SUPPLEMENTARY MATERIALS AND METHODS

ANTIBODIES

Mouse anti-human (h) PNPLA3 (clone 11C5) and rabbit anti-mouse (m) PNPLA3 monoclonal antibody (mAb) (clone 19A6) were developed as described.[1, 2] Rabbit anti-mouse 17β-hydroxysteroid dehydrogenase 13 (HSD17B13) antiserum was developed against a peptide from mHSD17B13 (residues 136-300). The antiserum was generated as described[3] except that it was not purified by Protein A affinity column. The following mAbs were obtained from commercial vendors: rabbit anti-perilipin 2 (PLIN2) (ab108323, abcam, Cambridge, MA), mouse anti-CGI-58 (Novus Biologicals, Centennial, CO), rabbit anti-hormone sensitive lipase (HSL) (#18381, Cell Signaling Technology, Danvers MA), mouse anti-green fluorescent protein (GFP) (Clontech, Mountain View, CA), rabbit anti-lactate dehydrogenase (LDH) (ab134187, abcam), mouse anti-β-actin (#3700, Cell Signaling Technology), and mouse anti-glutathione S-transferase (GST) (SAB4200237, Sigma-Aldrich, St. Louis, MO). A rabbit anti-ATGL polyclonal Ab (pAb) (#2138) was purchased from Cell Signaling Technology and a rabbit anti-Calnexin pAb was purchased from Enzo Life Sciences (Farmingdale, NY). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, goat anti-mouse IgG, goat anti-mouse light chain IgG, and mouse anti-rabbit light chain IgG were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). The Abs used for immunofluorescence included mouse anti-hPNPLA3 mAb (clone 11C5), rabbit anti-myc tag polyclonal Ab (ab9106, Abcam, Cambridge, UK), Alexa 488-conjugated goat-anti-rabbit IgG (A11034) and Alexa 555-conjugated goat-anti mouse IgG (A21422, Thermo Fisher Scientific, Waltham, MA).

LIPID QUANTIFICATION

Lipids were extracted from frozen liver samples (about 100 mg) using the method described by Folch.[4] Triglyceride (TG), phosphatidylcholine and total cholesterol were measured by enzymatic assays following the manufacturer's instructions (Thermo Fisher Scientific; Wako Chemicals USA, Inc., Richmond, VA). Values were normalized by sample weight.

MOUSE LIVER LD FRACTIONATION

Lipid droplets (LDs) from mouse livers were fractionated by size as described.[5] Briefly, liver homogenates were centrifuged at 500*g* for 5 min to remove the nuclei and an aliquot of the post-nuclear supernatant (PNS) (7 ml) was transferred to an ultracentrifuge tube (#344059, Beckman Coulter, Brea, CA). The PNS was overlaid with Buffer B (100 mM KCl, 2 mM $MgCl_2$, 20 mM HEPES, pH 7.4) (5 ml) and then centrifuged at 500*g* for 30 min. The top layer (white) was transferred to an Eppendorf tube (Fraction 1). Buffer B was added to the ultracentrifuge tube to restore the original volume (12 ml) and the tube was centrifuged at 2,000*g* for 30 min and the top layer was again collected (Fraction 2). After balancing the tubes with Buffer B, centrifugation was performed first at 8,000*g* (30 min) and then at 247,100*g* (30 min) to collect Fractions 3 and 4, respectively. Each of the isolated LD fractions was centrifuged again for 5 min at the original speed and the infranatants and pellets were removed. The process was repeated 2-3 times or until there was no pellet after centrifugation.

SIZE MEASUREMENT OF LD SUBPOPULATION BY DYNAMIC LIGHT SCATTERING (DLS)

The size distribution of LDs in the four LD fractions was measured by DLS. Triton X-100 was added to each LD fraction to a final concentration of 1% and placed on ice for 30 min with gentle vortexing every

10 min to limit aggregation. The Triton-treated LD fractions were diluted at least 100-fold in H_2O and then warmed to room temperature. The sizes of the LDs in each fraction were measured by DelsaMax PRO light scattering analyzer (Beckman Coulter).

QUANTITATIVE PCR

Total RNA were extracted from liver samples and the levels of selected transcripts were quantified by real-time PCR as described.[6] All reactions were performed in duplicate. Mouse cyclophilin A mRNA was used as internal control.

EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS

Recombinant PNPLA3(WT and 148M)-Flag-HisX10 were cloned into recombinant baculoviruses and expressed in *Sf9* cells as described.[7] After 72 h, cells were collected and stored at -80ºC. Cell pellets were resuspended in lysis buffer [0.5 mM sodium phosphate, pH 7.5, 10% glycerol, 1 mM EDTA, 1 mM EGTA plus leupeptin (4 µg/ml), pepstatin (4 µg/ml), and phenylmethylsulfonyl fluoride (PMSF, 4 mM)]. Lysates were homogenized using an electronic homogenizer at 35,000 rpm (Dremel 300 series, Dremel, Racine, WI) and then centrifuged at 500*g* for 10 min. Supernatants were collected and centrifuged at 100,000*g* for 2 h. Membrane pellets were collected, resuspended in solubilization buffer [Tris Buffer Saline (TBS): 50 mM Tris-Cl, pH 7.4, 150 mM NaCl; plus 10% glycerol, 2-mercaptoethanol (5 mM), and 1% DDM and proteinase inhibitors], and homogenized using a 100-ml glass homogenizer (Kontes, Kimble Chase, Rockwood, TN) (40 strokes). The solubilized membranes were stirred for 3 h at 4 °C, and then centrifuged at 100,000*g* for 1 h. Imidazole was added to the supernatant to a final concentration of

