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Supporting Information for

The Ubiquitin E3 Ligase BFAR Promotes Degradation of PNPLA3

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Supporting Information Text

Abbreviations:
BCA, Bicinchoninic acid; DMEM, Dulbecco′s Modified Eagle Medium; DMSO, Dimethyl Sulfoxide; FBS, Fetal Bovine Serum; FT, flow through; HEK293T, human embryonal kidney 293T; mAb, monoclonal antibody; pAb, polyclonal antibody; PBS, Phosphate Buffered Saline; PVDF, polyvinylidene difluoride; SDS, Sodium dodecyl-sulfate; SDS-PAGE, Sodium dodecyl-sulfate polyacrylamide gel electrophoresis; TECP, Tris(2-carboxyethyl)phosphine hydrochloride

Materials

REAGENT	SOURCE	IDENTIFIER
Antibodies (Ab)		
Goat anti-rabbit IgG 2º Ab, Alexa Fluor™ 488	Invitrogen	A-11034
Goat anti-mouse (m) IgG 2º Ab, Alexa Fluor™ 555	Invitrogen	A-21422
Mouse anti-mABHD5 monoclonal Ab (mAb)	Abnova	H00051099-M01
Mouse anti-human (h)PNPLA3 mAb (11C5)	(1)	
Mouse anti-ubiquitin mAb	Santa Cruz	P4D1
Mouse anti-hBFAR mAb (10E10)	This study- see Methods	
Mouse anti-mBFAR mAb (40H7)	This study-see Methods	
Rabbit anti-h/mATGL polyclonal Ab (pAb)	Cell Signaling Technology	2138S
Rabbit anti-h/mBI-1 pAb	Thermo Fisher Scientific	26782-1-AP
Rabbit anti-calnexin pAb	Abcam	ab10286
Rabbit anti-mPNPLA3 mAb (19A6)	(1)	
Rabbit anti-h p21 mAb	Cell Signaling Technology	2947S
Rabbit anti-h/m perilipin 2 pAb	Abcam	Ab108323
Rabbit anti-mSREBP-1 mAb (20B12)	(2)	
Bacterial and Virus Strains		
E. coli BL21 Star™ (DE3) pLysS	Thermo Fisher Scientific	C602003
E. coli DH5a	GoldBio	CC-101-TR
E. coli DH10Bac	Thermo Fisher Scientific	10361012
MAX Efficiency™ DH5α	Thermo Fisher Scientific	18258012
One Shot™ Stbl3™ *E. coli*	Thermo Fisher Scientific	C737303
Buffers		

4X Laemmli Sample Buffer	Bio Rad	1610747
6X Laemmli Sample Buffer	Thermo Fisher Scientific	J61337.AC
Dulbecco′s Phosphate Buffered Saline (PBS)	Sigma-Aldrich	D8537
E3 Ligase Reaction Buffer	R&D Systems	B-71
TRIS Buffered Saline (TBS)	Sigma-Aldrich	T6664

Cell Lines

HEK293T	ATCC	CRL-1573
HEK293S GnTI$^-$	ATCC	CRL-3022
HepG2	ATCC	HB-8065
HuH7	ATCC	PTA-4583
Sf9	ATCC	CRL-1711

Chemicals

3xFLAG peptide	ApexBio	A6001
cOmplete Mini EDTA-free Protease Inhibitor Cocktail	Sigma-Aldrich	11836170001
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich	D2650
Fetal Bovine Serum (FBS)	Cytiva	SH30396.03
Geneticin™ (G418 sulfate)	Thermo Fisher Scientific	10131035
Glycerol	Sigma-Aldrich	G9012
Isopropyl β-d-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich	I6758
Kanamycin sulfate	Sigma-Aldrich	K1377
Leupeptin	vivitide	ILP-4041
MLN4924	Sigma-Aldrich	5.05477
MG132	Peptide Institute, INC	3178-v
Mg-ATP (100 mM)	R&D Systems	B-20
n-Dodecyl-β-D-Maltopyranoside (DDM)	Anatrace	D310
Penicillin and streptomycin	Corning	30-002-Cl
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich	93482
PR-619	Sigma-Aldrich	SML0430
Puromycin dihydrochloride	Sigma-Aldrich	P8833
Sodium butyrate	Sigma-Aldrich	303410
Tris(2-carboxyethyl)phosphine hydrochloride (TCEP)	Sigma-Aldrich	75259
Tris hydrochloride	Sigma-Aldrich	10812846001

Enzymes

Benzonase nuclease	Sigma-Aldrich	E1014-25KU
T4 DNA Ligase	New England Biolabs	M0202S

Kits

Anti-FLAG® M2 Affinity Gel	Sigma-Aldrich	A2220
Anti-FLAG® M2 Magnetic Beads	Sigma-Aldrich	M8823
DNeasy Blood & Tissue Kits	Qiagen	69504
HiBiT Pull-down Kit	Promega	CS1967B02
Nano-Glo HiBiT Blotting System	Promega	N2410
Nano-Glo HiBiT Lytic Detection System	Promega	N3030
Nano-Glo Live Cell Assay System	Promega	N2011
Ni-NTA Agarose	Qiagen	30210
NucleoBond Xtra Maxi	Takara	740414.1
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific	23224
Pierce c-Myc-Tag Magnetic IP/Co-IP Kit	Thermo Fisher Scientific	88844X
Tandem Ubiquitin Binding Entities (TUBEs)	LifeSensors	UM402

Medium

Dulbecco′s Modified Eagle Medium (DMEM) with L-Glutamine, 4.5g/L Glucose	Thermo Fisher Scientific	MT10013CV
Freestyle 293 Expression Medium	Gibco	12338018
Luria Broth (LB)	Fisher Scientific	BP1426-500
Opti-MEM™ I Reduced Serum Medium	Thermo Fisher Scientific	31985070
Sf-900 III Serum Free Medium (SFM)	Gibco	12658027

Oligonucleotides

See Table S1

Recombinant DNA

pSpCas9(BB)-2A-Puro (PX459) V2.0	Addgene	62988
BFAR-Myc-DDK in pLenti-C-Myc-DDK	Origene	RC200078L1
pBiT3.1-C [CMV/HiBiT/Blast]	Promega	N2361
pcDNA3.1-PNPLA3-3XFLAG	This study (see Methods)	
pCMV3-Myc-BFAR (1-450)	This study (see Methods)	
pCMV3-Myc-BFAR (1-140)	This study (see Methods)	
pCMV3-Myc-BFAR (140-450)	This study (see Methods)	
pEG BacMam	Addgene	160451
pEG BacMam-FLAG-PNPLA3	This paper	
pET28a	Novagen	69864
pET28a-BFAR-His_6	This paper	
pLVX-Ef1a-IRES-Puro	Clontech	631988

