

## **Accumulation of PNPLA3 on Lipid Droplets is the Basis of Associated Hepatic Steatosis**

Soumik BasuRay<sup>a,b</sup>, Yang Wang<sup>a,b</sup>, Eriks Smagris<sup>a, b</sup>, Jonathan C. Cohen<sup>b,1</sup>, and Helen H. Hobbs<sup>a,b,c,1</sup>

Helen H. Hobbs & Jonathan C. Cohen

E-mail: [helen.hobbs@utsouthwestern.edu](mailto:helen.hobbs@utsouthwestern.edu) and [jonathan.cohen@utsouthwestern.edu](mailto:jonathan.cohen@utsouthwestern.edu)

### **This PDF file includes:**

Supplementary text

Figs. S1 to S5

## Supplementary Methods

### Mice

Transgenic mice (*PNPLA3Tg<sup>WT</sup>* and *PNPLA3Tg<sup>148M</sup>*) were offspring of heterozygotes for the transgene and WT mice. Non-*Tg* littermates were used as controls (1). The KI mice were backcrossed with C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) for 6 generations (N6). Mice were housed in micro-isolator and shoebox cages with 7090 sani-chip bedding (Harlan Teklad, Houston, TX).

Mice were maintained on a 12-h light (8AM to 8PM)/12-h and dark (8PM to 8AM) cycle and fed Teklad Mouse Diet 7001 chow diet (Harlan Teklad, Houston, TX) *ad libitum*. For the dietary challenge studies, mice were fed a HSD (no. 901683; 74% kCal from sucrose; MP Biomedicals, Santa Ana, CA) for 4 wks or a high-fructose diet (no. TD89247; 67% kCal from fructose; Teklad Envigo Diet) for 2 wks. Prior to each experiment, mice were metabolically synchronized for 3 days on a standard protocol (synchronization protocol): food was removed during the day (8AM to 8PM) and provided at night (8PM to 8AM). Mice were killed at the end of the last feeding cycle.

### Plasmids

Human PNPLA3(WT), PNPLA3(PAT) (residues 1-276) and PNPLA3(C-ter) (residues 277-481) were subcloned into pCMV-XL5 (Origene, Rockville, MD) with a Flag tag inserted at the C-ter. Plasmids used for immunofluorescence included the following: 1) PNPLA3 (WT and S47A) were cloned into pcDNA-V5-His-TOPO using TA cloning (Invitrogen, Carlsbad, CA), 2) PNPLA3(K→R) was cloned into pCMV-XL5 (Origene, Rockville, MD) with a Flag tag inserted at the C-ter, 3) CGI-58-myc was cloned into pcDNA3.1(+) (Invitrogen) using restriction ligation (*HindIII* & *BamHI*), 4) PNPLA3-T1 (residues 1-276), PNPLA3-T2 (residues 1-404) and PNPLA3-T3 (residues 1-438) were all cloned into pcDNA-V5-His-TOPO using TA according to the manufacturer's protocol. Constructs were sequenced to corroborate the integrity of the cloned gene cassettes.

### Antibodies

A mouse mAb (11C5) against human PNPLA3 was developed as described (2). A rabbit anti-mouse PNPLA3 mAb (19A6) was developed against a peptide corresponding to residues 152-309 of PNPLA3. The following commercial Abs were used at the indicated dilutions for immunoblotting: anti-Ub mAb (P4D1, 1:500; Santa Cruz Biotech, Inc.), rabbit anti-mATGL mAb (1:1,000, Cell Signaling Technology, MA), guinea pig anti-mPLIN2 polyclonal Ab (1:5000, Fitzgerald Industries International, Acton, MA), anti-hCGI-58 mAb (1:5,000, Abnova, Walnut CA), and anti-V5 mAb (1:3,000, Invitrogen, Carlsbad, CA). Secondary Abs used for immunoblotting included goat anti-rabbit IgG, goat anti-mouse IgG, or F(ab) fragment-specific horseradish peroxidase-conjugated Ab (1:3,000, Jackson Laboratories, West Grove, PA) (2). An anti-myc rabbit polyclonal Ab was purchased from Abcam (Cambridge, MA). The Alexa 488-conjugated goat anti-rabbit IgG (A11034) and Alexa 555-conjugated goat-anti mouse IgG (A21422) were purchased from Thermo Fisher Scientific (Waltham, MA).

