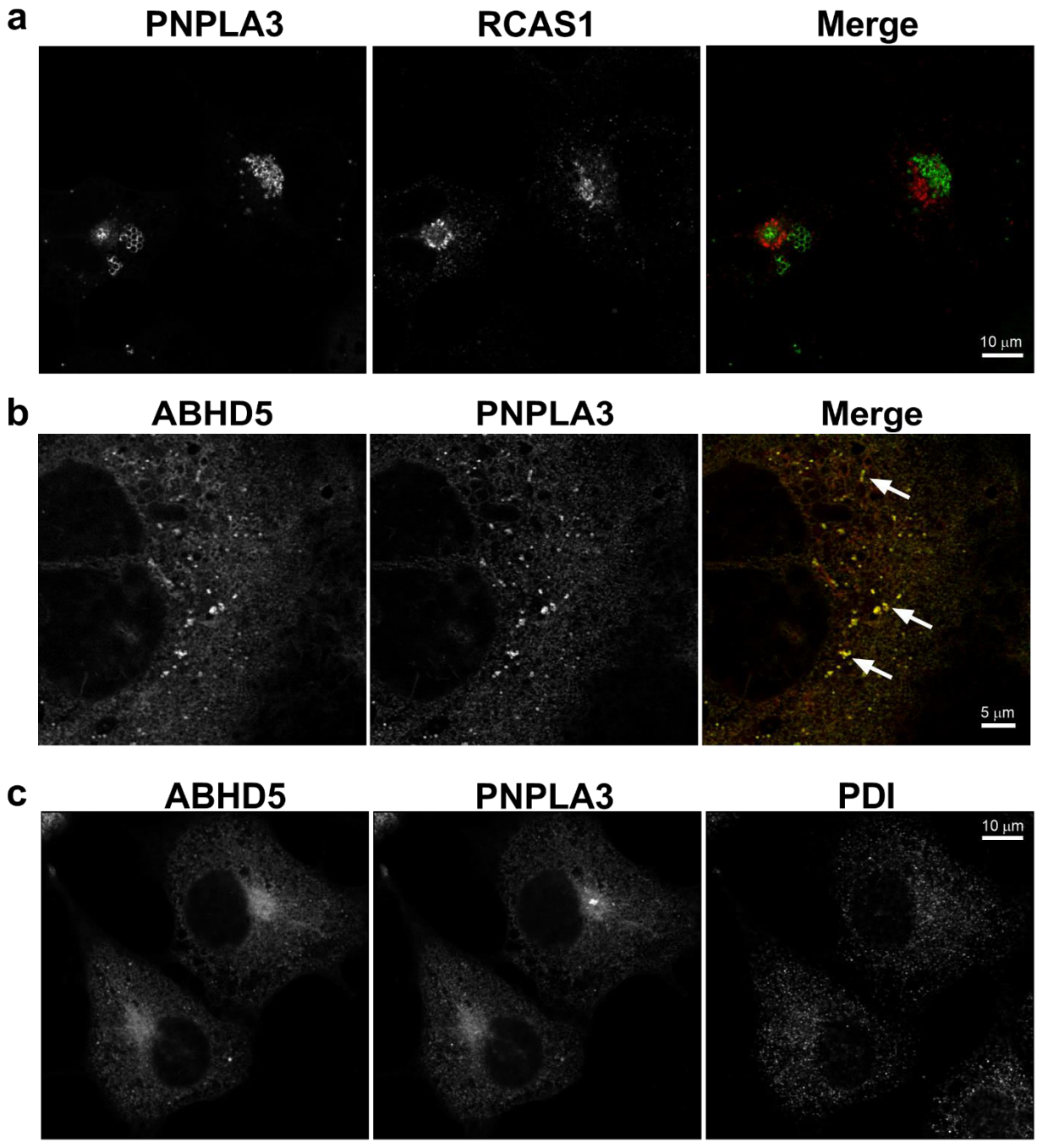
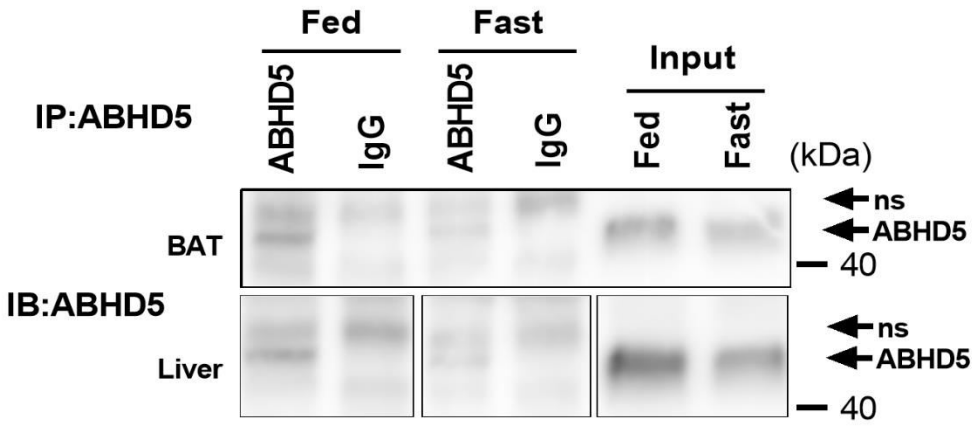
Supplementary Figures

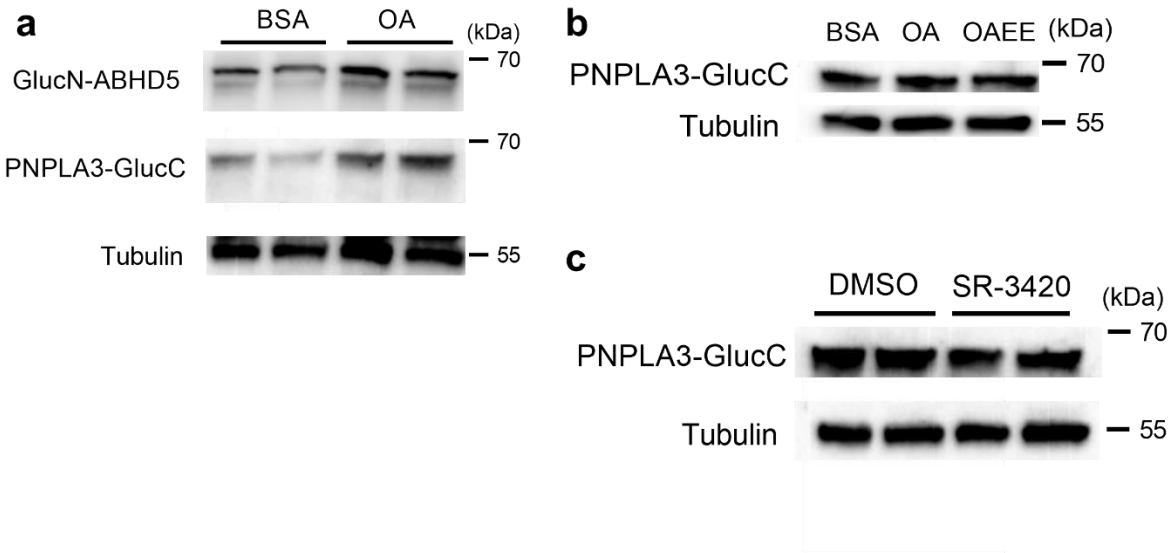


Supplementary Fig. 1. Golgi and endoplasmic reticulum staining and ABHD5 and PNPLA3 co-localization. Cells were transfected with the indicated constructs, loaded overnight with 200 μM oleic acid and fixed with 4% paraformaldehyde in PBS. Fixed cells were then stained for Golgi and ER markers by immunofluorescence and imaged on a Zeiss LSM 800 laser scanning microscope equipped with an Airyscan detector. (**a**) Localization of PNPLA3-EYFP (pseudocoloured green) with Golgi marker Receptor-Binding Cancer-Associated Surface