Recombinant Proteins

His_6-Ubiquitin-Activating Enzyme (UBE1)	R&D Systems	E-304
Human His_6-BFAR (1-331aa)	Proteintech	ag7755
Human UbcH5b/UBE2D2	R&D Systems	E2-622-100

Software and Algorithms

FlowJo v10	BD Biosciences	
Image Studio Lite v5.2	LI-COR Odyssey system	
Prism 8	GraphPad	

Other

AKTA Pure Chromatography System	Cytiva	29018224
BD FACSCalibur	BD Biosciences	HLA-B27
C57Bl/6J mice	The Jackson Laboratory	
Chow diet (rodent) 16% protein	Teklad Global	
Corning Costar TC-Treated 6-Well Plates	Sigma-Aldrich	CLS3516
Criterion™ TGX™ Precast Midi Protein Gel	Bio-Rad	5671085
FuGENE® HD Transfection Reagent	Promega	E2311
High Sucrose Diet: 74% kCal from sucrose	MP Biomedicals	901683
HCS LipidTOX™ Deep Red Neutral Lipid Stain	Invitrogen	H34477
HisTrap HP, 5ml	Cytiva	17524802
Instant nonfat dry milk	Nestle	
Lenti-X™ Concentrator	Clontech	631232
Lipofectamine™ 3000 Transfection Reagent	Thermo Fisher Scientific	L3000008
Lipofectamine™ RNAiMAX Transfection Reagent	Thermo Fisher Scientific	13778075
Nalgene Cryogenic vials	Thermo Fisher Scientific	5000-0012
Millex-HV Syringe Filter Unit (4mm, 0.45 µm pore)	Sigma-Aldrich	SLHV033RS
Nonfat dry milk (powder)	Nestle	
Polybrene Infection/Transfection Reagent	Sigma-Aldrich	TR-1003-G
Precast Protein Gels 10% Mini-PROTEAN® TGX™	Bio-Rad	4561036S
Sonic Dismembrator	Thermo Fisher Scientific	Model 100
Superdex 200 Increase 10/300 GL column	Cytiva	28990944
Superose 6 Increase 10/300 GL column	Cytiva	29091596
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	Thermo Fisher Scientific	34577
Trans-Blot Turbo RTA Midi 0.2 µm Nitrocellulose Membrane & Transfer Kit	Bio-Rad	1704271

METHODS

Generation of Lentiviruses. Human PNPLA3 was cloned into pBiT3.1-C [CMV/HiBiT/Blast] using restriction ligation (*EcoR*I and *Sac*I). A 12 bp polylinker was included between the coding regions of PNPLA3 and HiBiT. PNPLA3-HiBiT was then subcloned into the *EcoR*I and *Xba*I restriction sites in pLVX-Ef1a-IRES-Puro and pLVX-Ef1a-IRES-ZsGreen. Plasmids were propagated in *E. coli* and sequence integrity was confirmed by Sanger sequencing. Plasmids were then packaged into lentiviruses in human embryonal kidney (HEK) 293T cells, cultured in DMEM-high glucose containing 5% FBS and 2% Penicillin-Streptomycin (10,000 U/mL). Cells were plated in 6-well plates at 5 x 10^5 cells per well and grown until 70% confluent. The lentiviral plasmids and two envelope plasmids, psPAX2 and PMD2.G, were resuspended in OptiMEM (110 µL/well) at a ratio of 1:0.9:0.1 in the presence of FuGENE® HD Transfection Reagent (2 µg/well). Solutions were incubated for 15 min at room temperature and used to transfect 293T cells. The medium was replenished with FBS (700 µL) for 24 h after transfection and collected for virus propagation 48 h and 72 h later. The medium was filtered through a polyvinylidene difluoride (PVDF) filter (0.45 µm) using a Millex-HV Syringe Filter Unit and then stored at 4ºC. The media was recovered at 48 h and 72 h, pooled and concentrated 3:1 (Lenti-X™ Concentrator). Viral particles were pelleted by centrifugation at 1500*g* for 45 min, resuspended in 400 µL of DMEM, and stored at -80ºC.

Human Liver-derived HuH7 Cells Stably Expressing PNPLA3. Clonal HuH7 cells stably expressing PNPLA3-HiBiT were generated as follows: on day 0, HuH7 cells were plated at a density of 2 x 10^5 cells/well in 6-well plates and incubated for 24 h with concentrated lentivirus encoding PNPLA3-HiBiT. Cells were switched to virus-free medium for 24 h then incubated for 10 d in medium containing FBS (5%) and puromycin dihydrochloride (2.5 µg/ml). Single colonies were harvested using cloning cylinders. Expression of PNPLA3-HiBiT was verified using the NanoBiT® blotting system and immunoblotting with anti-hPNPLA3 mAb (11C5).

NanoBiT® Blotting and Immunoblotting. Cells were lysed using Lysis Buffer (10 mM Tris-Cl, pH 7.4, 100 mM NaCl, 1% SDS, 20% glycerol plus protease inhibitors and benzonase nuclease). Lysates were homogenized with an ultrasonic homogenizer, suspended in 4X Laemmli Sample Buffer and boiled at 95ºC for 5 min. Samples were size-fractionated on SDS-PAGE (4-15%) followed by transfer to a nitrocellulose membrane. Membranes were blocked by incubating in TBST [20 mM TrisCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 plus nonfat dry milk (5%)] for 1 h and then incubated for 30 min with LgBiT and furimazine substrate according to the instructions of the manufacturer. The signal was quantified using the LI-COR Odyssey System.

For immunoblotting, cells were lysed in Lysis Buffer, homogenized with an ultrasonic homogenizer, mixed with 4X Laemmli Sample Buffer and boiled. Proteins were size-fractionated on polyacrylamide gels and transferred to nitrocellulose filters. Alternatively, cells were grown in 6-well plates and then washed once with PBS. Cells were then lysed by adding 120 µl of RIPA buffer (25 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS plus protease inhibitors and

benzonase nuclease). After shaking for 30 min at room temperature, cell lysates were mixed with 6X Laemmli Sample Buffer (30 µl) and boiled at 95ºC for 5 min. A total of 15 µl of sample was size-fractionated by SDS-PAGE and subjected to immunoblotting. The membrane was incubated overnight with the primary antibody, washed with TBST (3x10 min), and then incubated for 1 h with the secondary antibody. Blots were washed with TBST (3x10 min) before signals were revealed using SuperSignal West Pico PLUS Chemiluminescent Substrate and quantified using the LI-COR Odyssey System.