### Adeno-associated Viruses

The shRNA sequences targeting PNPLA3 were designed using the Invitrogen BLOCK-iT™ RNAi Designer online tool. Twenty one-nucleotide (nt) sequences starting at nt 652, 678, 829, 830, 852, 916, 947, 979, 983 and 984 of the *Pnpla3* open reading frame were synthesized as complementary anti-parallel oligonucleotides with a loop sequence (5'-CGAA-3') and *PstI/BglII* overhangs. The forward and reverse oligonucleotides were annealed and ligated into the *PstI/BbsI* site of the AAV *cis* plasmid downstream of the human H1 promoter (pAAVsc-

CB6\_PI\_EGF). To evaluate the potency of the shRNAs, the 10 pAAV-shRNAs were transfected into QBI-293-A2 cells that transiently expressed mouse PNPLA3-V5 using Lipofectamine 2000 (Invitrogen). An AAV *cis* plasmid containing the H1 promoter and termination sequence was used as a negative control. After 48 h, cells were harvested and PNPLA3 expression was measured by RT-PCR and immunoblot analysis using an anti-V5 Ab. shRNA8 (5'-TCAGGATCCTGTCCTACATccgaAGATGTAGGACAGGATCCTGC-3') the shRNA that most potently inhibited PNPLA3 expression, was used for AAV production by the University of Massachusetts Medical School Viral Vector Core as previously described (3).

The optimal dose of AAV for knockdown of PNPLA3 in mouse livers was determined by injecting 10-week old C57BL/6J male mice with increasing amounts of pAAV.shRNA PNPLA3 [1.0, 2.0 3.0 and 4.0 X 10<sup>11</sup> genome copies (GC)]. Mice were fed a high-fructose diet for 14 days, metabolically synchronized for 3 days, and sacrificed at end of the last feeding cycle. LDs were isolated, proteins extracted, and PNPLA3 was detected by immunoblotting.

The following AAV constructs were ordered from Vector Biolabs Inc. 1) Atg7 (AAV8-GFP-U6-M-ATG7-shRNA, Genbank RefSeq: NM\_028835). 2) A virus encoding a scrambled mRNA (Scr): AAV8-CMV/GFP-U6-scr-shRNA). 3) Human AAV-PNPLA3 expression constructs that were subcloned into a pAAV-thyroxine binding globulin (TBG) vector backbone (Addgene, Watertown, MA) to provide liver specificity. The sequences of the constructs are available from the vendor.

### **Analysis of Lipid Droplets**

LDs were suspended in solubilization buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, Fos-choline13 (0.1%) supplemented with protease inhibitor (Roche)] and incubated at 4°C with mild shaking for 4 h. The suspension was centrifuged at 20,000xg for 30 min at 4°C and the LD proteins were incubated with 50 µl Flag-agarose (Sigma Aldrich) for 4 h at 4°C. Beads were washed 3X in solubilization buffer without detergent. Beads were boiled with 2X sample buffer at 95°C for 5 min and the supernatant was applied to a 12% SDS-PAGE gel and analyzed by immunoblotting. To avoid interference with the immunoglobulin heavy chain, goat anti-rabbit light chain IgG, goat anti-mouse light chain IgG, and Fc fragment-specific horseradish peroxidase-conjugated secondary Abs were used for immunoblotting. The blots were quantified using the LI-COR Odyssey system.

### **Immunofluorescence Microscopy**

QBI-293A cells were cultured in complete medium (DMEM with high glucose, 5% fetal calf serum) and transfected with the indicated plasmids using FuGENE 6 (Promega, Madison, WI). The medium was replaced with new complete medium plus OA (200 µM) 5 h after transfection. After 24 h, cells were rinsed with PBS and then fixed for 15 min in paraformaldehyde (4%). Cells were then incubated with 50 mM NH<sub>4</sub>Cl for 15 min and permeabilized for 2 min using Triton X-100 (0.1%) in FSG buffer [PBS containing fish skin gelatin (0.2%)] (MilliporeSigma, Burlington, MA). After blocking in FSG buffer for 15 min, cells were incubated with the primary Abs in FSG buffer at 4°C overnight. Cells were rinsed with FSG buffer twice before incubating with the secondary Ab for 35 min at room temperature. LDs were stained using monodansylpentane (1:1,000, MDH, AutoDOT, Abgent, San Diego, CA) for 30 min. Cells on coverslips were immersed in Vectashield anti-fade mounting medium (H-1400, Vector Laboratories, Burlingame, CA) and placed on slides. Cells were visualized using a confocal microscope (Zeiss LSM 880).