10 mM. Solubilized membranes from 2 L cells were incubated with 2 ml of washed Ni-NTA resin (Qiagen, Germantown, MD) at 4ºC overnight. The incubated resins were transferred to an Econo-Column chromatography column (#7371512, Bio-Rad, Hercules, CA) and washed with 100 column volumes (CV) of wash buffer-II [wash buffer-I (TBS, 0.1% DDM, 0.05% cholate, 20 mM imidazole) plus 150 mM NaCl and 2 mM 2-mercaptoethanol]. The bound proteins were eluted with 5 CV of elution buffer (wash buffer-I plus 300 mM imidazole). Proteins that eluted from the Ni-NTA resin were diluted 2-fold with wash buffer-I, incubated with 2 ml of anti-FLAG affinity gel (Sigma-Aldrich) at 4 °C overnight and then applied to an Econo-Column chromatography column. Beads were washed with 50 CV of wash buffer-I plus 0.6% cholate and then washed with 50 CV of wash buffer-III (TBS, 150 mM NaCl and 0.1% DDM) and the proteins were eluted in 5 CV Flag elution buffer [TBS, 0.1% DDM, 2 mM 2-mercaptoethanol and Flag peptide (200 μg/ml, Apexbio Technology LLC, Houston, TX)]. Proteins were mixed with glycerol (15%), flash frozen in liquid N_2 and stored at -80ºC.

Competent BL21(DE3) *E. coli* cells were transfected with recombinant plasmids (pGEX-4T3) encoding GST or GST-CGI-58 fusion protein and cultured at 37ºC. When the optical density of the suspended cells reached 0.6, isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.25 mM) was added to the medium, and the temperature was reduced to 30°C for 5-6 h. Cells were pelleted by centrifugation (4,000 rpm for 15 min) lysed in cold phosphate-buffered saline (PBS) containing 1 mg/ml lysozyme, 4 mM PMSF, and protease inhibitors (cOmplete, Sigma-Aldrich), and disrupted by sonication. Lysates were centrifuged at 100,000*g* for 45 min and the supernatants were collected and incubated with glutathione-agarose beads (G4510, Sigma-Aldrich) prewashed with PBS at 4ºC for 2 h. After incubation, the column was washed with 40 CV of PBS and the bound proteins were eluted with buffer containing 50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 15% glycerol, protease inhibitors, and 10 mM reduced glutathione (Sigma-Aldrich).

CO-IMMUNOPRECIPITATION OF CGI-58 AND PNPLA3

LDs were suspended in immunoprecipitation (IP) buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.1% DDM plus protease inhibitor) and incubated on ice for 30 min with gentle vortexing every 10 min. The mixture was centrifuged at 10,000*g* for 3 min and the protein concentration of the infranatant was determined by BCA assay. Equal amounts of LD protein from each group were pre-cleared using protein A agarose beads (No. 2001, Santa Cruz Biotechnology, Inc., Dallas, TX) at 4ºC for 1 h. Samples were incubated with anti-V5 agarose beads (A7345, Sigma-Aldrich) or protein A beads coated with an irrelevant Ab (2001)[8] at 4ºC overnight and then at 37ºC for 30 min. Beads were washed 4 times with IP buffer (1 ml), incubated at 95°C for 5 min in Laemmli sample buffer (2X), and loaded onto PAGE gels. Immunoblot analysis was performed using HRP-conjugated goat anti-mouse light chain and mouse anti-rabbit light chain IgG to avoid interference by the IgG heavy chain.

For GST pulldown assays, GST or GST-CGI-58 (2 μg) was mixed with recombinant PNPLA3(WT or 148M)-Flag/HisX10 (1 μg) and incubated at 37ºC for 1 h. Glutathione agarose gel (10 μl) was added and the incubation was continued for another 30 minutes. Beads were then pelleted by centrifugation (2,000*g* for 2 min) and washed 4X with 1 ml of pulldown buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.1% DDM plus protease inhibitor). The beads were mixed with Laemmli sample buffer (2X) and heated to 95ºC for 5 min prior to immunoblot analysis.

Legends to Supplementary Figures

Fig. S1. Cellular localization of recombinant PNPLA3(WT and 148M). Recombinant human PNPLA3(WT and 148M) were expressed as fusion proteins with green fluorescent protein (GFP) at the N-terminus (ter) or C-ter in QBI-293A cells treated with 200 μM oleic acid (OA) for 24 h. Sections were

processed for visualization as described in the *Methods*. LDs were stained with monodansylpentane (MDH, cyan). The dashed lines indicate the outline of the cells. Scale bar = 10 μm.

Fig. S2. Cellular localization of recombinant ATGL(WT, 148M, and 47A) in cells treated with OA (200 μM). Recombinant mouse (m)ATGL(WT and 148M) (A) and human (h)ATGL (B), both fused with GFP at the N-ter or C-ter were expressed in QBI-293A cells. Cells were treated with 200 μM OA for 24 h prior to processing for visualization as described in the *Methods*. LDs were stained with MDH (cyan). The dashed lines indicate the outline of the cells. Scale bar = 10 μm.