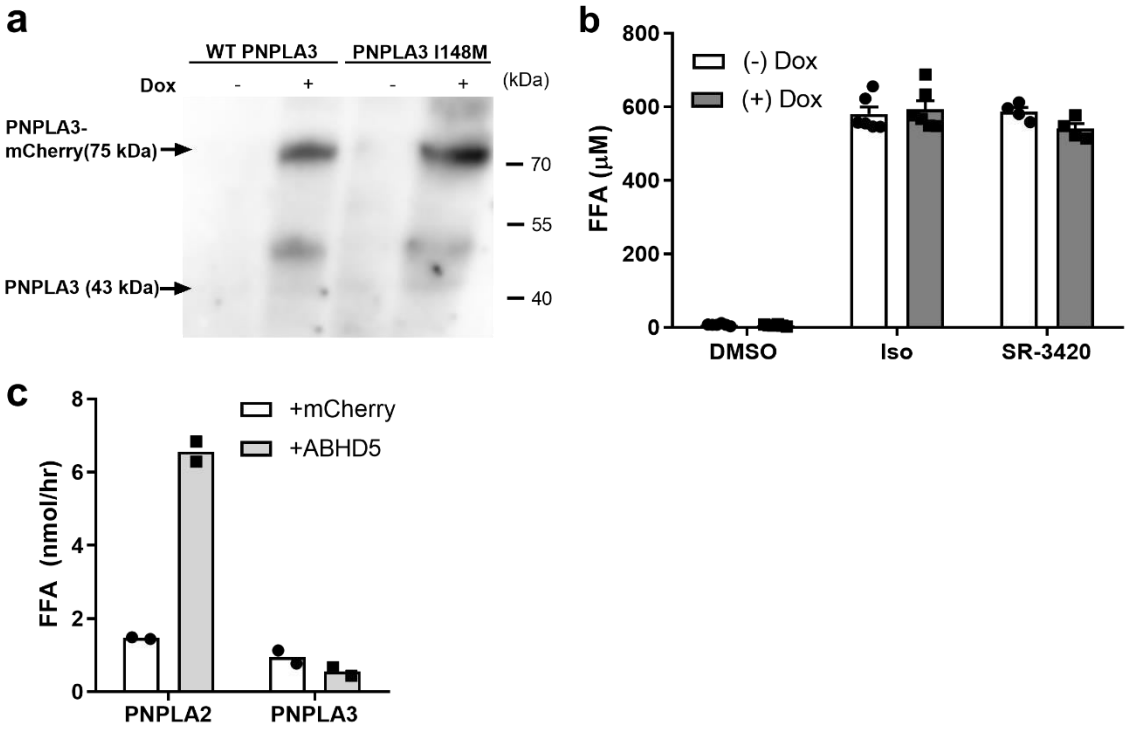
antigen-1 (RCAS1, pseudocoloured red) in COS7 lipid loaded cells. (**b**) Co-localization of ABHD5-mCherry (pseudocoloured red) and PNPLA3-EYFP (pseudocoloured green) to ER and lipid droplets (white arrows) in lipid loaded COS7 cells. (**c**) Localization of ABHD5-mCherry and PNPLA3-EYFP with ER marker protein disulfide isomerase (PDI). Results are from one experiment and are representative of three independent experiments.



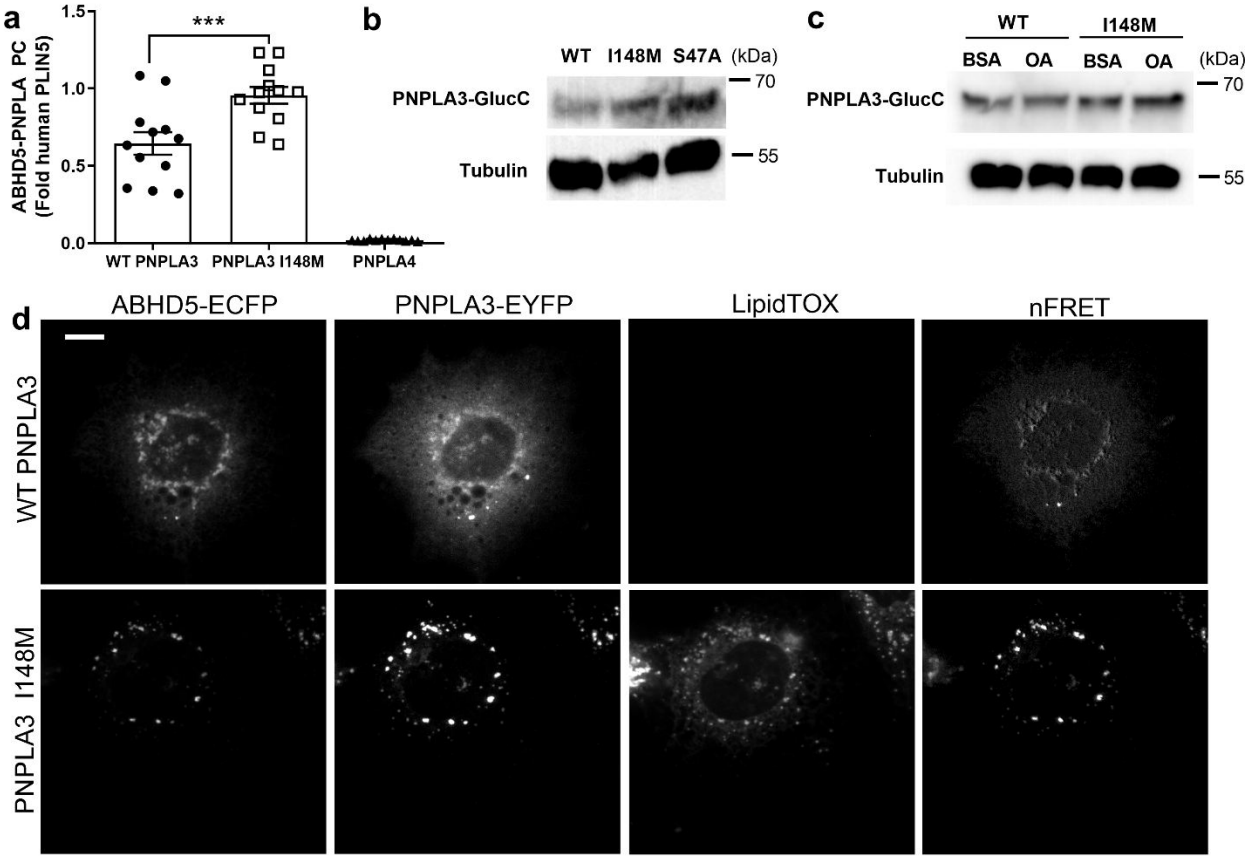
Supplementary Fig. 2. Immunoprecipitation of ABHD5 from brown adipose tissue and liver lipid droplets. Immunoprecipitation (IP) of ABHD5 from solubilized lipid droplets from BAT and liver as in **Fig. 2e**. Non-specific band (ns). Size markers (in kDa) are depicted on western blot panels. Blots were first probed and detected for PNPLA3 (**Fig. 2e**), then stripped and immunoblotted (IB) for ABHD5. IP results are representative of three independent experiments.