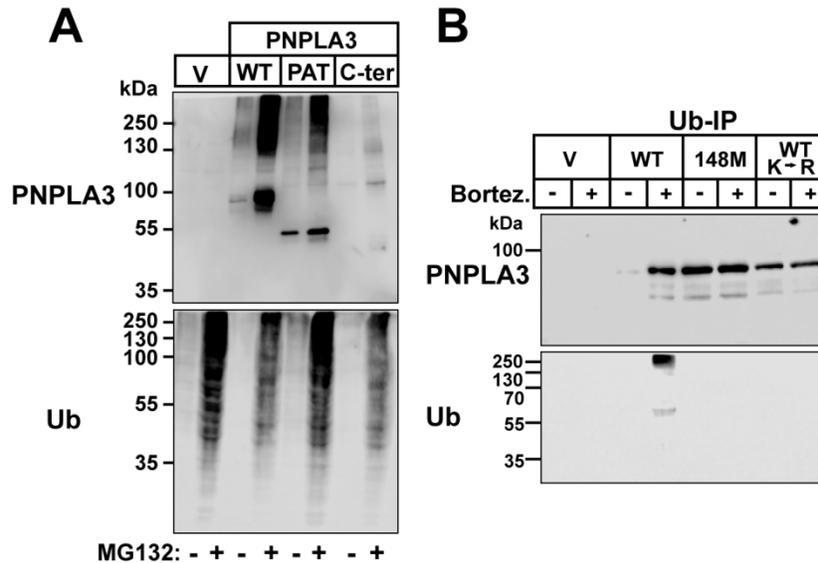
### **Bortezomib Treatment.**

Mice fed a HSD *ad libitum* for 4 weeks were subjected to the synchronization protocol. After the last refeeding cycle, mice were injected via the tail vein with bortezomib (LC Laboratories, Woburn, MA) that was dissolved in ethanol (10 mg/ml) and then diluted 1:20 in saline (0.9% NaCl (w/v)). Mice were injected with bortezomib (1 mg/kg) in a volume of 200 µl. Control mice

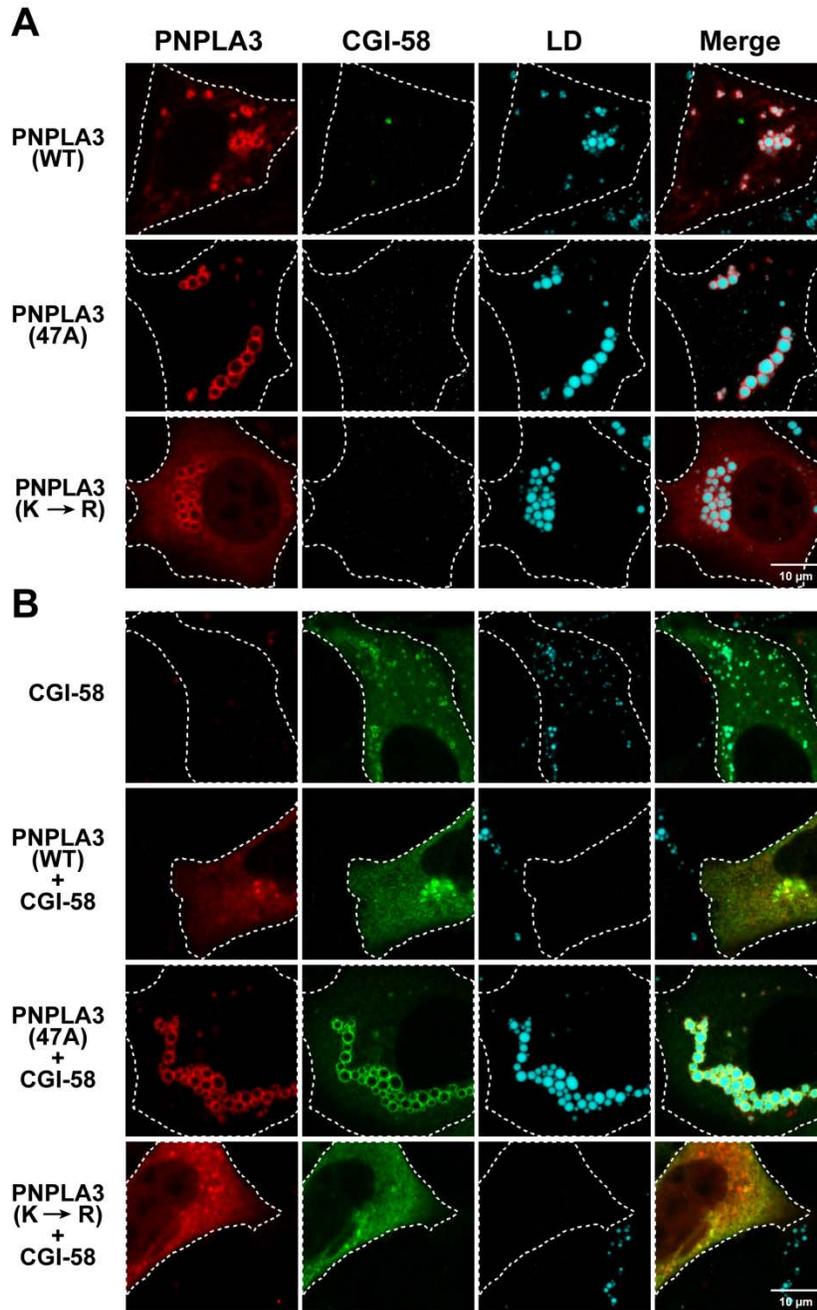
were injected with an equal volume of vehicle alone. The mice were killed 5 h after the injection and livers were harvested for lipid and immunoblot analysis.