Treatment of Cultured Heptocytes with MG-132 and MLN4924. A 10 mM stock solution of MG132 was prepared in DMSO. Cells were plated as described above and treated with MG-132 or DMSO in culture medium at a final concentration of 10 µM. After 8 h, cells were harvested and immunoblotting was performed on cell lysates using antibodies, as listed above.

A stock solution of the NAE inhibitor, MLN4924 (10 mM), was prepared in DMSO. Cells were plated and grown as described above and then treated with MLN4924 (10 µM) or an equivalent amount of DMSO in culture medium. Cells were harvested after 8 h and cell lysates were subjected to immunoblotting.

Ubiquitin E3 Ligase siRNA Library Screening. To identify ubiquitin E3 ligases that target PNPLA3 for degradation, we screened a commercially available, arrayed library of siRNAs targeting ubiquitination-related proteins, including 643 ubiquitin E3 ligases. The library was arrayed in 384-well plates with each well containing a pool of four siRNAs targeting each gene. The siRNAs were resuspended in Nuclease-Free Water to a concentration of 10 µM. The library was screened in HuH7 cells stably expressing PNPLA3-HiBiT. Cells from the same passage were expanded and cryo-frozen in Cryogenic Vials. Cells from each vial were thawed and plated in 24-well plates at a density of 5 x 10^4 cells/well. Each well was transfected with a pool of siRNA (15 pmol/well) using Lipofectamine™ RNAiMAX Transfection Reagent in Opti-MEM™ I Reduced Serum Medium to a volume of 110 µL/well. Each siRNA pool was assayed in quadruplicate wells. Assays included cells treated with MG-132 (10 µM/ml) or DMSO as positive and negative controls, respectively, and cells treated with ON-TARGETplus Human PNPLA3 siRNA and ON-TARGETplus Non-targeting Control siRNA (15 pM/well) as calibrators. After 48 h incubation at 37°C, PNPLA3 levels were analyzed in HuH7 cell lysates using the NanoBiT® Blotting assay.

Inactivation of BFAR in Cultured Cells. Cloned HuH7 cells lacking BFAR were generated as follows: LentiCRISPRv2 encoding two sgRNAs targeting BFAR were generated (Genbank Reference Sequence: NM_016561.3). Targeting exon 6 of BFAR (amino acids Q69 to Q404) by sgRNA1 (Table S2) introduced a frameshift mutation followed by a premature stop codon at residue 361, while use of sgRNA2 (Table S2) introduced a premature termination codon in exon 4 at residue 209. On day 0, HuH7 cells were plated (2 x 10^5 cells per well) in 6-well plates. Separate dishes of cells were then infected with an sgRNA1- or sgRNA2-containing lentivirus. After 24 h, cells were switched to virus-free medium. After 24 h, the medium was changed to Selection Medium (DMEM medium plus 5% FBS and 2.5 µg/ml of puromycin) and grown for 10 days. Single colonies were harvested using cloning cylinders. Genomic DNA was

extracted using DNeasy Blood & Tissue Kits according to the manufacturer's instructions. Colonies lacking BFAR expression were verified by DNA sequencing of genomic DNA. Single cell colonies selected from HuH7 cells infected with CRISPR-Lenti Non-Targeting Control Plasmid were used as a negative control. A similar strategy was used to create a genetically engineered HepG2 cell line lacking endogenous BFAR.

Real Time PCR to Quantify Messenger RNA Levels. Total RNA was prepared from cells and mouse tissues and levels of selected transcripts were measured using real-time PCR as described (2). The relative amount of messenger RNAs (mRNAs) was calculated using the comparative threshold cycle method using mouse 36B4 as an internal control (3). All reactions were performed in triplicate.

Expression BFAR in HuH7 Cells. A fragment encoding full length human BFAR (1-450), N-terminal BFAR (1-140), or C-terminal BFAR (140-450) was placed in the *Kpn*I/*Not*I cloning sites downstream of a myc tag. The plasmids were expressed in HuH7 cells in 6-well plates using Lipofectamine 3000 according to the instructions of the manufacturer. After incubation for 48 h at 37ºC in CO_2 (8.8%), cells were washed once with PBS and then lysed by adding 120 µl of RIPA buffer. After shaking 30 min at room temperature, cell lysates were mixed with 6X Laemmli Sample Buffer (30 µl) and boiled at 95ºC for 5 min. For BFAR blotting, equal amount of 2x high reducing buffer (62.5 mM Tris-Cl, pH 6.8, 15% SDS, 8 M urea, 10% glycerol, 100 mM dithiothreitol) was added to cell lysates prior to addition of Laemmili Sample buffer. A total of 15 µl of sample was size-fractionated by SDS-PAGE and subjected to immunoblotting. The membrane was incubated overnight with the primary antibody, washed (3x10 min) with TBST, and then incubated for 1 h with secondary antibody. Blots were washed with TBST (3x10 min) before the signal was revealed using SuperSignal West Pico PLUS Chemiluminescent Substrate and quantified using the LI-COR Odyssey System.

Purification of Human PNPLA3. Human PNPLA3 was purified as described previously (4) with the following modifications: the cDNA encoding human PNPLA3 (NCBI Reference Sequence: NM_025225.3)was inserted into pEG BacMam vector with a FLAG tag at the N terminus. The coding region of plasmid was validated by DNA sequencing. The BacMam was expressed in mammalian HEK293S GnTI$^-$ cells using baculovirus-mediated transduction as previously described (5). After 8 h, sodium butyrate (10 mM) was added and the cells were placed at 30℃ for 72 h. Cells were homogenized in Buffer A (20 mM HEPES, pH 7.5, 150 mM NaCl) supplemented with leupeptin (10 µg/mL) and PMSF (1 mM) and then lysed by sonication. Cellular debris was removed by low-speed centrifugation. The supernatant was then incubated with 1% (w/v) n-Dodecyl-β-D-Maltopyranoside (DDM) for 1 h at 4℃. The insoluble fraction was removed by centrifugation (15,000*g*, 4℃, 30 min), and the supernatant was loaded onto anti-FLAG M2 resin, pre-equilibrated with Buffer A plus 0.02% DDM (Buffer B). The resin was washed with 15 mL Buffer B plus 2 mM ATP and 4 mM $MgCl_2$, and then again with Buffer B by gravity flow. Protein was eluted in 7.5 mL of Elution Buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.02% DDM, 0.1 mg/mL 3x FLAG peptide) and further purified by size-exclusion chromatography using a Superose 6 Increase, 10/300 GL

column pre-equilibrated with Buffer B. Peak fractions containing target proteins were concentrated and stored in -80 ℃ for further analysis.