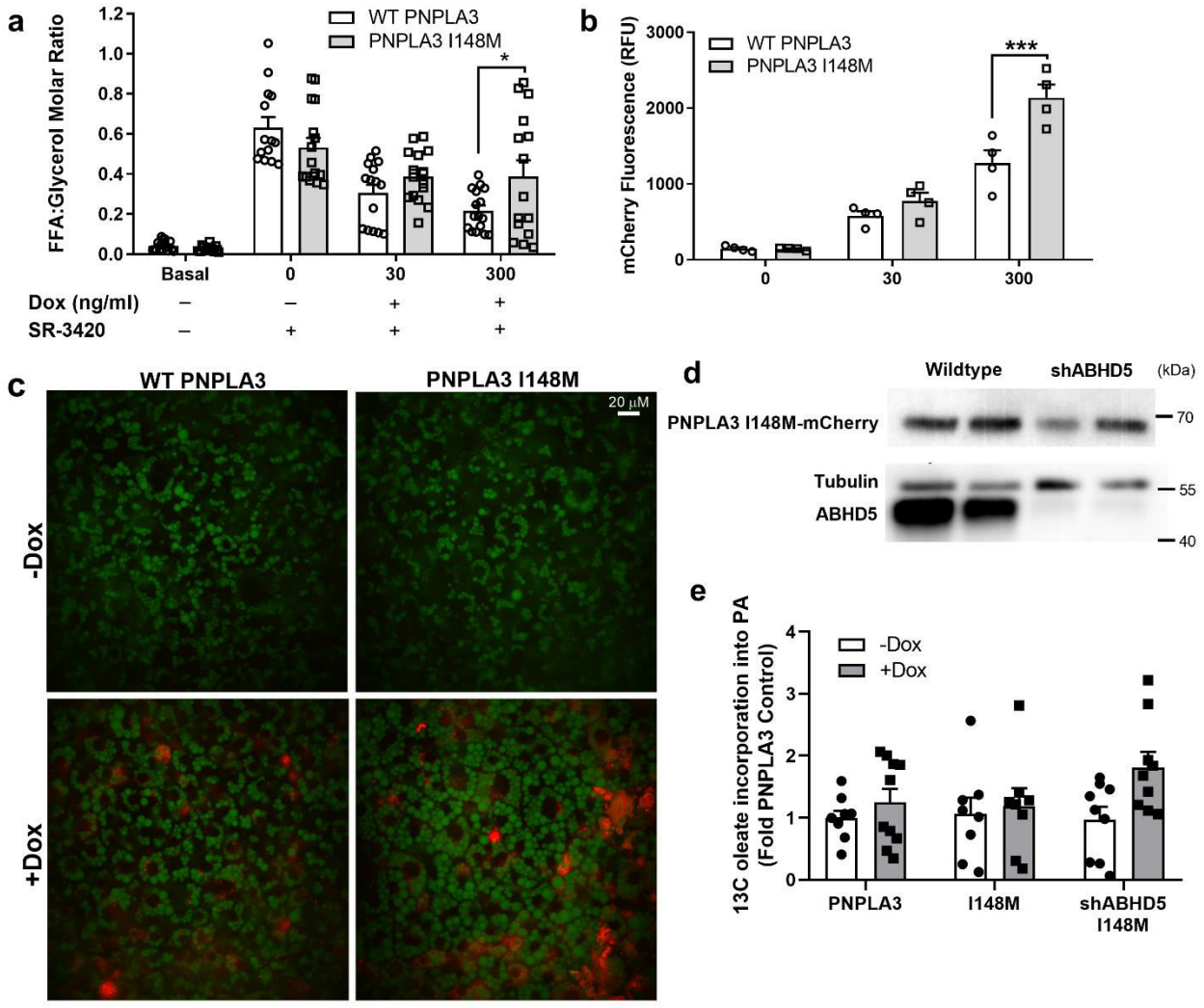
Supplementary Fig. 3. Immunoblot analysis of ABHD5 and PNPLA3 fusion proteins. (**a**) Protein levels of PNPLA3-GlucC, GlucN-ABHD5 and α/β-Tubulin in HEK293A cells following three hours of oleic acid (OA) or bovine serum albumin (BSA) as determined by immunoblotting with *Gaussia* Luciferase and Tubulin antibodies. (**b**) Protein levels of PNPLA3-GlucC and Tubulin in HEK293A cells following treatment with either BSA, OA, or oleic acid ethyl ester (OAEE) for 30 minutes as determined by western blotting with *Gaussia* Luciferase and Tubulin antibodies. (**c**) Protein levels of PNPLA3-GlucC and Tubulin in HEK293A cells following treatment with either DMSO or SR-3420 for 1 hr as determined by western blotting with *Gaussia* Luciferase and Tubulin antibodies. Size markers (in kDa) are depicted on western blot panels. (**a**-**c**) Immunoblotting results are representative of three independent experiments.



Supplementary Fig. 4. PNPLA3 immunoblotting in an immortalized brown adipocyte (BA) cell line and the effect of doxycycline on lipolysis in a control cell line. (**a**) levels of PNPLA3-mCherry fusion protein as detected with a PNPLA3 antibody showing no detectable endogenous levels of PNPLA3 in BA cells. Size markers (in kDa) are depicted on western blot panels. Data are from one experiment and results representative of three independent experiments. (**b**) A control BA cell line was treated with doxycycline as in **Fig. 4b** and stimulated with DMSO, 10 nM Isoproterenol, or 20 μM SR-3420 for three hours. Data are from two (SR-3420) or three (Iso) independent experiments performed with duplicate biological samples. (**c**) *In vitro* lipase assay of triolein substrate incubated with transfected COS-7 lysates expressing mCherry or ABHD5-mCherry and either PNPLA2-EYFP or PNPLA3-EYFP for one hour. Results are from one experiment performed in duplicate and is representative of three independent experiments. Data are expressed as means +/- SEM.



Supplementary Fig. 5. Human ABHD5 and PNPLA3 interact, protein levels of PNPLA3, and FRET in AML12 cells. (**a**) Gluc protein complementation assay of human proteins in HEK293A cells transiently transfected with complementary fragments of human GlucN-ABHD5 and either WT PNPLA3-GlucC, PNPLA3 I148M-GlucC, PLIN5-GlucC, or PNPLA4-GlucC. Data are from three independent experiments performed with biological quadruplicate samples. ***p=0.001 indicates a significant difference between PNPLA3 I148M and WT PNPLA3 as determined by one-way ANOVA. Data are expressed as means +/- SEM. (**b**) Protein levels of WT PNPLA3-GlucC, PNPLA3I148M-GlucC, and PNPLA3 S47A-GlucC and Tubulin as determined by western blot using *Gaussia* luciferase and Tubulin antibody. (**c**) Protein levels of WT PNPLA3-GlucC and PNPLA3 I148M-GlucC treated with either BSA or OA for 30 minutes as determined by western blot with *Gaussia* luciferase antibody. Size markers (in kDa) are depicted on western blot panels. (**d**) FRET assay in AML12 cells expressing ECFP-ABHD5 and either WT PNPLA3-EYFP or PNPLA3-I148M-EYFP and loaded with 200 μM OA overnight. Lipid droplets were visualized using LipidTox Red stain. (**b**-**d**) Data are from one experiment and results are representative of three independent experiments.