Fig. S3. Detergent-mediated dissociation of proteins from hepatic LD. Hepatic LDs were purified from *PNPLA3Tg*$^{WT/+}$ and *PNPLA3Tg*$^{148M/+}$ mice. A total of 100 μl of LDs (containing 20 μg protein) was incubated in serial dilutions of n-dodecyl β-D-maltoside (DDM) and maintained on ice for 30 min. After the incubation, the mixture was centrifuged at 10,000*g* for 3 min and the infranatant was collected. (A) Proteins were analyzed by immunoblotting as described in the *Methods*. Proteins from the LDs that were not incubated with detergent served as controls (lane 6 and 12). (B) The signals corresponding to each protein were quantified using LI-COR (LI-COR Biosciences), and expressed as a fraction of the detergent-free control. Graphs show the percentage of dissociated protein (lanes 1-5, 7-11) to total protein (lane 6 and 12) at each concentration of DDM. The experiment was repeated once and the results were similar.

Fig. S4. Distribution of PNPLA3(WT and 148M) among LD fractions of different sizes. Hepatic LDs from WT, *PNPLA3Tg*$^{WT/+}$ and *PNPLA3Tg*$^{148M/+}$ mice were isolated using rate-differential centrifugation (see Supplementary Materials and Methods). (A) The mean diameters (± SD) of LDs from each fraction were determined by dynamic light scattering (DLS) using a light scattering analyzer (DelsaPRO MAX). (B) Protein from the LD fractions (2 μg) was size-fractionated by SDS-PAGE and analyzed by

immunoblotting as described in the *Methods*. Signals were detected and quantified using LI-COR. M, membrane. PNS, post-nuclear supernatant. (C) Signals in panel B were normalized to the levels of PLIN2. The experiment was repeated once in transgenic mice and twice in knock-in (*Pnpla3*$^{148M/M}$) mice and the results were similar.

Fig. S5. Chow-fed male *Cgi58*$^{fl/fl}$ and Ls-*Cgi58*$^{-/-}$ mice were infected with recombinant adenoviruses expressing no insert (RR5), V5-tagged PNPLA3(WT or 148M). Messenger RNA levels of selected transcripts were quantified by real-time PCR. The values were normalized to the levels of mouse cyclophilin A and expressed relative to the levels in *Cgi58*$^{fl/fl}$ mice infected with empty virus (RR5). (B) Body weights, liver weights and lipid levels in mice described in the legend to Fig. 6. Phospholipids and total cholesterol levels were measured by enzymatic assays and normalized to sample weight. Values are means ± SD. This experiment was repeated twice and the results were similar. The data in Panel B and C were pooled from 3 independent experiments under similar conditions.

Fig. S6. Recombinant GST, GST-CGI-58, PNPLA3(WT or 148M)-Flag/HisX10 were purified as described in the *Methods*. A total of 2 μg of GST or GST-CGI-58 was incubated with 1 μg of PNPLA3(WT or 148M)-Flag/HisX10 at 37ºC for 1 h. The mixtures were processed as described in the *Methods* and silver staining and immunoblotting was performed using 1/10th of the sample (PNPLA3) or 1/120th of the sample (CGI-58, GST). The experiments were repeated once and the results were similar.

Supplementary Table

Table S1. Plasmid description and cloning methods.

Name	Description	Cloning method*
PNPLA3(WT, 47A, or 148M)	Untagged hPNPLA3 (WT, 47A or 148M)	Cloned into pcDNA-V5-His-TOPO using TA cloning (Invitrogen, Carlsbad, CA). A stop codon was inserted at the end of PNPLA3 using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA).
mC-PNPLA3(WT or 148M)	hPNPLA3 (WT or 148M) with mC at N-ter	Cloned into pMXs-CMV-mCherry† using Gateway cloning (Invitrogen)
PNPLA3(WT or 148M)-mC	hPNPLA3 (WT or 148M) with mC at C-ter	Restriction ligation (*Nhe*I & *Hind*III) into pmCherry-N1 (Clontech, Mountain View, CA)
GFP-PNPLA3(WT or 148M)	hPNPLA3 (WT or 148M) with GFP at the N-ter	Cloned into pcDNA-DEST53 (Invitrogen) using Gateway cloning
PNPLA3(WT or 148M)-GFP	hPNPLA3 (WT or 148M) with GFP at the C-ter	Cloned by restriction ligation (*Nhe*I & *Hind*III) into pAcGFP1-N1 (Clontech)
ssBFP-KDEL‡	BFP with ER import signal sequence (PDI signal sequence) at N-ter and KDEL at C-ter	
GFP-mATGL(WT or 148M)	mATGL(WT or 148M) with GFP at the N-ter	Cloned into pcDNA-DEST53 using Gateway cloning
GFP-hATGL(WT or 47A)	hATGL(WT or 47A) with GFP at the N-ter	Cloned into pcDNA-DEST53 using Gateway cloning
mATGL(WT or 148M)-GFP	mATGL with GFP at the C-ter	Cloned into pAcGFP1-N1 using restriction ligation (*Nhe*I & *Sac*II)
hATGL(WT or 47A)-GFP	hATGL with GFP at the C-ter	Cloned into pAcGFP1-N1 using restriction ligation (*Hind*III & *Bam*HI)
mC-mATGL(WT or 148M)	mATGL with mC at the N-ter	Cloned into pMXs-CMV-mCherry using Gateway cloning
EGFP	Enhanced GFP	pEGFP-N1 (Clontech)
hHSD17B13-EGFP	hHSD17B13 with EGFP at the C-ter	Cloned via restriction ligation (*Hind*III & *BamH*I) into pEGFP-N1
hCGI-58-myc	hCGI-58 with myc tag at the C-ter	Cloned via restriction ligation (*Hind*III & *BamH*I) into pcDNA3.1(+)

*All restriction enzymes and T4 ligase were purchased from New England Biolabs (Ipswich, MA). All

variant substitutions were performed using QuikChange II XL Site-Directed Mutagenesis Kit.