Co-immunoprecipitation of BFAR and PNPLA3 *In Vitro*. Purified His_6-BFAR (1-331 aa) and FLAG- PNPLA3 were incubated at molar ratio of 1.4:1. Briefly, A total of 5 μg each protein [His_6-BFAR(1-331 aa) (0.14 nmol) and FLAG-PNPLA3 (0.10 nmol)] was incubated in 500 μl TBST at room temperature for 1 h. A total of 25 μl was retained (input). The remainder was added in equal parts (237.5 μl each) to 20 μl of pre-washed Ni-NTA or FLAG-beads and rotated at 4ºC overnight. Beads were pelleted by centrifugation (1000*g* x 3 min) and the supernatant was saved (flow through, FT). Pelleted beads were washed X 3 for 5 min each. After the final wash, beads were resuspended in 2x Sample Buffer (50 μl) and then boiled at 95ºC for 10 min. A total of 20% of both the input and flow through and all of the immunoprecipiates were size-fractionated using SDS-PAGE (4-15%) and transferred to nitrocellulose membranes prior to immunoblotting.

Co-immunoprecipitation of Recombinant BFAR and PNPLA3 in HuH7 cells. A total of 15 μg of pcDNA3.1-PNPLA3-3xFLAG and pCMV3-Myc-BFAR or empty vector were co-transfected in HuH7 cells in 100 mm dishes using Lipofectamine 3000 (Invitrogen) according to the instructions of the manufacturer. Cells were harvested after 48 h and lysed in lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Triton® X-100, 0.1% sodium deoxycholate, plus protease inhibitors and benzonase nuclease). For immunoprecipitation, 50 μg of pre-washed magnetic beads were incubated with 240 μg cell lysate for 16 h at 4ºC. Beads were washed three times (10 min/each) in the same buffer and after the final wash, beads were mixed with 2X Laemmli Sample Buffer (60 μl) and boiled at 95ºC for 5 min prior to immunoblotting.

***In Vitro* Reconstitution of Ubiquitin Conjugation Reaction.** All recombinant proteins were purchased from a commercial company except BFAR and PNPLA3. Complementary DNA encoding truncated human BFAR (1-331 aa, NCBI Reference Sequence: NM_016561.3) was cloned into pET28a vector at *BamH*I/*Not*I sites, downstream of His_6 tag. The plasmid was transfected into *E.coli* (BL21) and cultured in LB plus kanamycin (50 μg/ml). Cells were induced by adding 0.5 mM IPTG and incubating at 16℃ overnight. Cell pellets were lysed by sonication in Lysis Buffer (50 ml) containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) plus protease inhibitor. Supernatants were collected by centrifugation at 17,000*g* for 30 min. Cell lysates were diluted in Lysis Buffer (to 150 ml) and applied to HisTrap column (5 ml) by AKTA pure chromatography system. His_6-BFAR was eluted using a linear NaCl (0-300 mM) gradient (100 ml) in Lysis Buffer. His_6-BFAR containing fractions were collected and further concentrated by size exclusion chromatography using Superdex 200 10/300 GL column. His_6-BFAR was added to 20% glycerol for storage at -80℃.

In vitro ubiquitination of PNPLA3 was performed by combining His_6-Ubiquitin-activating Enzyme/UBE (15 μM) in 50 mM HEPES, pH 8.0, 50 mM NaCl, 1 mM TCEP, Ubiquitin-conjugating Enzyme E2 D2/UBE2D2 (25 μM) in 50 mM HEPES, pH 7.5, 200 mM NaCl, 10% glycerol, 1 mM TCEP, His_6-BFAR(1-331 aa) (5 μM) in 50 mM Tris

buffer, FLAG-PNPLA3 (5 μM) in 20 mM HEPES buffer, pH 7.5, 150 mM NaCl, 0.02% DDM and ubiquitin (2.5 mM) in sterile water. The final concentrations of the reactions (in 50 μl) were as follows: 1 x E3 Ligase Buffer, 10 mM MgATP, 100 μM Ub, 0.1 μM E1, 1 μM E2, 1 μM BFAR, and 1 μM PNPLA3 or as indicated. Reactions were incubated at 37ºC for the indicated times and then terminated by adding 4x Laemmli Sample Buffer. Samples were resolved by 4-15% SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was performed as described above.

Ubiquitination of PNPLA3 in HuH7 Cells. HuH7 cells stably expressing PNPLA3-HiBiT (3 x 10^6 cells/100 mm dish) were transfected with a scramble siRNA or anti-BFAR siRNA. After 32 h, medium was replaced with DMEM plus MG132 (10 μM) and incubated 16 h before harvesting. For cell lysates, 1 ml of Lysis Buffer plus 20 μM PR619 (deubiquitylation inhibitor) was added to the cell pellets. Lysates were rotated at 4ºC for 1 h and centrifuged at 15,000*g* for 10 min and supernatants were collected. A total of 1.5 mg of lysate was incubated with pre-washed TUBE agarose beads at 4ºC overnight. Beads were pelleted by centrifugation at 1000*g* for 3 min to collect flow through (FT). The beads were washed thrice with TBST at room temperature for 5 min and then resuspended in Sample Buffer (50 μl), boiled at 95ºC for 10 min, before loading 15 μl (100%) was subjected to SDS-PAGE (4-15%), together with 22.5 □g of lysate (6%) and proportional flow through samples (6%).

Generation of Anti-Human and -Mouse mAb to BFAR. DNA fragments encoding human and mouse BFAR (1-140 amino acids) were cloned into pET28a at *BamH*I/*Not*I sites, downstream of the His_6 tag. Recombinant BFAR (1-140) that was purified from *E.coli* (BL21) were used to immunize mice, as described (1) to develop mouse mAb against human BFAR (mAb 10E10) and mouse BFAR (mAb 40H7).

