 weeks.
(E) ABHD6 knockdown causes reduction of certain short chain TAG species in mouse liver.
All data represent the mean ± SEM (n = 5-6); * = *P* < 0.05 (vs. control ASO group).

Table S1. Intestinal fatty acid absorption, Related to Figure 2, was determined by the sucrose polybehenate method as described in methods. Measurements were taken in mice treated with either Control ASO or ABHD6 ASOβ, and fed a high fat diet for 9 weeks. Data shown are % absorption values and represent the mean ± S.E.M (n=14-15). * = P < 0.05 (vs. Control ASO within each diet group).

	Control ASO	ABHD6 ASOβ
Total Fatty Acids	0.826 ± 0.01	0.856 ± 0.007
C14:0	0.944 ± 0.005	0.954 ± 0.005
C14:1	0.407 ± 0.07	0.495 ± 0.05
C15:0	0.752 ± 0.02	0.827 ± 0.01 *
C16:0	0.839 ± 0.01	0.876 ± 0.008 *
C16:1 trans	0.958 ± 0.004	0.975 ± 0.001 *
C16:1 w7	0.978 ±0.002	0.985 ± 0.001 *
C17:0	0.595 ± 0.03	0.68 ± 0.02 *
C17:1	0.959 ± 0.007	0.979 ± 0.004 *
C18:0	0.351 ± 0.05	0.442 ± 0.03
C18:1 trans	0.491 ± 0.02	0.575 ± 0.02 *
C18:1 delta 9 (Oleic)	0.939 ± 0.004	0.953 ± 0.003 *
C18:1 delta 11	0.925 ± 0.005	0.942 ± 0.003 *
C18:2 w6	0.972 ± 0.002	0.978 ± 0.001 *
C18:3 w3	0.978 ± 0.001	0.984 ± 0.001 *
C18:4 w3	0.878 ± 0.02	0.878 ± 0.02
C20:0	N.D.	N.D.
C20:1 w9	0.785 ± 0.02	0.823 ± 0.013
C20:2 w6	0.941 ± 0.005	0.951 ± 0.005
C22:4 w6	0.925 ± 0.007	0.922 ± 0.004

EXTENDED EXPERIMENTAL PROCEDURES

Mice

For ABHD6 knockdown studies, at 6-8 weeks of age male C57BL/6N mice (Harlan) were either maintained on standard rodent chow or switched to a high fat diet for a period of 4-12 weeks, and simultaneously injected with antisense oligonucleotides (ASOs) biweekly (25 mg/kg BW) targeting knockdown of ABHD6 as previously described (Brown et al., 2008a; Brown et al., 2008b; Brown et al., 2010; Lord et al., 2011). The high fat diet (HFD) was prepared by the Wake Forest School of Medicine institutional diet core, and contains ~45% of energy as lard (16:0 = 23.3%, 18:0 = 15.9%, 18:1 = 34.8%, 18:2 = 18.7%); this diet has been previously described (Brown et al., 2010; Lord et al., 2011). The 20-mer phosphorothioate ASOs were designed to contain 2'-0-methoxyethyl groups at positions 1 to 5 and 15 to 20 and were synthesized, screened, and purified as described previously (Crooke et al., 2005) by ISIS Pharmaceuticals, Inc. (Carlsbad, CA). For tissue distribution studies, at 6-8 weeks of age male C57BL/6N mice (Harlan) were either maintained on standard rodent chow or switched to a high fat diet for a period of 10 weeks without ASO treatment. For WWL-70 treatment studies, 6 week old male C57BL/6N mice were switched to a high fat diet for a period of 8 weeks, and were simultaneously injected intraperitoneally with either a vehicle (9:1 dimethylsulfoxide:saline) or WWL-70 (10 mg/kg BW) once daily. All mice were maintained in an American Association for Accreditation of Laboratory Animal Care (AALAC) approved specific pathogen-free environment on a 12:12 h light:dark cycle and allowed free access to food and water. All experiments were performed with the approval of the institutional animal care and use committee at Wake Forest School of Medicine.