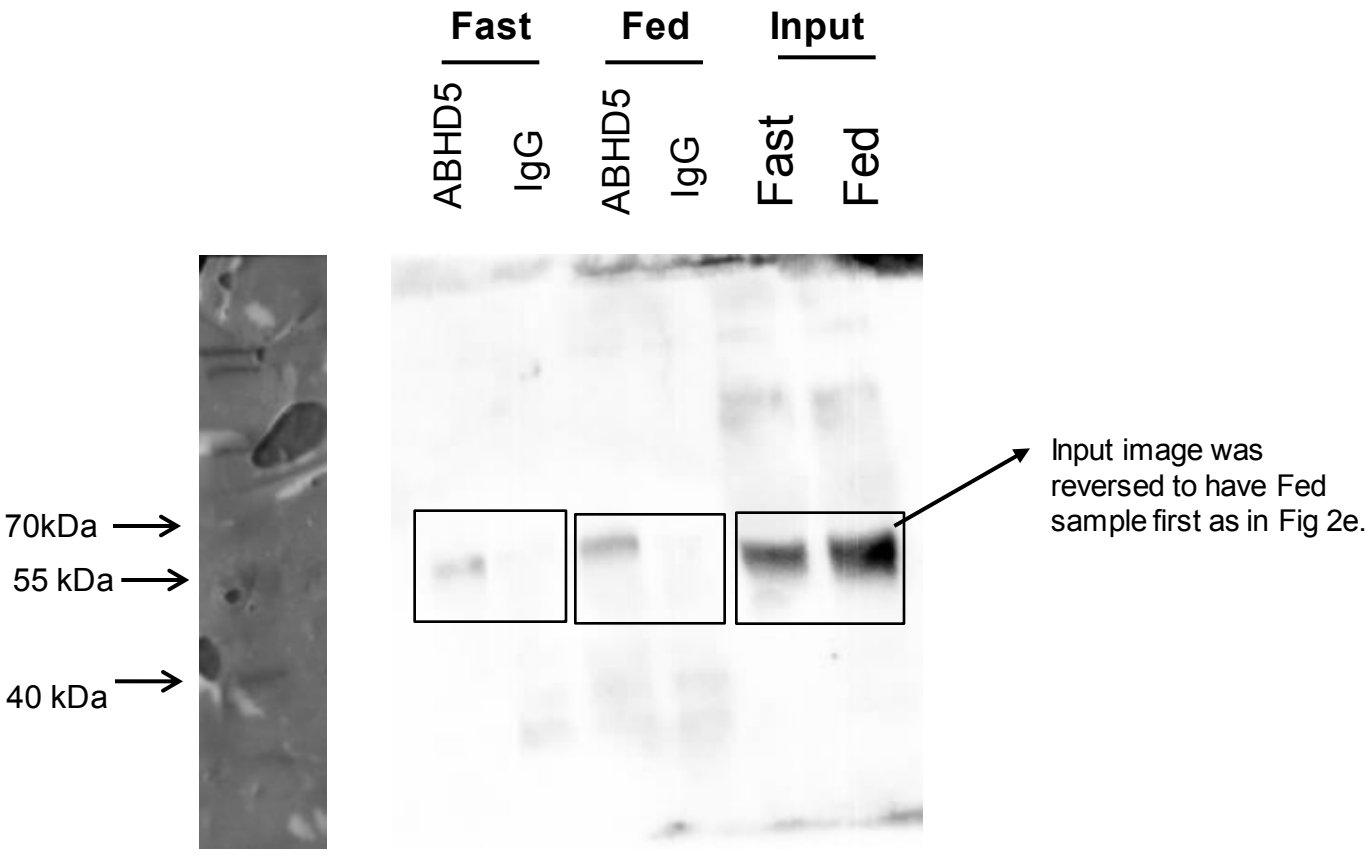


Supplementary Fig. 6. Lipolysis Molar Ratio, analysis of the I148M variant expression, and Lysophosphatidic acid acyltransferase (LPAAT) assay. (**a**) Molar ratio of free fatty acid: glycerol released from Basal (no Dox, DMSO) or SR-3420 (20 μM, 1 hour) stimulated WT PNPLA3-mCherry or PNPLA3 I148M-mCherry brown adipocytes (BA) treated with differing doses of doxycycline (Dox; 0, 30, and 300 ng/ml) from **Fig. 6b** and **c**. *p= 0.0177, indicates a significant difference in the molar ratio between WT PNPLA3 and PNPLA3 I148M as determined by two-way ANOVA with Bonferroni post *t*-test. Data are biological quadruplicates from four independent experiments. (**b**) mCherry fluorescent intensity measured from doxycycline treated (0, 30, or 300 ng/mL) WT PNPLA3-mCherry or PNPLA3-I148M-mCherry BA from **Fig. 6b**. ***p=0.0001 indicates a significant difference between WT PNPLA3-mCherry and PNPLA3 I148M-mCherry expression as determined by two-way ANOVA with Bonferroni post *t*-test. Data are the averages of biological quadruplicates from four independent experiments. (**c**) Fluorescent images at 40x magnification of lipid droplets (Autodot, pseudocoluored green) and mCherry (red) from control (no doxycycline, -Dox) or doxycycline (2 μg/mL; +Dox) treated brown adipocytes expressing WT PNPLA3-mCherry and PNPLA3 I148M-mCherry in a Dox inducible manner. Lipid droplets were stained with Autodot (Abgent) as suggested by the manufacturer. Autodot staining results are from one experiment and are representative of observations from three independent experiments. Scale bar denotes 20 μm. (**d**) Protein levels of exogenously

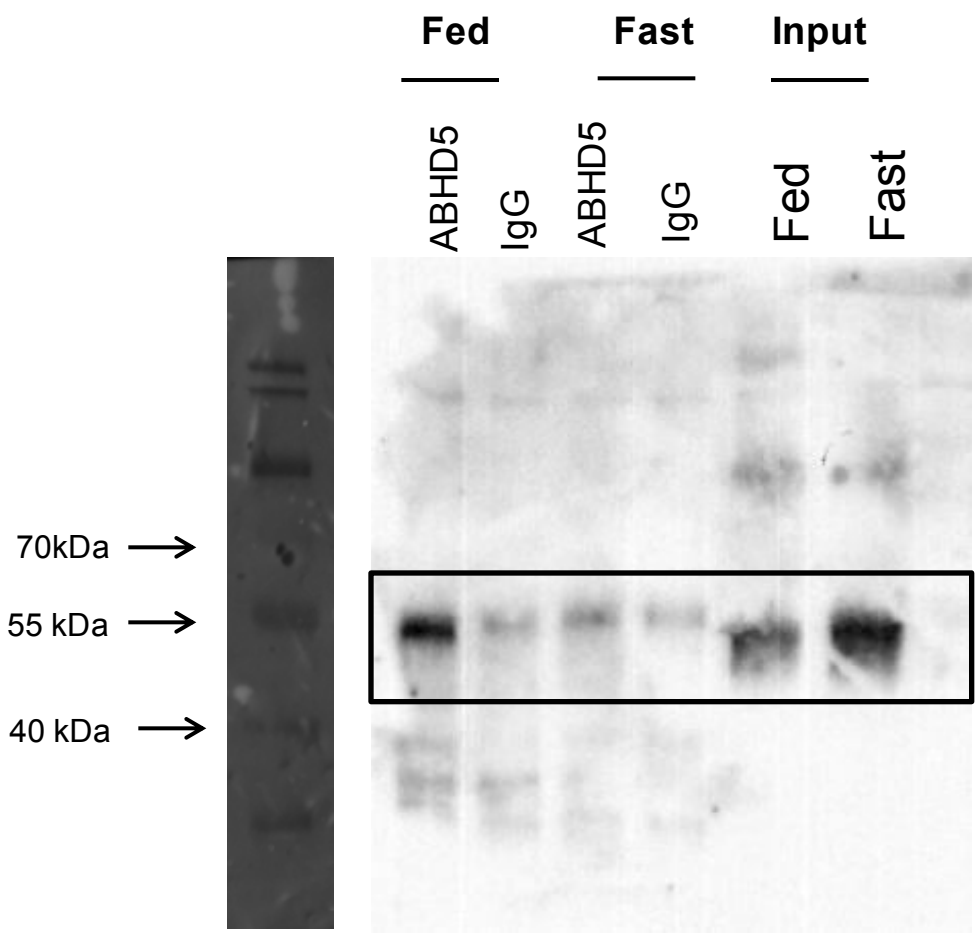
expressed PNPLA3-I148M mCherry in wildtype BA cells and cells with stable knockdown of ABHD5 (shABHD5). Samples are from two independent experiments and blotted once. Size markers (in kDa) are depicted on western blot panels. (**e**) Lysophosphatidic acid acyltransferase (LPAAT) assay in purified lipid droplets from brown adipocytes. A total of 10 μg of lipid droplets purified from WT PNPLA3-mCherry, PNPLA3I148M-mCherry, and PNPLA3-I148M-mCherry shRNA BA cells was incubated with [13C] oleoyl-CoA, lysophosphatidic acid (LPA), and unlabeled oleoyl-CoA for 15 minutes. Incorporation of [13C] oleoyl-CoA into phosphatidic acid was determined by mass spectrometry. Data are from four independent experiments performed in duplicate or triplicate. Data are expressed as means +/- SEM.

Supplementary Fig. 7 (11 pages). Uncropped western blot images from indicated figures. Black boxes indicate the cropped area in the main figures. Markers from white light images of blots are shown to the left.