Inactivation of BFAR in Mice. C57BL/6J BFAR knockout mice were generated using CRISPR-Cas9 technology by UT Southwestern Transgenic Technology Core facility as described previously(6). Two guide (g) gRNAs (sequences provided in Table S1) were designed to delete a ~760 bp region that includes exon 3. Both gRNAs were synthesized as crRNAs, annealed to the trans-activating CRISPR RNA (tracrRNA), and then co-injected with Cas9 protein into the cytoplasm of fertilized eggs (C57BL/6J) to produce F_0 founders, which were bred with WT C57BL/6J mice to obtain F_1 lines of mice heterozygous for the deleted *Bfar* allele ($Bfar^{+/-}$). Two independent lines of $Bfar^{-/-}$ mice (with deletion of 794 bp and 773 bp, respectively) were chosen for further studies and both produced identical results. These deletion resulted in the splicing of exon 2 to exon 4, causing a frameshift and a premature stop codon. For all animal experiments, littermate wild-type ($Bfar^{+/+}$) and knockout ($Bfar^{-/-}$) mice produced from intercrosses of $Bfar^{+/-}$ mice were used. The $Bfar^{-/-}$ mice were genotyped by PCR using primers provided in Table S1 to amplify a fragment of 1143 bp (wild-type) or of 349 bp (or 370 bp) with deletion of 794 bp (or 773 bp), respectively). The knockout mice were backcrossed with C57BL/6J mice for 3 generations. Mice were maintained at 21–23°C on a 12-h dark/light cycle (lights on: 6:00 AM–6:00 PM), and fed a rodent chow died. For the dietary challenge studies, mice were fed a high sucrose diet (HSD) for 3 wks. Prior to each experiment, mice were metabolically synchronized for 3 days by removing food during

the night for 15 h (6:00 PM–9:00 AM) and providing food at 9:00 AM. Samples were collected 6 h after the mice were provided food at the end of the last feeding cycle. All animal experiments has been approved and conducted under the oversight of the UT Southwestern Institutional Animal Care and Use Committee.

Isolation of Hepatic LDs from Livers of Mice. Lipid droplets (LDs) from mouse livers were fractionated as described (7). Briefly, livers were homogenized in 20 mM Tricine, pH 7.8, 250 mM sucrose (7 ml). Lysates were centrifuged at 500*g* for 5 min to remove nuclei and an aliquot of post-nuclear supernatant (PNS) (7 ml) was transferred to an ultracentrifuge tube. The PNS was overlaid with 5 ml of Buffer C (100 mM KCl, 2 mM $MgCl_2$, 20 mM HEPES, pH 7.4) and then centrifuged at 10,000*g* for 1 h. The top layer (white) was transferred to an Eppendorf tube and subjected to centrifugation at 10,000*g* for 5 min. The infranatant under the LDs was removed using a gel loading tip and the LDs were suspended in Buffer C (300 µl) and then subjected to gentle votexing. The wash step was repeated thrice. After the last wash, the LDs were vortexed vigorously in 1 ml acetone and then stored at -20ºC overnight. Proteins from LDs were pelleted at 15,000*g* for 15 min at 4ºC. The organic solvent was removed and pellets were washed twice by adding 1 ml each of acetone:ethyl ether (1:1)(v/v) and then ethyl ether. The protein pellets were dried by air in a chemical hood and then solubilized in 50-150 µl LD Protein Dissolving Buffer (2% SDS, 1 M urea in PBS). The protein concentration was measured by BCA and proteins (1 µg) were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was performed as described above.

Figures and Figure Legends

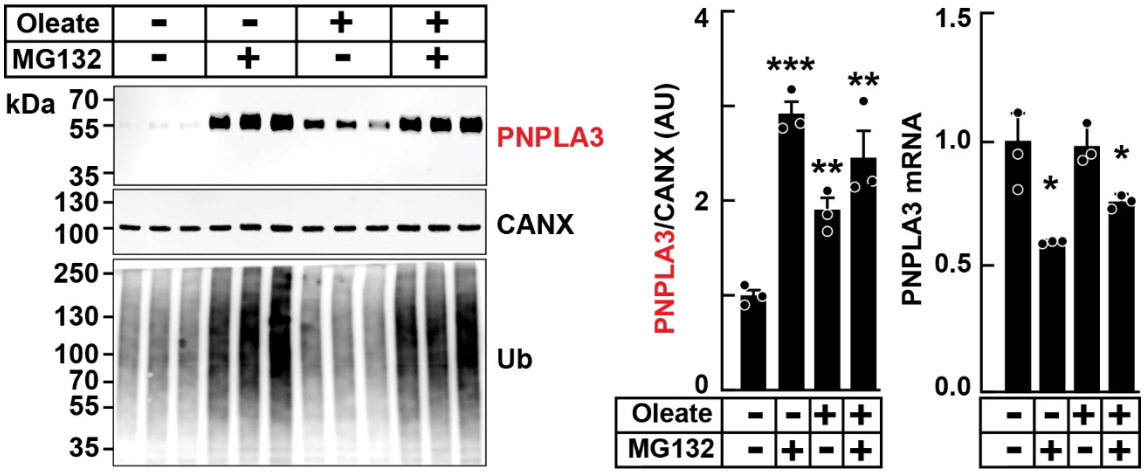

Fig. S1. NanoBiT blotting assay of PNPLA3-HiBiT cells in the presence or absence of oleate and MG132. HuH7 cells stably expressing PNPLA3-HiBiT were incubated for 24 h in the presence or absence of oleate (400 μM) and MG132 (10 μM) diluted in DMSO, or with DMSO alone. After 8 h, levels of PNPLA3 were quantified in cell lysates (5 μg) using the NanoBiT blotting assay (see Methods). Levels of calnexin (CANX) and ubiquitin (Ub) were assayed using antibodies listed in *SI Appendix*. The signals corresponding to PNPLA3 were quantified by LI-COR, normalized to CANX levels, and expressed as arbitrary units (AUs). PNPLA3 mRNA levels were quantified using RT-PCR as described in the Methods. Values represent means ± SEM. The levels of PNPLA3 protein and mRNA were compared using student t-test. *, P<0.05, **, P<.01, ***, P<.001. The experiment was repeated twice and the results were similar.