Necropsy, Tissue Histology, and Plasma Analyses

At necropsy, all mice were terminally anesthetized with ketamine/xylazine (100-160 mg/kg ketamine and 20-32 mg/kg xylazine), and a midline laparotomy was performed. Blood was collected by heart puncture. Following blood collection, a whole body perfusion was conducted by puncturing the inferior vena cava and slowly delivering 10 ml of saline into the heart to remove blood from tissues. Tissues were collected and snap frozen in liquid nitrogen for most analyses. For histology, tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and

eosin. Plasma glucose levels were measured using a glucometer (Ascensia Countour, Bayer Healthcare, Leverkusen, Germany). Plasma insulin levels were measured by ELISA (Crystal Chem. Inc., Downers Grove, IL, USA). Plasma non-esterified fatty acids (NEFA) were quantified enzymatically (NEFA-HR; Wako Diagnostics, Richmond, VA, USA). Plasma triacylglycerol levels were quantified enzymatically (L-Type TG M, Wako Diagnostics, Richmond, VA, USA). A detailed description of total plasma cholesterol and lipoprotein cholesterol distribution analyses has been previously described (Brown et al., 2008a; Brown et al., 2008b; Brown, et al., 2010).

Body Composition Analysis by Magnetic Resonance Imaging

A subset of mice was treated with ASO for 8 weeks prior to magnetic resonance imaging. Isoflurane anesthetized mice were imaged in a Bruker 7T MRI used for small animals as previously described (Olson et al., 2012). Slices of $1mm^2$ were analyzed using ImageJ software using the threshold adjustment method to calculate adipose mass and lean body mass.

Food Intake Analysis

At 6 weeks of age, mice were switched from rodent chow onto a high fat diet and injected with either a control or ABHD6 ASO biweekly (25 mg/kg BW) for 2 weeks to initiate knockdown. Following 2 weeks of diet/ASO treatment, mice were moved into individual cages fitted with wire bottom inserts to avoid coprophagy. These mice continued to receive high fat diet and biweekly ASO injections, and quantitative food recovery was determined once daily for 28 consecutive days. Food intake is expressed as cumulative food intake for this 4-week period.

Glucose and Insulin Tolerance Tests

Intraperitoneal glucose tolerance tests and insulin tolerance tests were performed essentially as previously described (Brown et al., 2008a) in mice treated with diet and ASO for 10-11 weeks. Briefly, glucose tolerance tests (GTT) were performed after an overnight (10 hour, 11:00 pm – 9:00 am) fast by injecting 1 mg/kg body weight of glucose into the peritoneal cavity. Insulin tolerance tests (ITT) were performed after a short-term fast (4 hour, 9:00 am – 1:00 pm) by injecting 0.75 U/kg body weight into the peritoneal cavity. Plasma glucose levels were measured using a commercial glucometer (Ascensia Countour, Bayer).

In Vivo Cannabinoid Receptor (CB1) Receptor Signaling Analyses

Mice were injected with control ASO or ABHD6 ASOβ and maintained on a HFD for a period of 8 weeks. After an overnight fast (9:00 p.m. - 9:00 a.m.), mice were anesthetized with isoflurane (4% for induction, 2% for maintenance), and were maintained on a 37^{o}C heating pad to control body temperature. Mice received either vehicle (cremophor EL:ethanol:saline at a 1:1:18 ratio) or the CB1 agonist (CP-55,490, Cayman Chemical # 13241) at a dose of 0.1 mg/kg body weight directly into the portal vein. Exactly 5 minutes later the liver was excised and snap frozen in liquid nitrogen. Protein extracts were analyzed by Western blotting to examine CB1 signaling.

In Vivo Determination of Very Low Density Lipoprotein (VLDL) Secretion

Mice were injected with control ASO or ABHD6 ASOβ and maintained on a HFD for a period of 11 weeks prior to experiment. After a 4-hour fast, male mice were anesthetized with isoflurane (4% for induction, 2% for maintenance) and Triton WR 1339 (500 mg/kg body weight; Sigma) was delivered via retro-orbital injection to block lipolysis. Thereafter, blood samples were collected from anesthetized mice by retro-orbital bleeding at 0, 0.5, 1, 2, and 3 h after injection. Plasma was harvested from the blood samples and used to quantify TG mass by enzymatic assay.

Quantification of the Absorption of Dietary Fat

Mice were injected with control ASO or ABHD6 ASOβ and maintained on a HFD for a period of 9 weeks prior to experiment. Thereafter, fatty acid absorption was measured using the Olestra® method (Jandacek et al., 2004). Briefly, mice were fed the same HFD containing the nonabsorbable fat sucrose polybehenate (1.2%; wt/wt) for 4 consecutive days. Feces were collected from mice housed individually in cages with wire mesh floors to avoid coprophagy. Weighed samples of the synthetic diet and feces were saponified with methanolic NaOH, extracted with hexane, converted to methyl esters, and analyzed by gas chromatography (Brown et al., 2008a) to quantitate behenic acid (BA, 22:0) and saturated (14:0, 16:0, 18:0), monounsaturated (16:1, 18:1), and polyunsaturated (18:1, 18:2, 18:3ω3, 20:5ω3, 22:6ω3) fatty acids. The coefficient of absorption for each FA was computed as $\{1 - (FA/BA)_{feces} / (FA/BA)_{diet}\} \times 100$.