† pMXs-CMV-mCherry was obtained from Dr. Orion D. Weiner (University of California at San Francisco).

‡ ssBFP-KDEL was gifted by Farese and Walther laboratory (Harvard School of Public Health).

Abbreviations: ATGL, adipose triglyceride lipase; BFP, blue fluorescent protein; CGI-58-myc, comparative gene identification-58; GFP, green fluorescent protein (EGFP, enhanced GFP); HSD17B13, 17β-hydroxysteroid dehydrogenase 13; h, human; m, mouse; mC, mCherry; PNPLA3, patatin-domain containing phospholipase-like protein 3; ter, terminus

References

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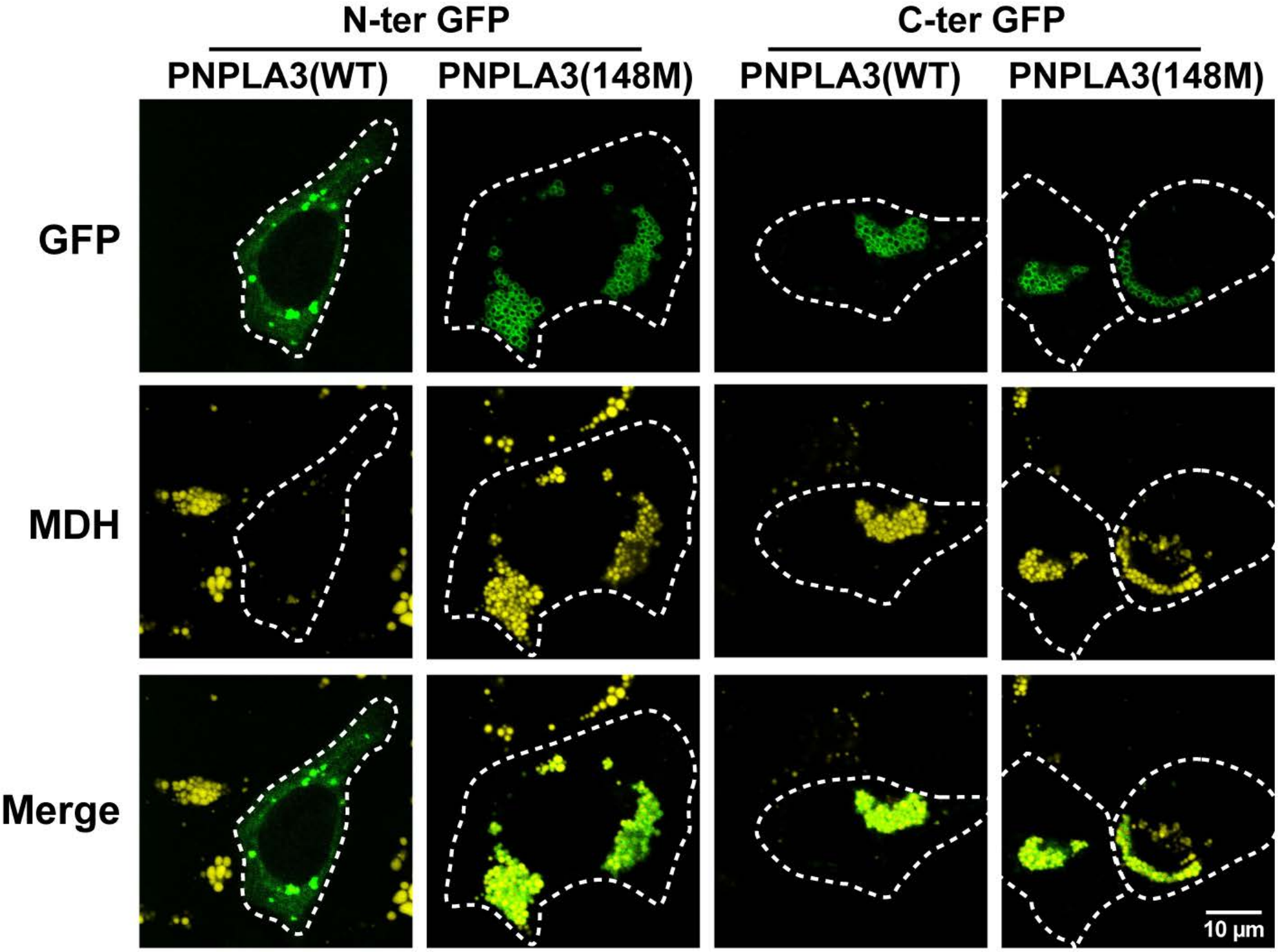
Fig. S1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C-ter GFP
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PNPLA3(148M)
PNPLA3(WT)
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GFP
MDH
Merge
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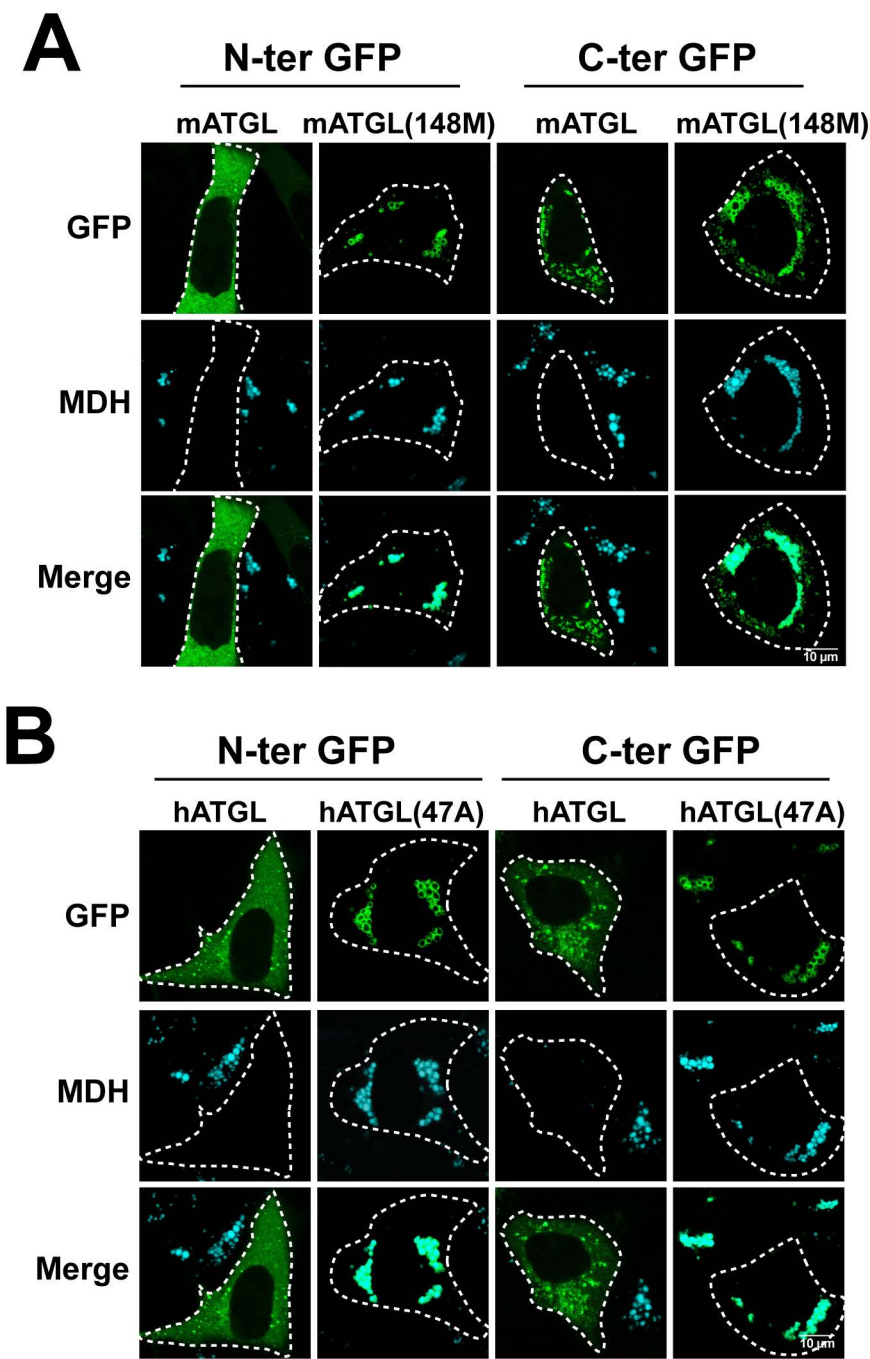
Fig. S2