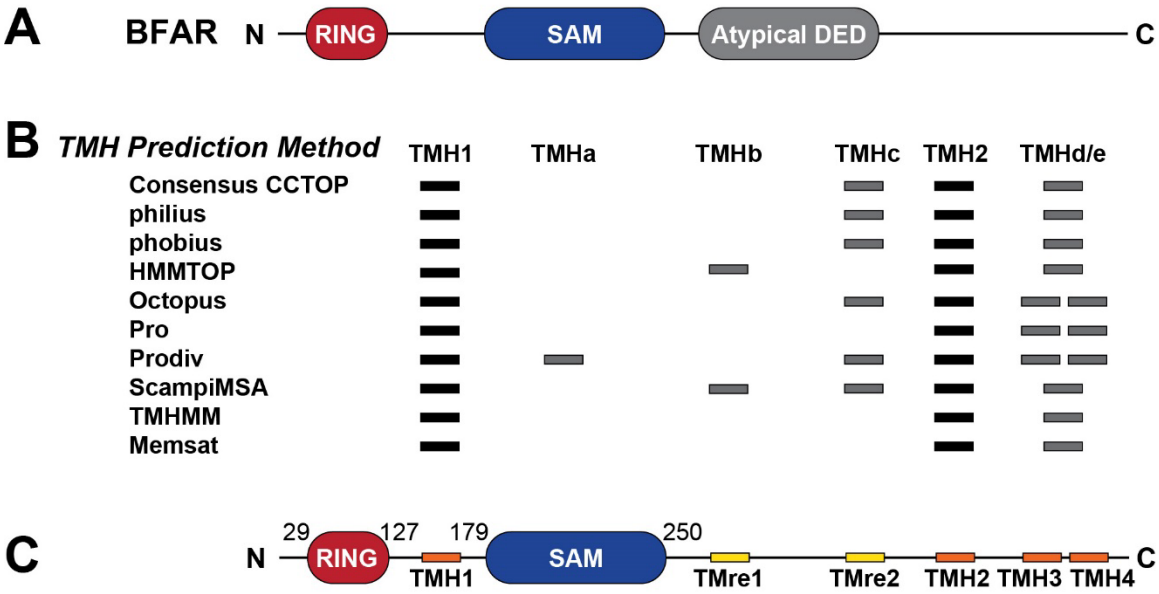

Fig. S2. Schematic of predicted structural domains of BFAR. (*A*) Previously described BFAR domains (ovals) are depicted: RING (red), SAM (blue), and atypical DED (gray). (*B*) Consensus Constrained TAPology prediction (CCTOP) was used to predict transmembrane helix (TMH) positions (rectangles colored according to consistency: black and gray predicted using all or some algorithms, respectively), with consensus TMH on top and additional methods below (labeled to the left).

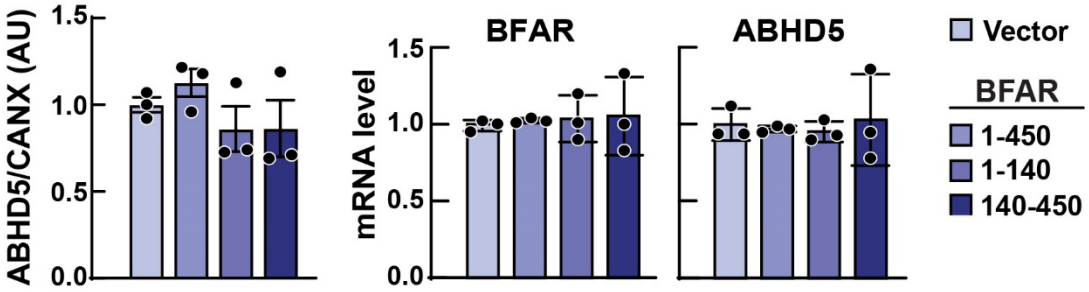

Fig. S3. Levels of selected proteins and mRNAs from the experiment shown in Fig. 2*B*. Levels of immunodetectable ABHD5 protein, and BFAR and ABHD5 mRNA were quantified as describe in the legend to Fig. 2*B*. Values represent means ± SEM.

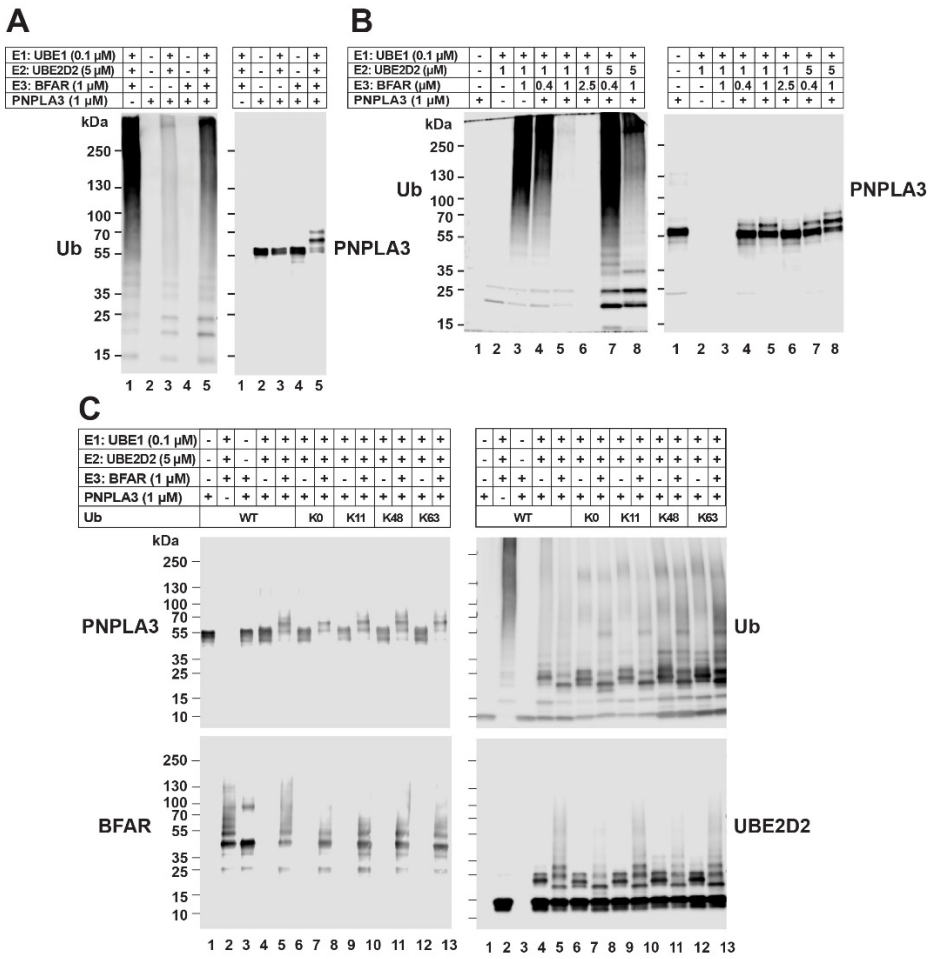

Fig. S4. *In vitro* reconstitution of BFAR-mediated ubiquitylation of PNPLA3. Proteins were incubated in E3 ligase buffer (50 μl) to reconstitute the ubiquitin conjugation reaction *in vitro* as described in the Methods. Immunoblotting was performed on 15% of the volume of each reaction using mAb to ubiquitin, PNPLA3, and BFAR, or rabbit pAb to UBE2D2. *(A) In vitro* reconstitution of BFAR-mediated ubiquitylation of PNPLA3 showed multiple ubiquitinated bands in Lane 5 compared to control sets (Lane 1-4). *(B)* Titration of BFAR and E2 (UBE2D2) in reconstitution of BFAR-mediated ubiquitylation of PNPLA3 *in vitro*. Increasing concentration of BFAR did not increase ubiquitination of PNPLA3 (Lane 4-6). Increasing concentration of E2 (UBE2D2) increases ubiquitination of PNPLA3 (Lane 7 and 8 vs Lane 4 and 5). (C) BFAR-mediated mono-ubiquitination of PNPLA3. WT, K0, K11, K48 and K63-ubiquitin were added as indicated in the figure. PNPLA3 immunoblots showed that ubiquitin chains were added in each reaction. Ub, BFAR and UBE2D2 blots indicate ubiquitination on each components of the reaction. The experiment was repeated twice and the results were similar.