Indirect Calorimetry and Metabolic Cage Measurements

Mice were injected with control ASO or ABHD6 ASOβ and maintained on a HFD for a

period of 7-8 weeks prior to the experiment. Mice were acclimated to metabolic cages for 24 h, and on day 2 were injected with ASO. Thereafter, physical activity, oxygen consumption (VO_2), and the respiratory exchange ratio (RER) were continually monitored for an additional 72 hours using the Oxymax CLAMS system (Columbus Instruments) at a temperature of 22^{o}C. Data represent the last 6 a.m. to 6 a.m. period after adequate acclimation.

In Vivo Quantification of *De Novo* Fatty Acid Synthesis Rates

Quantification of hepatic lipogenesis was carried out as described previously (Shimano et al., 1996). Briefly, non-fasted mice were intraperitoneally injected with 10 mCi of [^{3}H] water (Moravek Biochemicals and Radiochemicals, Brea, CA, USA). One hour post-injection liver and plasma were collected. Plasma was used to determine the plasma [^{3}H] water specific activity. A portion of the liver (200-300 mg) was digested in potassium hydroxide. Neutral sterols were extracted with petroleum ether and discarded. Fatty acids were acidified by adding hydrochloric acid to the aqueous phase, and were extracted with hexane. Aliquots of the hexane extract were dried down, and residual water was removed by incubating at 80°C in a vacuum oven. Methanol and scintillation fluid were added and cpm were counted. The rates of fatty acid synthesis were calculated as μmol of [^{3}H]-water incorporated into fatty acids per hour per gram of tissue.

Primary Hepatocyte Isolation and Determination of Lipogenic Rates

Chow fed C57BL/6 male mice were injected with control ASO or ABHD6 ASOβ for a period of 8 weeks prior to the experiment. After 8 weeks of ASO treatment, mouse primary hepatocytes were isolated by collagenase perfusion as previously described (Lord et al., 2011). Hepatocytes were pooled from 2 donor mice from each ASO group each day for quantification of lipogenic rates. Briefly, freshly isolated hepatocytes were initially seeded on collagen coated 6-well plates at a seeding density of 1x10^6 cells per well. Thereafter, cells were cultured for 3 h in serum free William's E Medium, and during the last hour (hour 2-3) all cells were supplemented with a lipase inhibitor cocktail (diethylumbelliferyl phosphate = DEUP + diethyl-p-nitrophenyl phosphate = E-600) to measure triacylglycerol synthesis without the complications of the simultaneous lipolysis/re-esterification reactions. To generate lipase inhibitor stocks, E600 was dissolved in water (2.5 mg/ml) and DEUP was solubilized in DMSO (50 mg/ml). These inhibitors were added to a final concentration of 50 μg/ml DEUP and 500 μM E-600 for 1

hour prior to radiotracer addition and were present throughout the labeling time course. Following lipase inhibition, cells were labeled over a time course with 5 µCi/ml [^{3}H]oleic acid and 0.5 µCi/ml [^{14}C]acetate. Following labeling, cells were gently washed twice with PBS and total lipid extracts were made from the remaining cells using the method of Bligh and Dyer (1959). Thereafter, carrier standards were added to samples and lipid classes were separated by thin layer chromatography (TLC) using Silica Gel 60 plates and a solvent system containing hexane-diethyl ether-acetic acid (70:30:1). Lipids were visualized by exposure to iodine vapor, and bands corresponding to CE, TG, and PL were scraped and counted. Protein concentration was determined using the bicinchoninic acid (BCA) assay.