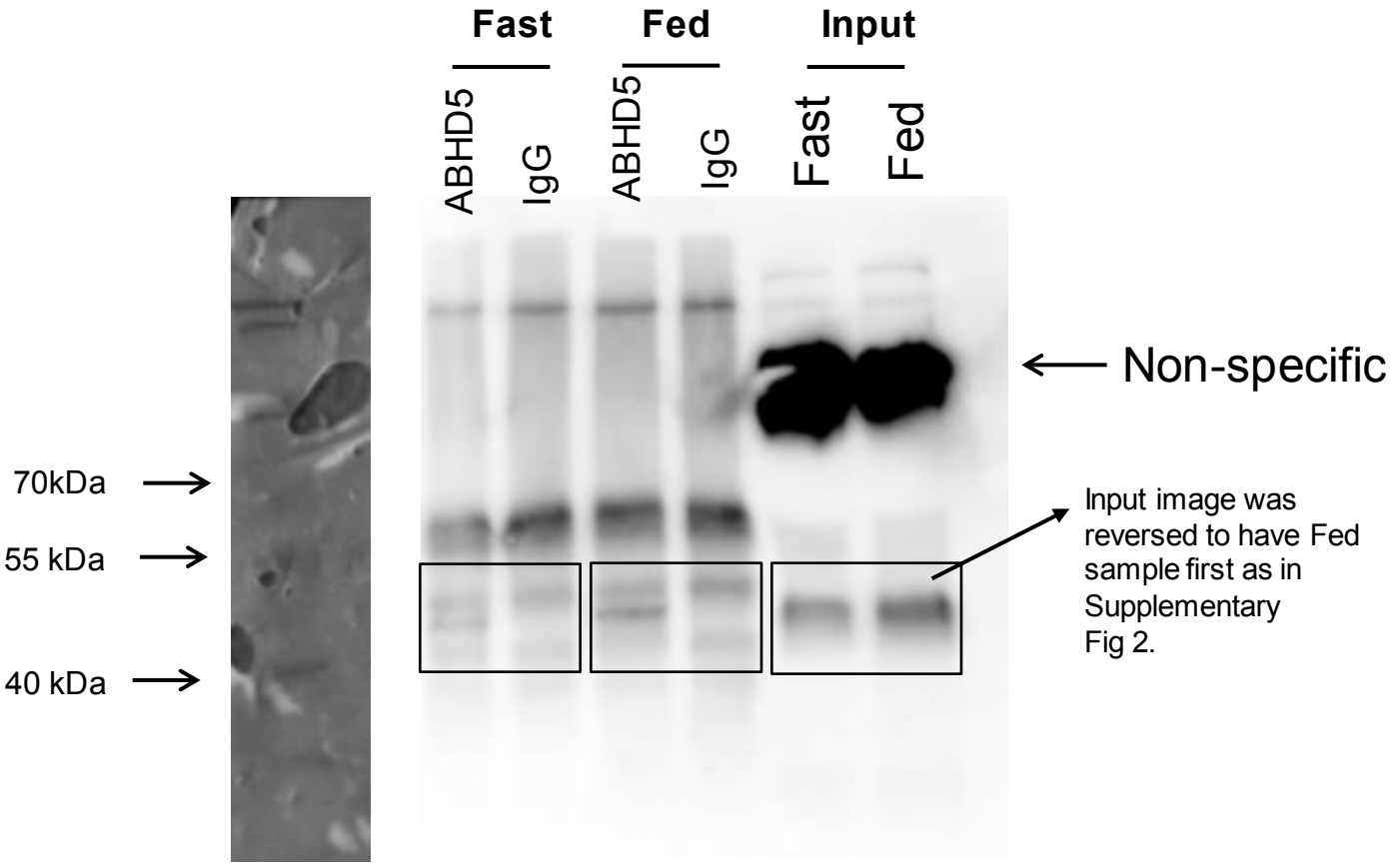
Liver PNPLA3 blot from Fig. 2e



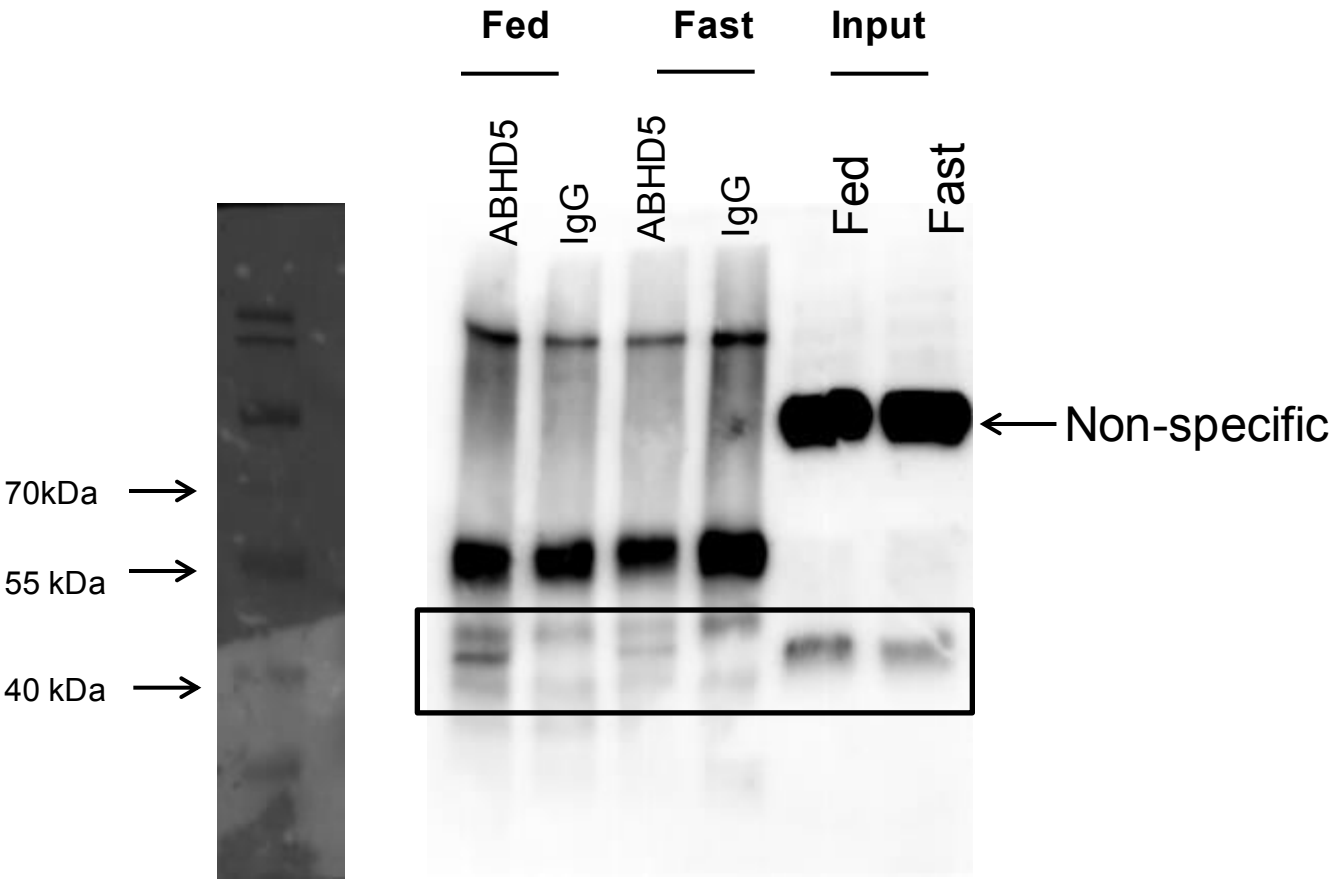
BAT PNPLA3 blot from Fig. 2e