A
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C-ter GFP
mATGL
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mATGL
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GFP
MDH
Merge
10 µm
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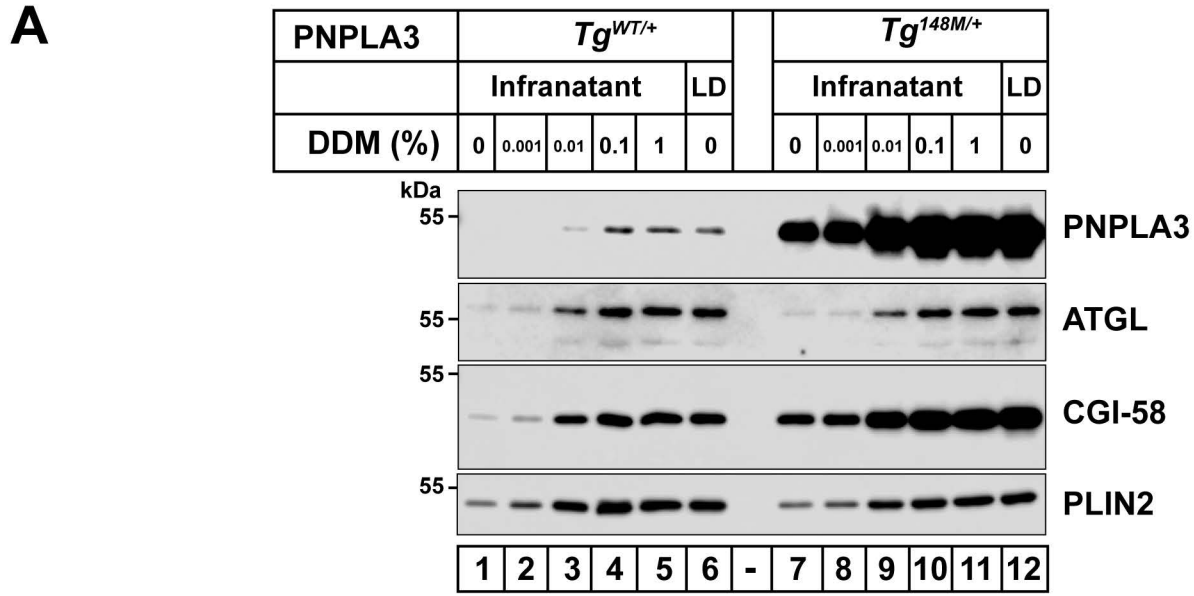
GFP
MDH
Merge
10 µm