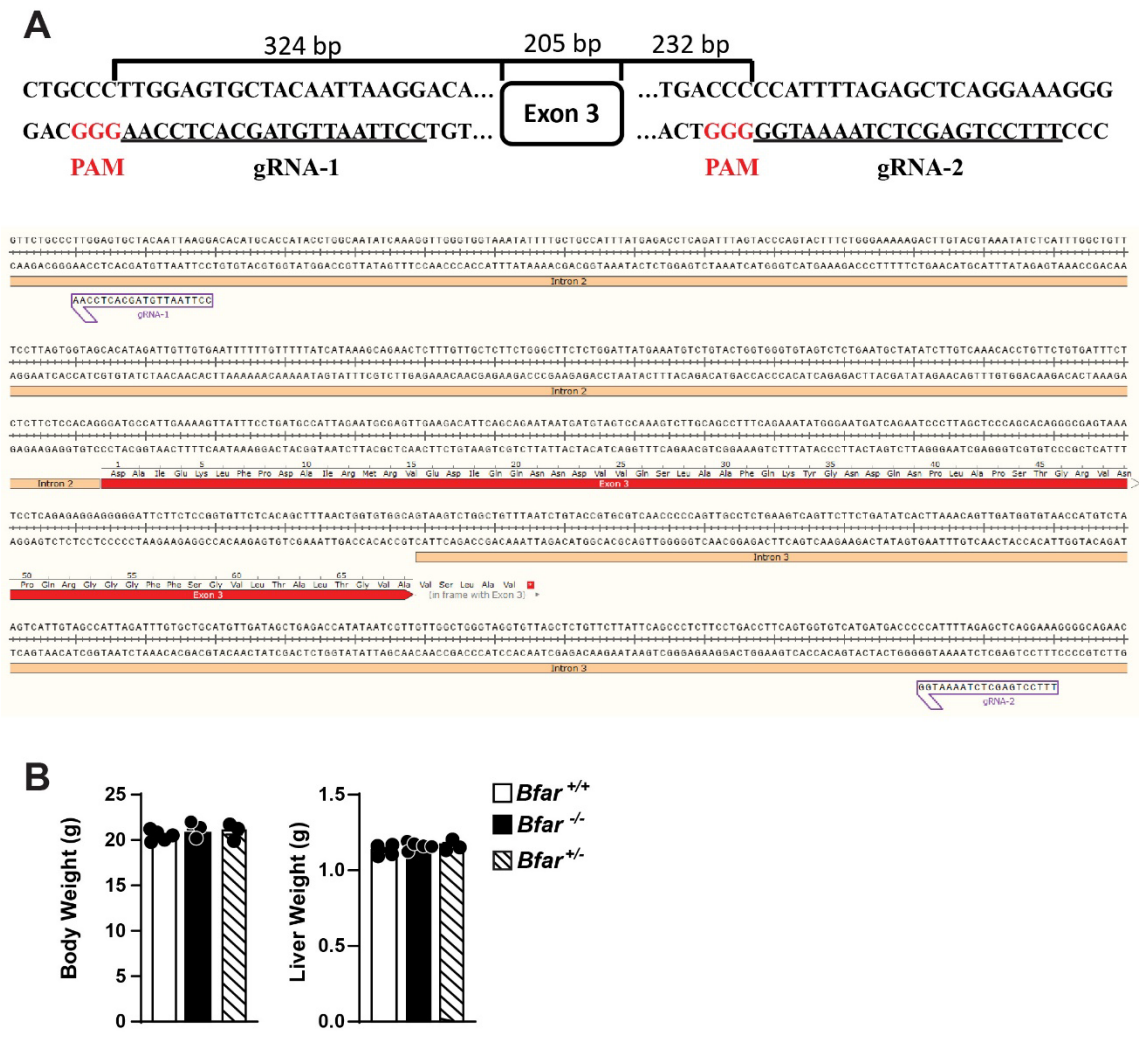

Fig. S5. Generation of *Bfar*$^{-/-}$ Mice using CRISP-Cas9. (*A*) Schematic for CRISPR-Cas9 inactivation of *Bfar* in mice. The location and sequence of two gRNAs used to generate *Bfar*$^{-/-}$ mice are shown. The gRNA are complimentary to sequences located in the introns flanking exon 3 (gRNA-1 and gRNA-2, respectively). PAM (NGG) sequences are highlighted. (***B***) Body and liver weights of WT, *Bfar*$^{+/-}$,and *Bfar*$^{-/-}$ mice. Male mice (5/group, 8 weeks old) were fed a HSD for 3 weeks. After 3 cycles of fasting and refeeding, mice were killed and livers were harvested 6 h after the last refeeding. Body weights and liver weights were measured. Values represent means ± SEM.

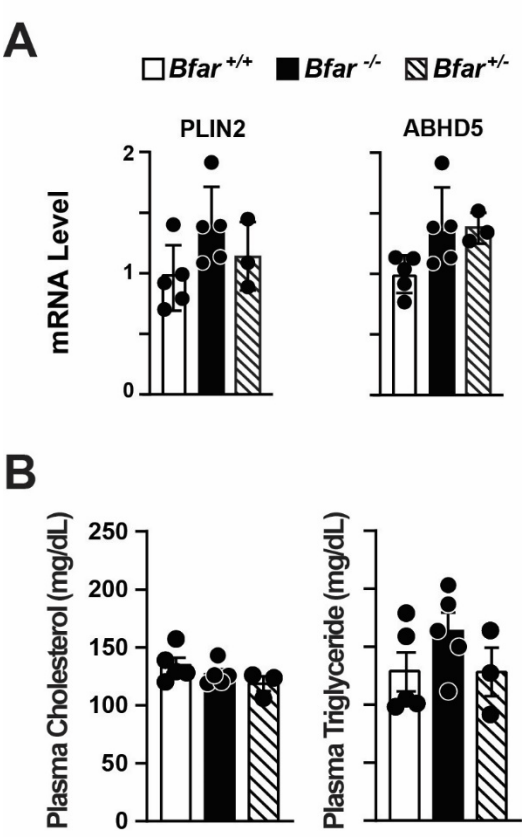

Fig. S6. Hepatic mRNA levels in livers of *Bfar*$^{-/-}$ mice. The mRNA levels of indicated genes (PLIN2 and ABHD5) were measured and normalized as described in Fig. 6 and in the Methods. (*B*) Mean levels of plasma cholesterol and triglyceride in offspring of *Bfar*$^{+/-}$ mice of the indicated genotypes. Values represent means ± SEM. The experiments were repeated twice and the results were similar.