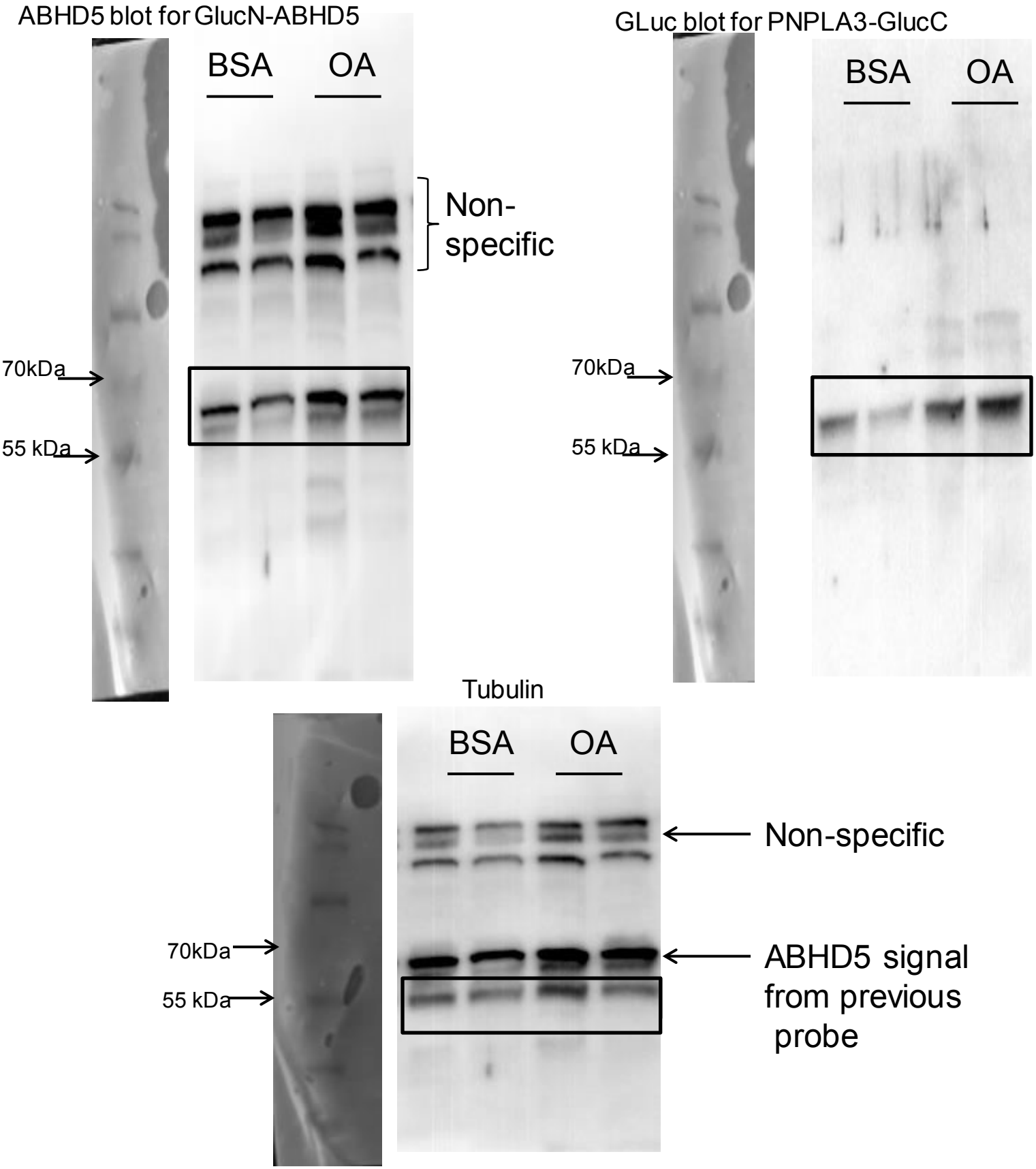
Liver ABHD5 blot from Supplementary Fig. 2



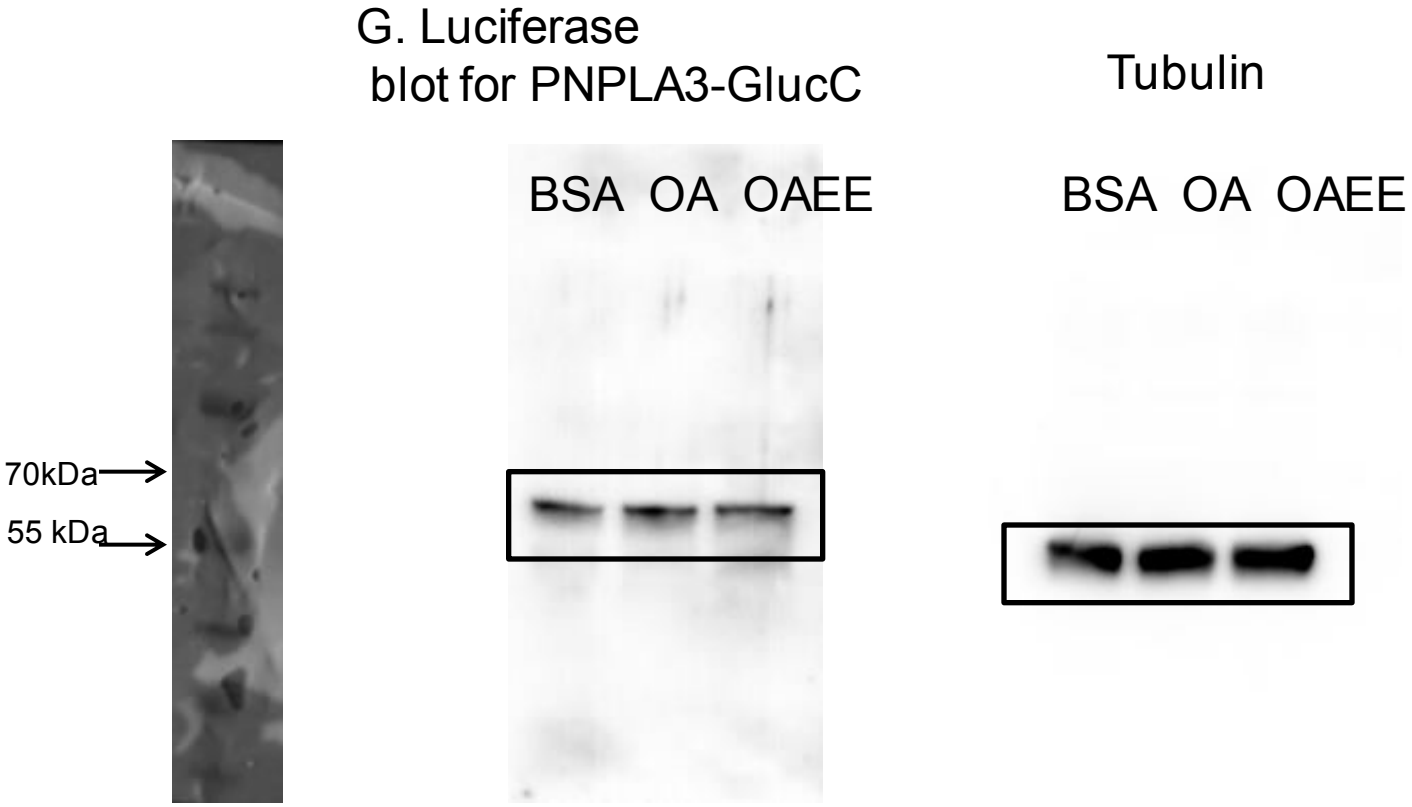
BAT ABHD5 blot from Supplementary Fig. 2