Fig. S3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PNPLA3
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Tg^148M/+
Infranatant
LD
DDM (%)
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1
0
kDa
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PNPLA3
ATGL
CGI-58
PLIN2
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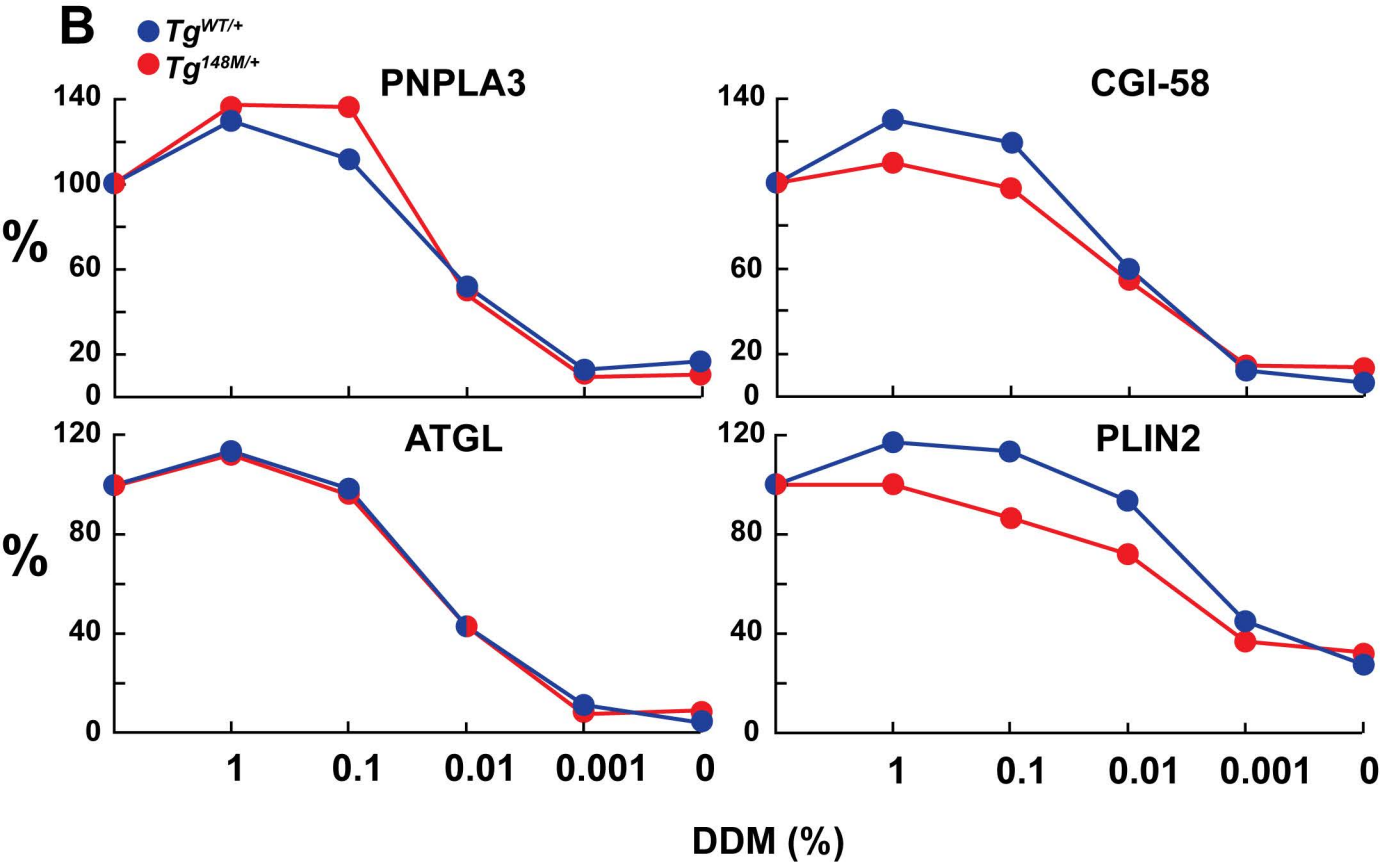
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PNPLA3
CGI-58
ATGL
PLIN2
%
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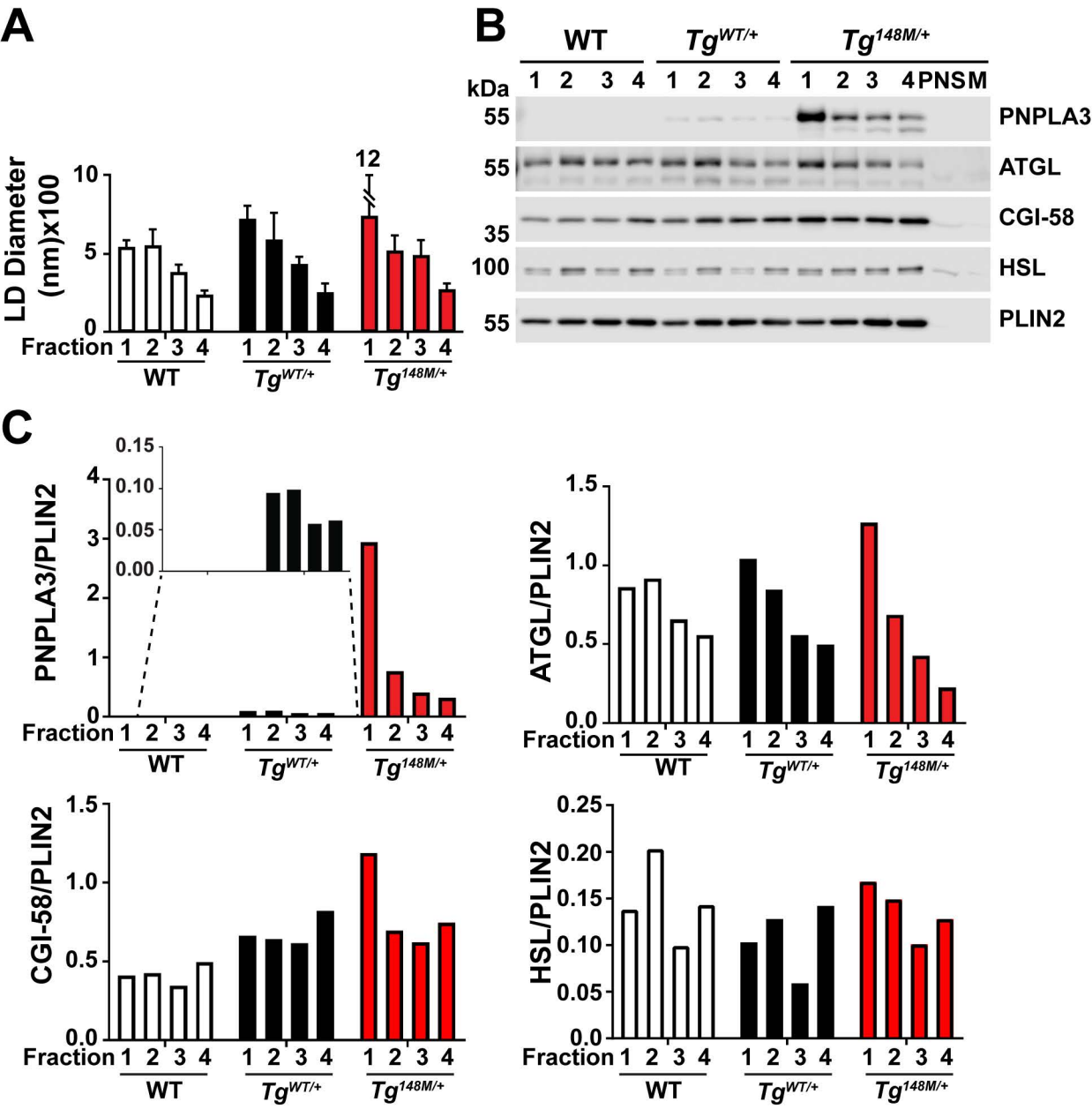
0.01
0.001
0
DDM (%)