### **Chloroquine Treatment**

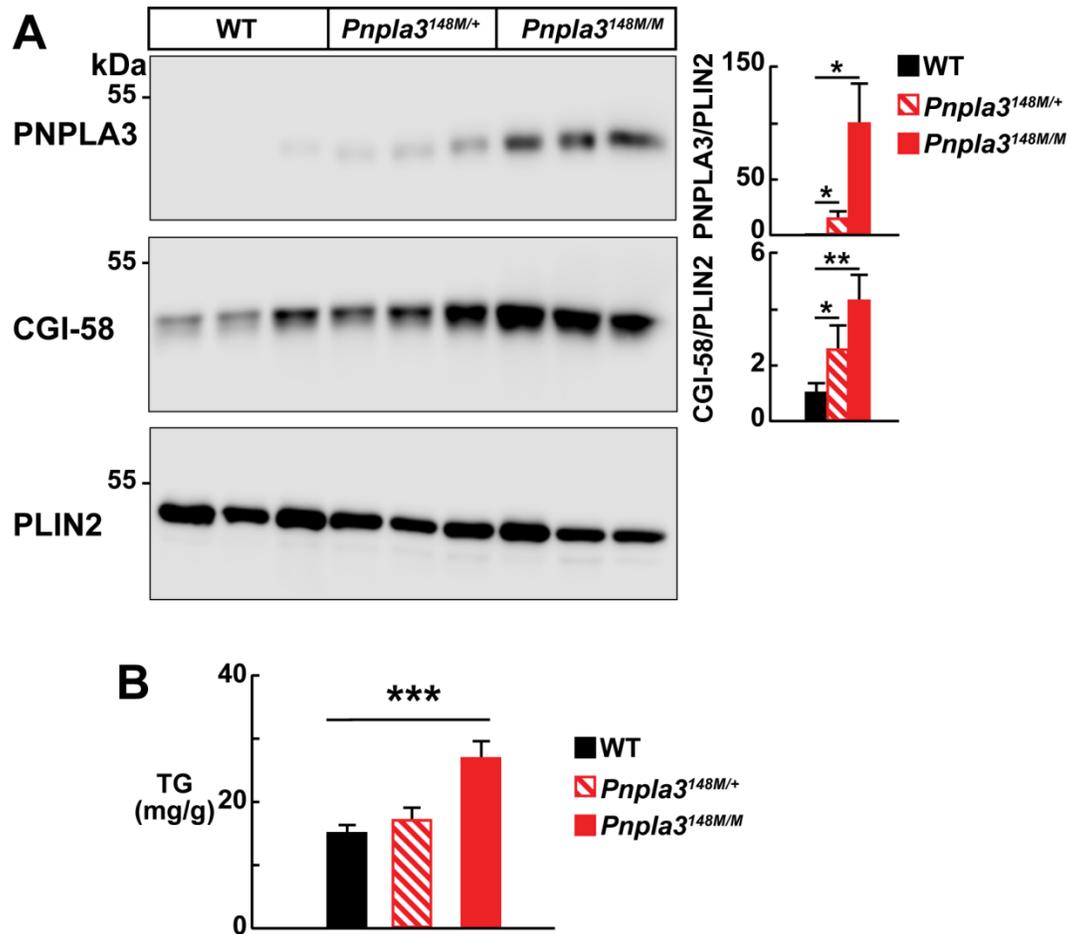
Mice fed a HSD *ad libitum* for 4 weeks were subjected to the synchronization protocol and then treated with chloroquine (25 mg/kg diluted in 200  $\mu$ l PBS) via the tail vein at the end of the last refeeding cycle as described (4). Mice were killed after 3 h of fasting since chloroquine-mediated inhibition of autophagic flux is maximal after 2-4 h (5, 6). Livers were harvested and LDs and lysates were prepared as described (7).