Generation and Purification of a Polyclonal Antibody Against ABHD6

A maltose binding protein (MBP) ABHD6 fusion protein construct was produced by inserting the DNA sequence encoding amino acids 119-315 of mouse ABHD6 into the pMAL-C2 vector (New England Biolabs) at BamHI and HindIII restriction sites. For PCR cloning an expression vector containing murine ABHD6 cDNA (pCMV-SPORT6-mABHD6) was purchased from Open Biosystems (#MMM1013-7512548) and used as a template for PCR cloning using the following primers: forward (5'-gcggatccgacctgtccatagtggggcaag-3') and reverse (5'-gcaagcttctatgtcttcctcggtctctccastcac-3'). The resulting pMAL-mABHD6$^{119\text{-}315}$ vector was sequence confirmed and then used then to drive fusion protein expression in Escherichia coli using an isopropyl-β-D-1-thiogalactopyranoside (IPTG) inducible expression system. Recombinant MBP-mABHD6$^{119\text{-}315}$ fusion proteins were purified by affinity chromatography using an amylose resin. Given that some degradation products were detected after amylose affinity purification, the full-length fusion protein was further purified by passing over an 8% agarose column. Three milligrams of the resulting affinity purified MBP-mABHD6$^{119\text{-}315}$ fusion protein was sent to Lampire (Pipersville, PA) for antibody production in 3 independent rabbits according to standard protocol. To obtain affinity purified antibodies, approximately 20mg of MBP-mABHD6$^{119\text{-}315}$ fusion protein was coupled to 4 ml Amino link coupling gel (Pierce) using the manufacturer's protocol to generate an affinity column. Then, 10 ml of rabbit antisera was placed on this affinity column and rocked overnight at 4^{o}C. The column was drained and washed, and antibody was eluted with 0.01 M glycine, 10% (v/v) ethylene glycol, pH 2.8. The eluted antibody was further purified by passing it over a MBP affinity column and collecting the flow-thru.

To obtain this antibody for research purposes, please contact Dr. J. Mark Brown (brownm5@ccf.org).

Immunoblotting and Immunohistochemistry

Whole tissue homogenates were made from multiple tissues in a modified RIPA buffer as previously described[8]. Proteins were separated by 4–12% SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and proteins were detected after incubation with specific antibodies as previously described (Brown et al., 2004). Antibodies used included: affinity-purified anti-ABHD6 rabbit polyclonal (described above), anti-histone 3 rabbit monoclonal (Cell Signaling Technologies #4499), anti-β actin rabbit monoclonal (Cell Signaling Technologies #4970), anti-BIP rabbit monoclonal (Cell Signaling Technologies #3177), anti-FAS rabbit monoclonal, anti-SCD1 rabbit monoclonal (Cell Signaling Technologies # 2794), anti-phospho-p44/p42 MAPK$^{Thr202/Tyr204}$ rabbit monoclonal (Cell Signaling Technologies # 4370), anti-total-p44/p42 MAPK (Cell Signaling Technologies # 4695), anti-phospho-AMPKαThr^{172} rabbit monoclonal (Cell Signaling # 2535), anti-total AMPKα rabbit monoclonal (Cell Signaling Technologies # 2603), anti-phospho-AMPKβSer^{108} rabbit polyclonal (Cell Signaling Technologies # 4181), anti-total AMPKβ rabbit monoclonal (Cell Signaling Technologies # 4150), anti-phospho-ACCSer^{79} rabbit polyclonal (Cell Signaling Technologies # 3661), anti-total ACC rabbit monoclonal (Cell Signaling Technologies # 3676), anti-rabbit IgG-HRP linked secondary (Cell Signaling Technologies #7074), and anti-mouse IgG-HRP linked secondary (Cell Signaling Technologies #7076). For immunohistochemical (IHC) detection of ABHD6 in liver and brain section, tissues were mounted in OCT and frozen in dry ice and methylbutane. Sections of 6 µm thickness were prepared using a cryostat and air dried overnight. Slides were subsequently fixed in cold acetone for 5 minutes, and rinsed 3x for 2 minutes with TBS. Thereafter, endogenous peroxidase was quenched by the addition of 3% hydrogen peroxide for 10 minutes, followed by 3x rinsing with TBS for 2 minutes each. Slides were then blocked with Cyto-Q Background Buster (Innovex Biosciences), and subsequently incubated with affinity purified ABHD6 rabbit polyclonal antibody for 1 hour at room temperature (1:100 dilution). Following 3 rinses with with TBS for 2 minutes each, a goat anti-rabbit HRP secondary antibody was added (1:500) and incubated for 30 minutes at room temperature, followed by sequential rinsing and application of the chromagen DAB.