Fig. S4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Tg^WT/+
Tg^148M/+
B
WT
Tg^WT/+
Tg^148M/+
kDa
1 2 3 4 1 2 3 4 1 2 3 4 P N S M
55
PNPLA3
55
ATGL
35
CGI-58
100
HSL
55
PLIN2
C
PNPLA3/PLIN2
0.15
0.10
0.05
0.00
4
3
2
1
0
ATGL/PLIN2
1.5
1.0
0.5
0.0
CGI-58/PLIN2
1.5
1.0
0.5
0.0
HSL/PLIN2
0.25
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0.05
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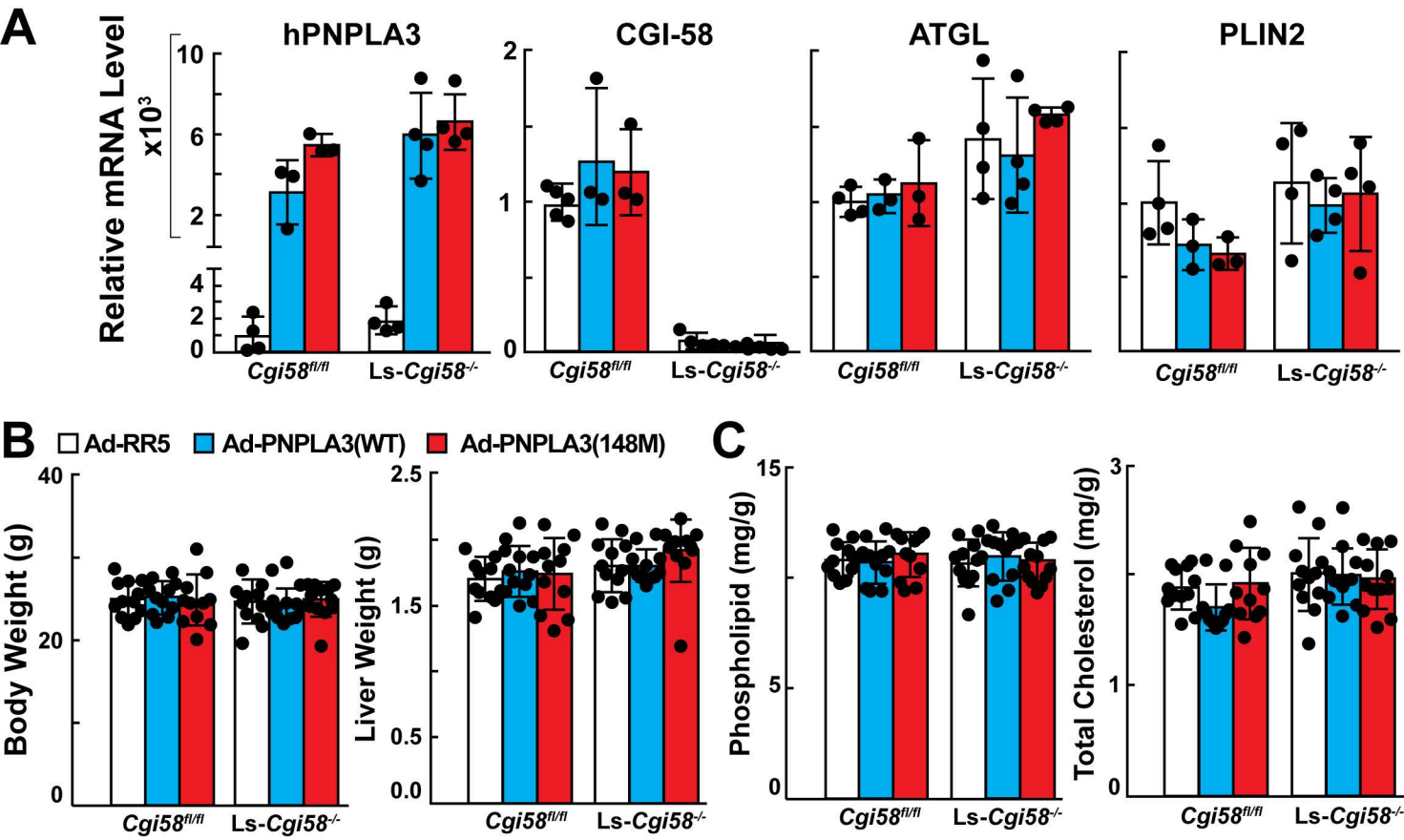
Tg^WT/+
Tg^148M/+