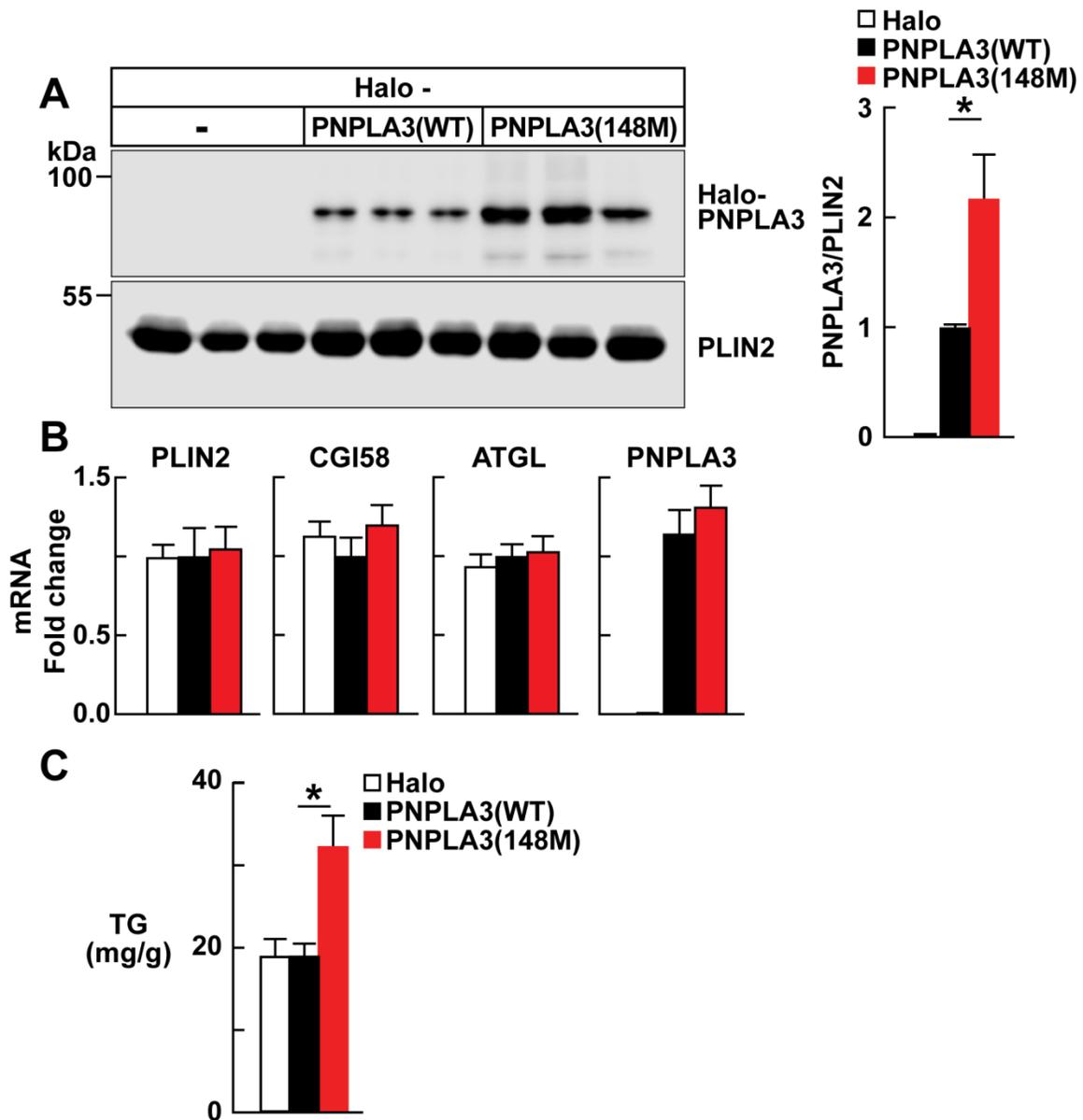
**Fig. S1. Inhibition of proteasome-mediated degradation in cultured hepatocytes (A) or in livers of mice (B) results in accumulation of ubiquitylated PNPLA3.** (A) HepG2 cells were transfected with empty plasmid (pCMV-XL5) (V), and with plasmids expressing human PNPLA3(WT), the patatin domain (residues 1-276) [PNPLA3(PAT)], and the C-terminal domain (residues 277-481) of PNPLA3 [PNPLA3(C-ter)]. All constructs were tagged at the C-terminus with a FLAG epitope. After 2 days, cells were treated with MG132 (10  $\mu$ M) for 8 h prior to lysis in lysis buffer [Triton X-100 (1%) SDS (0.1%), PBS, 10 mM NEM and protease inhibitor cocktail (Roche)]. Proteins from cell lysates (25  $\mu$ g) were resolved by 10% SDS-PAGE and immunoblotted with anti-FLAG (MBL International Corporation, Woburn, MA) and anti-Ub Abs (Santa Cruz Biotechnology). (B) Mice were infected with AAVs encoding PNPLA3(WT), PNPLA3(148M), and a synthetic PNPLA3 isoform in which all 19 lysines in the protein were substituted with arginines (see Fig. 3, top panel). LDs were isolated and solubilized, and PNPLA3 was immunoprecipitated from a total of 25  $\mu$ g of the protein as described in the *Methods*. A total of one-tenth of the immunoprecipitate was subjected to immunoblotting. The experiment was repeated twice and the result was similar.