Microarray and Quantitative Real-Time PCR Analysis of Hepatic Gene Expression

Tissue RNA extraction was performed as previously described for all mRNA analyses (Brown et al., 2008a; Brown et al., 2008b; Brown, et al., 2010; Lord et al., 2012). For microarray analyses, hepatic total RNA samples were further purified using the RNeasy MinElute Cleanup Kit (Qiagen # 74204) followed by quality assessment using an Agilent 2100 bioanalyzer. Samples with RIN values > 8.0 were carried forward for cRNA synthesis and hybridization to GeneAtlas MG-430 PM Array Strips (Affymetrix, Santa Clara, CA) following the manufacturer's recommended protocol. Microarray analyses were performed by the Wake Forest School of Medicine Microarray Shared Resource Core using standard operating procedures. The raw data generated were normalized using the robust multi-array average (RMA) method (Irizarry et al., 2003), and functional annotation to gene ontology was performed using Ingenuity-IPA software (Ingenuity Systems, Inc., Redwood City, CA). Quantitative real-time PCR (qPCR) analyses were conducted as previously described (Brown et al., 2008a; Brown et al., 2008b; Brown, et al., 2010; Lord et al., 2011). mRNA expression levels were calculated based on the ΔΔ-CT method. Q-PCR was conducted using the Applied Biosystems 7500 Real-Time PCR System. Primers used for Q-PCR are available on request.

Analysis of Hypothalamic Gene Expression in ABHD6 ASO Treated Mice

Mice were sacrificed at 8 weeks post-injection of either control versus ABHD6 ASOβ, and brains were dissected and placed in an ice-cold brain matrix. The hypothalamus was isolated, placed on a chilled dissecting block, and 1-mm diameter circular brain punches were collected from the basal lateral hypothalamus. Total RNA was isolated from each punch and purified using an RNeasy Mini Kit (Qiagen). RNA quantification and purity were determined with purity set at 260/280 and 260/230 ≥ 2.0 (NanoDrop 1000 Spectrophotometer, Thermo Scientific, DE, USA). Total RNA (1μg) was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). Real-time qPCR was performed using FastStart Universal SYBR Green Master Mix (ROX) and specific Forward and Reverse primer probe assay sets. Data were analyzed using the ΔΔCT method with eno2 serving as the reference standard.

Hepatic Lipid Analyses

Extraction of liver lipids and quantification of molecular species by mass spectrometry were performed essentially as previously described (Lord et al., 2011; Myers et al., 2011; Ivanova et al., 2007; Callender et al., 2007). For glycerophospholipid analyses, liver tissue was extracted using a modified Bligh and Dyer procedure (Bligh and Dyer 1959). Approximately 10 mg of frozen mouse liver was homogenized in 800 µl of ice-cold 0.1 N HCl:CH_3OH (1:1) using a tight-fit glass homogenizer (Kimble/Kontes Glass Co, Vineland, NJ) for approximately 1 min on ice. The suspension was then transferred to cold 1.5 ml Eppendorf tubes and vortexed with 400 µl of cold $CHCl_3$ for 1 min. The extraction proceeded with centrifugation (5 min, 4^{o}C, 18,000 x g) to separate the two phases. The lower organic layer was collected, and the solvent was evaporated. The resulting lipid film was dissolved in 100 µl of isopropanol:hexane:100 mM NH_4COOH(aq) 58:40:2 (mobile phase A). Quantification of glycerophospholipids was achieved by the use of an LC-MS technique employing synthetic odd-carbon diacyl and lysophospholipid standards. Typically, 200 ng of each odd-carbon standard was added per 10-20 mg tissue. Glycerophospholipids were analyzed on an Applied Biosystems/MDS SCIEX 4000 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) and a Shimadzu high pressure liquid chromatography system with a Phenomenex Luna Silica column (2 × 250 mm, 5-µm particle size) using a gradient elution as previously described (Ivanova et al., 2007; Myers et al. 2011). The identification of the individual species, achieved by LC/MS/MS, was based on their chromatographic and mass spectral characteristics. This analysis allows identification of the two fatty acid moieties but does not determine their position on the glycerol backbone (*sn-1* versus *sn-2*). Neutral lipids from frozen mouse liver tissue (10-15 mg) were extracted by homogenizing tissue in the presence of internal standards (14:0 monoacylglycerol, 24:0 diacylglycerol, and 42:0 triacylglycerol) in 2 ml 1X PBS and extracting with 2 ml ethyl acetate:trimethylpentane (25:75). After drying the extracts the lipid film was dissolved in 1 ml hexane:isopropanol (4:1) and passed through a bed of Silica gel 60 Å to remove any remaining polar phospholipids. Solvent from the collected fractions was evaporated and the lipid film was redissolved in 100 µl 9:1 CH_3OH:$CHCl_3$, containing 10 µl of 100 mM CH_3COONa for MS analysis essentially as described (Lord et al., 2011; Callendar et al., 2007). The specific analysis of endocannabinoid lipids such as 2-arachidonylglycerol and anandamide was conducted as previously described (Long, et al., 2009).