Table S1. Sequences of oligonucleotides and siRNAs

Subcloning:	
PNPLA3/PNPLA3 (I148M) cDNA --> pBiT3.1	5'-CCGGAATTCATGTACGACGCAGAGCGCGGC-3' 5'-CCCGAGCTCTCCAGACTCTTCTCTAGTGA-3'
PNPLA3-HiBiT/PNPLA3 (I148M)-HiBiT cDNA --> pLVX-EF1alpha-IRES-Puro	5'-CCGGAATTCATGTACGACGCAGAGCGCGGC-3' 5'-TGCTCTAGACAGCTAATCTTCTTGAACAGCCG CCAGCCGCTCACCAGACTCTT-3'
BFAR cDNA --> pET28a	5'-CGCGGATCCATGGAGGAACCTCAGAAAAGC-3' 5'-TTTTCCTTTTGCGGCCGCTCACAACACCTGGGT TTCCAGCC-3'
FLAG-PNPLA3 cDNA --> pEG BacMam	5'-CCGGAATTCATGGATTACAAGGACGACGATGAC AAGTACGACGCAGAGCGCGG-3' 5'-ATAAGAATGCGGCCGCTCACAGACTCTTCTCTAG TGAAAAACTG-3'
Quantitative PCR:	
BFAR-F(1333-1412)	5'-TGGATCCCTTTCTCTGAATGGA-3'
BFAR-R(1333-1412)	5'-TGAATAACATGAAGGCCACTAAATCT-3'
PNPLA3-F(623-693)	5'-CTACAGTGGCCTTATCCCTCCTT-3'
PNPLA3-R(623-693)	5'-GTACGTTGTCACTCACTCCTCCAT-3'
ATGL-F(1952-2042)	5'-CCCTTTACTCCTGAGAACTTTGCA-3'
ATGL-R(1952-2042)	5'-GCGAAAATAAATAATTCTTCACACACAT-3'
SREBP-1c-F(176-177)	5'-GGGCGGGCGCAGAT-3'
SREBP-1c-R(176-177)	5'-TTGTTGATAAGCTGAAGCATGTCTT-3'
36b4-F(164-233)	5'-GGCCTGAGCTCCCTGTCTCT-3'
36b4-R(164-233)	5'-GCGGTGCGTCAGGGATT-3'

sgRNAs for BFAR inactivation in cells:	
sgRNA1 (Exon 6)	5'-GTTTGACTACACCGACACCT-3'
sgRNA2 (Exon 4)	5'-GTGATAGACGAGCAGGACCA-3'
Oligos to confirm BFAR inactivation in cells:	
sgRNA1-F	5'-TCTTTATACAATGTAACCCCAAATG-3'
sgRNA1-R	5'-GCATGTGACTTGATATGAACAGG-3'
sgRNA2-F	5'-GTCCTTTTCTCGCCTCTGTG-3'
sgRNA2-R	5'-GAAAGCTGTTTTCTAAAATGTCCA-3'
sgRNAs for generating *Bfar*$^{-/-}$ mice	
gRNA-1	5'-CCTTAATTGTAGCACTCCAA-3'
gRNA-2	5'-TTTCCTGAGCTCTAAAATGG-3'
Oligos to genotype *Bfar*$^{-/-}$ mice by PCR	
Forward	5'-CATGAACCCTTTAACCCAACA-3'
Reverse	5'-CCGTAACAGTGCAGACATGG-3'

Table S2: Silencing RNAs (siRNAs) that caused ≥ 2-fold increase in PNPLA3 in PNPLA3-HiBiT cells.

siRNA	Fold change in PNPLA3/CANX	P value
RNF144A	10.3	0.395
MKRN2	8.9	0.001
RNF10	7.5	0.211
MIB2	7.5	0.166
MARCH9	5.6	0.049
RNFT1	4.2	0.080
RNF214	3.8	0.016
BIRC3	3.7	0.090
BMI1	3.7	0.126
KDM5B	3.1	0.157
NSD1	3	0.023
RNF34	2.7	0.277
RNF2	2.7	0.105
RNF170	2.7	0.178
RNF182	2.5	0.149
RNFT2	2.5	0.099
BFAR	**2.5**	**0.041**
RNF157	2.5	0.545
RNF144B	2.3	0.128
RNF128	2.2	0.352

PNPLA3 levels were quantified by LI-COR and expressed as arbitrary units (AUs) and normalized to levels of calnexin. The fold change of levels with each siRNA was compared to the corresponding non-siRNA treated cells. Four biological replicates were used for each siRNA and two for non-treated siRNA controls. Welch t-test was used to calculate the statistical significance of PNPLA3 fold change with siRNA knockdown.

Table S3a. Offspring of heterozygous matings of *BFAR*$^{-/-}$ mice (794 bp deletion)

	Males (n=91)				Females (n=59)					Total (n=150)			
	Observed		Expected		Observed		Expected			Observed		Expected	
	#	%	#	%	#	%	#	%		#	%	#	%
(+/+)	15	17	23	25	17	29	15	25	**Males**	91	61	75	50
(+/-)	45	49	45	50	29	49	29	50	**Females**	59	39	75	50
(-/-)	31	34	23	25	13	22	15	25					

Breedings			Pups		
Male	Female	No. of Litter	Pups per litter	Males	Females
(+/-)	(+/-)	26	6	91	59

Table S3b. Offspring of heterozygous matings of *BFAR*$^{-/-}$ mice (773 bp deletion)

	Males (n=42)				Females (n=42)					Total (n=84)			
	Observed		Expected		Observed		Expected			Observed		Expected	
	#	%	#	%	#	%	#	%		#	%	#	%
(+/+)	13	31	10	25	7	17	10	25	**Males**	42	50	42	50
(+/-)	26	62	22	50	22	52	22	50	**Females**	42	50	42	50
(-/-)	3	7	10	25	13	31	10	25					

Breedings			Pups		
Male	Female	No. of Litter	Pups per litter	Males	Females
(+/-)	(+/-)	18	4.6	42	42

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4. Huang Y, Cohen JC, & Hobbs HH (2011) Expression and characterization of a PNPLA3 protein isoform (I148M) associated with nonalcoholic fatty liver disease. *J Biol Chem* 286:37085-37093.
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