**Fig. S2. Intracellular localization and activity of PNPLA3(K→R) phenocopies PNPLA3(WT) in QBI-293A cells.** (A) PNPLA3(WT), PNPLA3(47A) and PNPLA3(K→R) were expressed in QBI-293A cells alone or (B) together with CGI-58, a co-factor for ATGL. Cells were grown in the presence of OA (200  $\mu$ M) for 24 h and then immunofluorescence localization was performed as described in the *Supplementary Methods* using an anti-PNPLA3 mAb (11C5) and anti-myc pAb. AutoDOT (Abgent, San Diego, CA) was used to stain LDs. The white dashed lines indicate sample cells that express the protein. Scale bar = 10  $\mu$ m. The experiment was repeated and the results were similar.



**Fig. S3. *Pnpla3*<sup>148M/M</sup> mice develop steatosis when subjected to fructose challenge.** Male *Pnpla3*<sup>+/+</sup> and *Pnpla3*<sup>148M/M</sup> mice (n=10/group, aged 7-9 weeks) were fed a high-fructose diet for 2 wks and then subjected to the diet synchronization protocol. LDs were isolated as described in the *Supplementary Methods* and the proteins (3  $\mu$ g) were analyzed by immunoblotting using antibodies directed against PNPLA3, CGI-58 and PLIN2. The signals were quantified using LICOR and the ratios of PNPLA3 and CGI-58 to PLIN2 are provided. Liver TGs were extracted from lysates and measured enzymatically as described in the *Methods*. The means  $\pm$  SE are given. \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001. The experiment was repeated once and the results were similar.



**Fig. S4. PROTAC3-mediated degradation of Halo-PNPLA3(148M).** (A) Male mice (n=4/group, aged 12 wks) were injected via the tail vein with AAVs expressing Halo, Halo-PNPLA3(WT) and Halo-PNPLA3(148M) ( $1.25 \times 10^{11}$ GC) and then fed a HSD for 4 wks. The diets were synchronized prior to the livers being harvested for lipid analysis and isolation of LDs as described in the *Methods*. (B) Total RNA was prepared from the livers of the mice and RT-PCR analysis was performed as described in the *Methods*. (C) Lipids were extracted from the liver lysates and TG's measured enzymatically. Means  $\pm$  SE are provided.