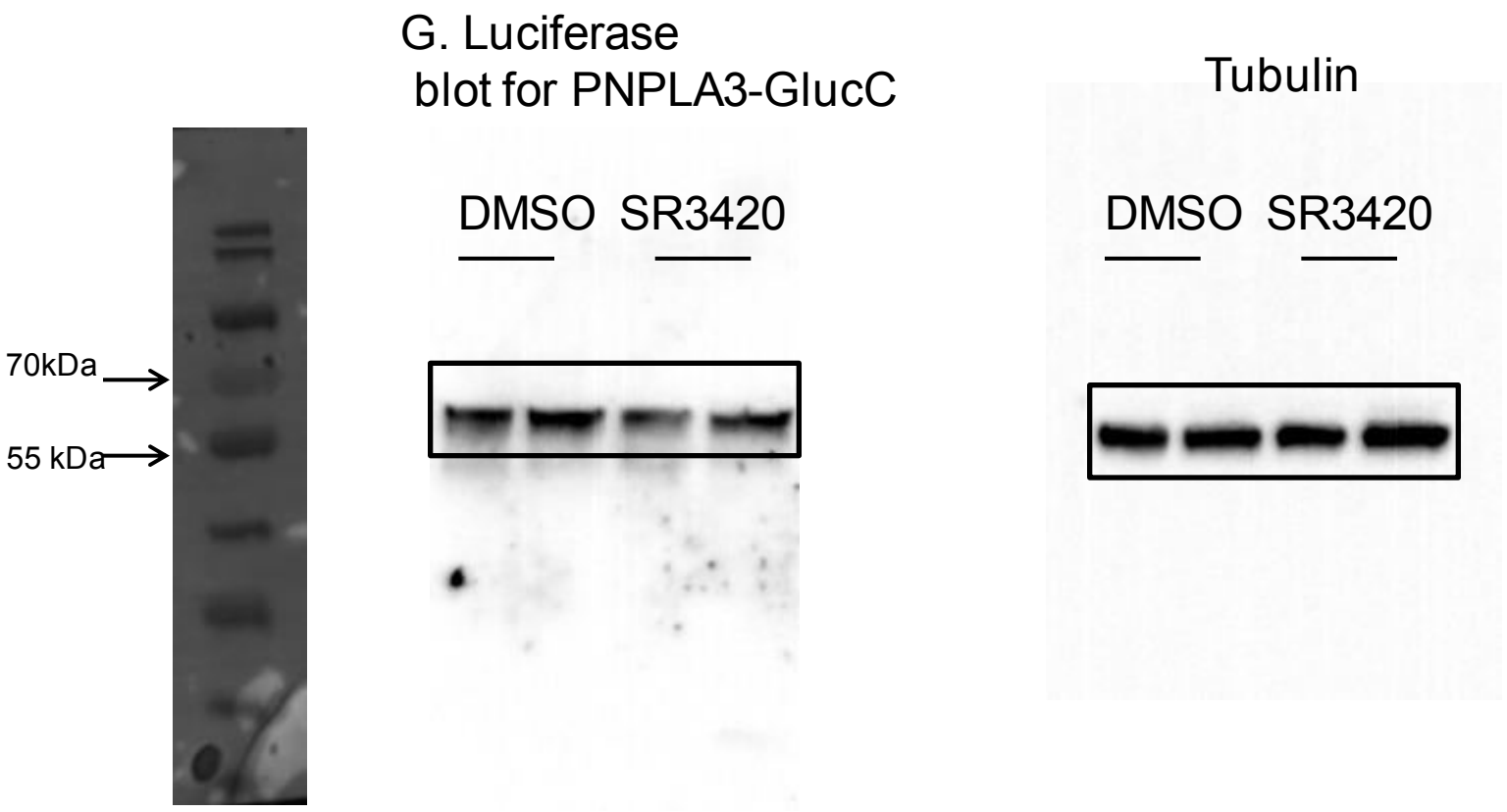
Supplementary Fig. 3a



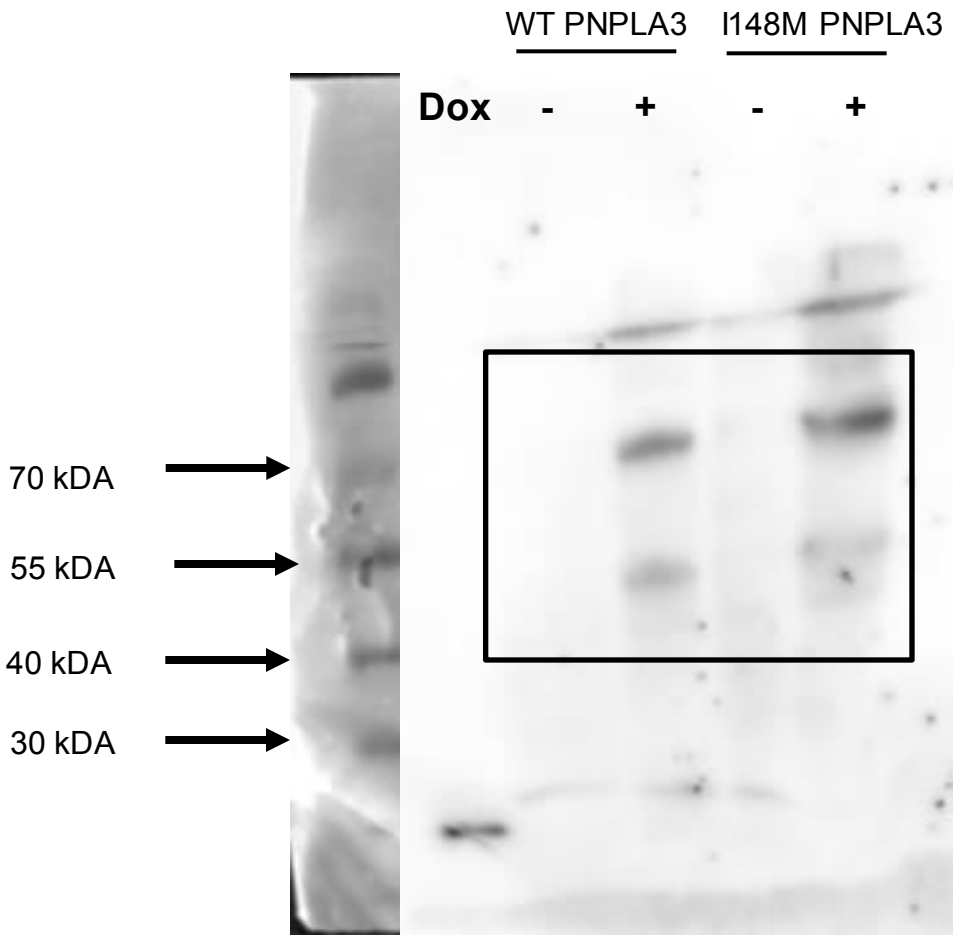
Supplementary Fig. 3b



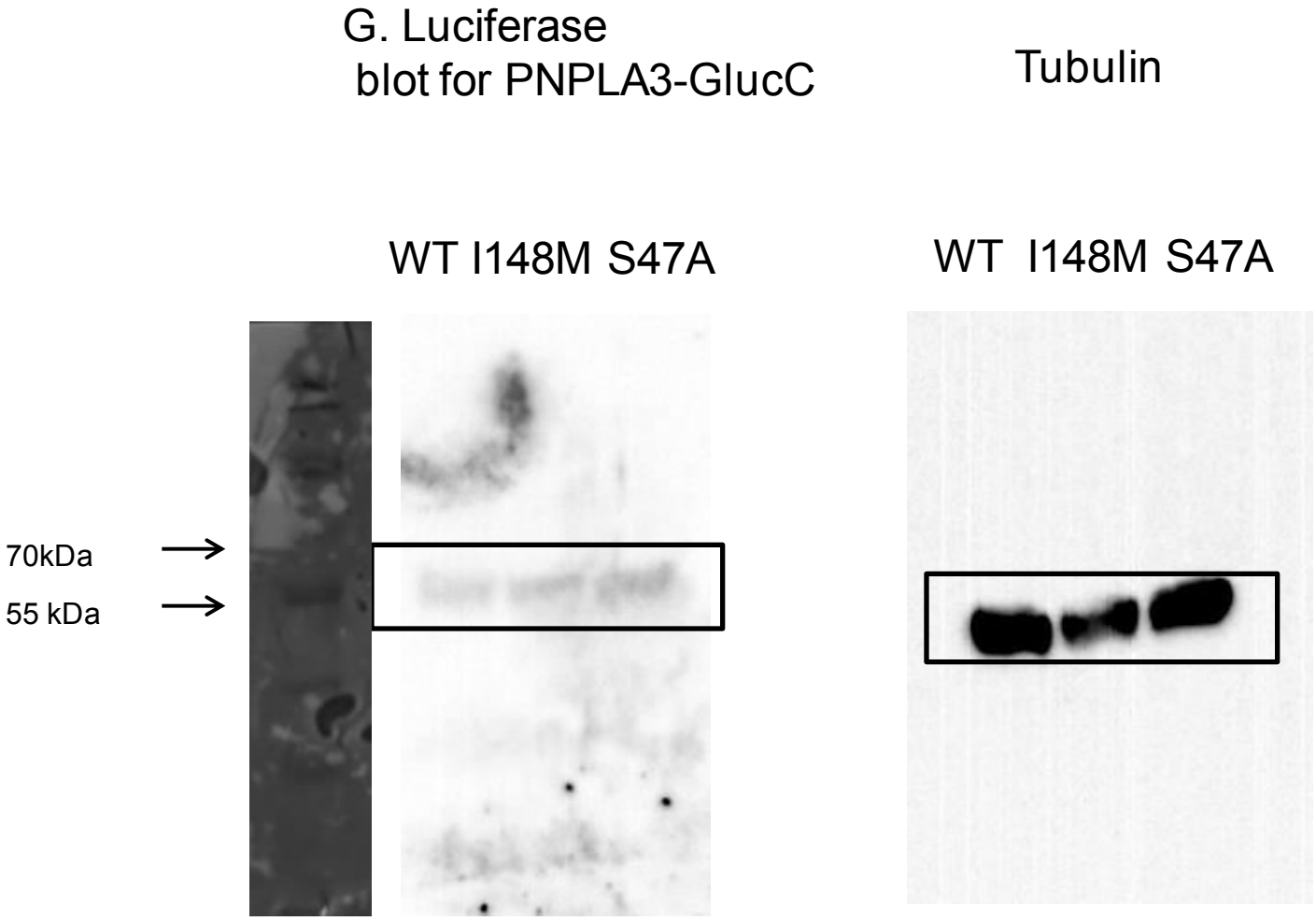
Supplementary Fig. 3c



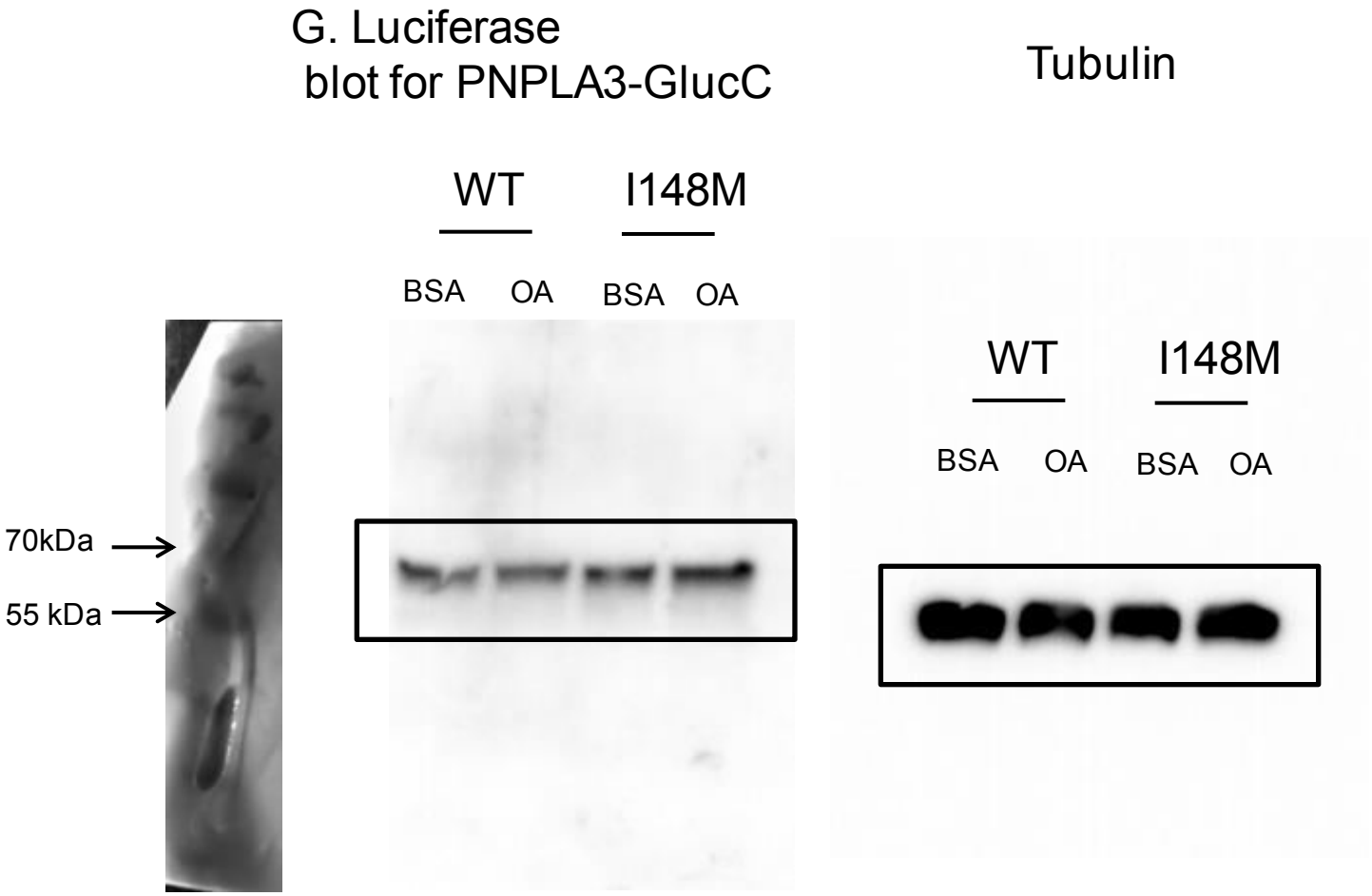