Fig. S5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Ls-Cgi58-/-
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Ad-PNPLA3(WT)
Ad-PNPLA3(148M)
Body Weight (g)
Liver Weight (g)
C
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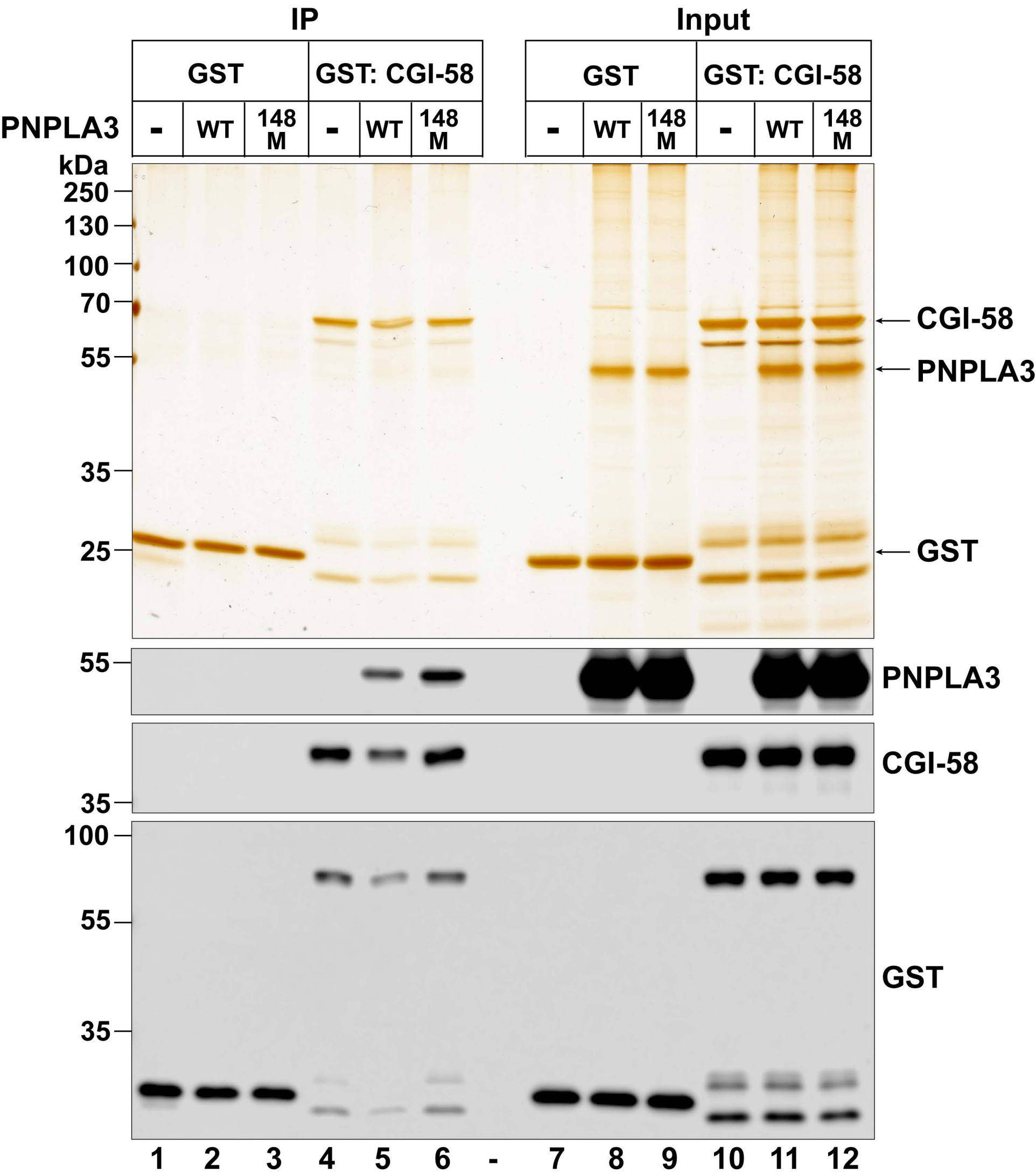
Total Cholesterol (mg/g)

Fig. S6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kDa
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PNPLA3
GST
55
PNPLA3
35
CGI-58
100
55
GST
35
1 2 3 4 5 6 - 7 8 9 10 11 12