Cloning and Purification of GST-Tagged Murine ABHD6

The coding sequence of mABHD6 was cloned into the yeast expression vector pYEX4T-1 (Clontech Laboratories Inc.; Mountain View, USA) creating an N-terminal GST-fusion protein under a copper-inducible promoter. The construct was verified by sequencing (Eurofins MWG Operon; Ebersberg, Germany) and transformed into *S.cerevisiae* wildtype BY4742 (MATa; his3Δ 1; leu2Δ 0; lys2Δ 0; ura3Δ 0) obtained from Euroscarf, Frankfurt, Germany. Expression of GST-mABHD6 was induced in mid-log phase at 30°C using 0.5 mM $CuSO_4$. Thereafter, cells were harvested and protoplasts were obtained by zymolyase treatment (Zymolyase ® 20T; AMS Biotechnology Limited, Germany). Cells were disrupted by sonication in 10 mM PBS (pH 7.4) and 0.2% NP-40. Subsequently, the lysate was centrifuged (10.000 x g, 15 min) and incubated with Glutathione-Sepharose beads (Glutathione-Sepharose 4B; GE Healthcare, Piscataway, NJ) for 30 minutes at room temperature. After extensive washing with PBS/0.2 % NP-40, the purified protein was eluted using a buffer containing 16 mM GSH, 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 0.2% NP-40 and dialyzed overnight against a buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.05 % NP-40, 250 mM sucrose, 1 mM EDTA and 20 µM DTT at 4°C. To generate the S148A mutant protein, site-directed mutagenesis was performed using the the GeneArt® Site-Directed Mutagenesis System (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instructions, and the protein was purified as described for the wild type protein.

ABHD6 Enzyme Activity Assays

For determination of monoacylglycerol hydrolase (MGH) activity, monoacylglycerol (MG) substrates [*rac*-1(3)-oleoylglycerol (*rac*-1(3)-OG) or 2-oleoylglycerol (Sigma Aldrich, St. Louis, USA)] were dried under nitrogen and dispersed by brief sonication in 50 mM Tris-HCl (pH 8.0), sodium acetate buffer (pH 4.5-5.5), MES buffer (pH 5.5-6.5) or bistrispropane buffer (pH 6.5-9), 100 mM NaCl, 0.1% NP-40, 1 mM defatted BSA, and various concentrations of MG. Then, 10 µl of purified ABHD6 (approx. 0.2 µg) was incubated with 90 µl of substrate for 20 min at 37°C. Thereafter, the reaction was terminated by addition of 100 µl $CHCl_3$ and glycerol release was quantified in the aqueous supernatant using a commercially available kit (Free glycerol kit; Sigma Aldrich, St.Louis, USA). Phospholipids and lysophospholipids [LPC, LPG, LPA, LPS, LPE (all 18:1)] were dried under nitrogen and solubilized in assay buffer by brief sonication (5

sec). Neutral lipids were emulsified on ice by sonication in the presence of PC at a molar ratio PC:RP (TO, CO, DO) of 1:7 (4x 1 min, 1 min break). The reaction buffer contained 1 mM substrate, 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 1 mM defatted BSA. Additionally, 10 µl of purified ABHD6 (approx. 0.2 µg) was incubated with 40 µl of substrate for 20 minutes at 37°C. Alternatively, LPG hydrolase activity was determined in a buffer containing 50 mM Tris/HCl (pH 8), 100 mM NaCl, 1 mM EDTA and 4.8 mM CHAPS. All reactions were terminated by heat inactivation of the enzyme at 70°C for 1 min. The release of free fatty acids was quantified enzymatically (Nefa C Kit; Wako Chemicals, Germany).

Statistical Analysis

All data are expressed as the mean ± S.E.M. or S.D., and were analyzed using either a one-way or two-way analysis of variance (ANOVA) followed by Student's t tests for post hoc analysis using JMP version 5.0.12 software (SAS Institute, Cary, NC).

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