PNPLA3-mCherry blot from Supplementary Fig. 4a



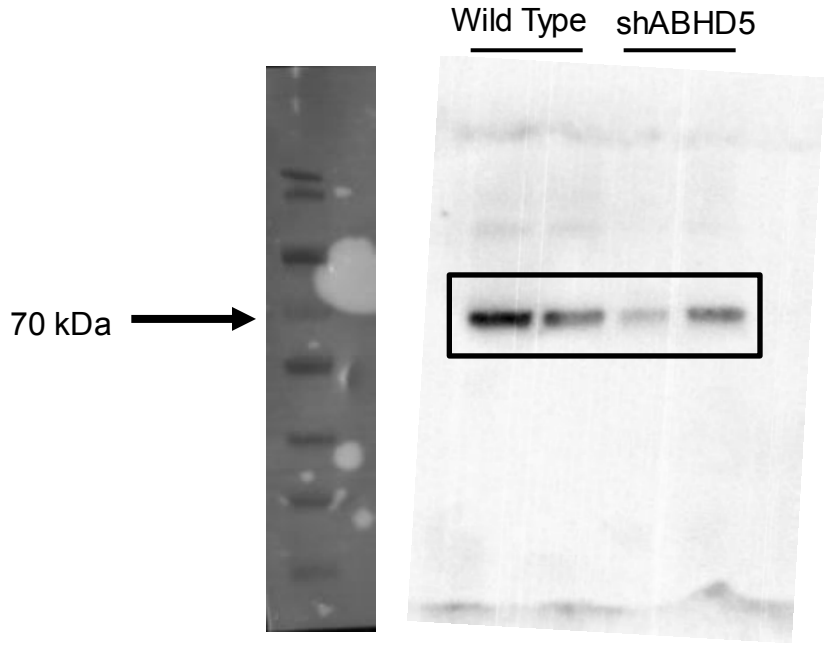
Supplementary Fig. 5b



Supplementary Fig. 5c



PNPLA3 I148M-mCherry blot from supplementary Fig.6d



Tubulin/ABHD5 blot from supplementary Fig.6d