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**Supplementary Information for**
**FAF1 Blocks Ferroptosis by Inhibiting Peroxidation of Polyunsaturated Fatty Acids**

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## Supplementary Information Text

### Supporting Materials and Methods

**Materials.** We obtained FAs and arachidonic ethyl from Nu-Chek Prep, Inc.; CellTiter-Glo luminescent cell viability assay kit from Promega; BODIPY 581/591 C11, BODIPY 493/503, Hoechst 33342, Biotin azide, and Streptavidin-HRP from Thermo Fisher Scientific; TBARS (TCA Method) Assay Kit, deferoxamine, (1S,3R)-RSL3, Erastin2, ferrostatin 1, necrosulfonamide, VX-765 and Nonidet P-40 alternative (NP-40) from

Cayman; Rabbit anti-actin, vitamin E (α-Tocopherol), FA-free BSA, Tris (2-carboxyethyl) phosphine hydrochloride, Liprostatin-1 and trypsin from Sigma-Aldrich; A939572 from Biovision; Ni-NTA agarose from Qiagen; Protease inhibitor cocktail and proteinase K from Roche; ω-alkynyl AA and ω-alkynyl PA from Avanti Polar Lipids; Rabbit anti-calnexin from Enzo Life Sciences; Rabbit anti-ACSL4 and Rabbit anti-FAF1 from abcam; $^{3}$H-Arachidonic acid from PerkinElmer. Hybridoma cells producing anti-Myc 9E10 was obtained from ATCC. Delipidated fetal calf serum (DFCS) was prepared from newborn calf serum by n-butyl alcohol and isopropyl ether extraction as previously described (1). All FAs added into culture media were conjugated to BSA as previously reported (1). For in vitro assays, FAs dissolved in ethanol were added into assay solutions.

**Cell cultures.** *FAF1*$^{-/-}$ and *ACSL4*$^{-/-}$ SV589 cells (human male transformed fibroblasts) were generated using the CRISPR/Cas9 approach by transfection of cells with pSpCas9(BB)-2A-Puro (PX459) encoding gRNA targeting *FAF1* or *ACSL4* followed by selection and single cell cloning in medium containing puromycin. *FAF1*$^{-/-}$; *ACSL4*$^{-/-}$ SV589 cells were generated using the CRISPR/Cas9 approach by transfection of *FAF1*$^{-/-}$ cells with pSpCas9(BB)-2A-Hygro (PX459) encoding gRNA targeting *ACSL4* followed by selection and single cell cloning in medium containing 500 µg/ml hygomycin. *FAF1*$^{-/-}$; *pFAF1* SV589 cells were generated by stably transfecting *FAF1*$^{-/-}$ cells with pCMV-FAF1-Myc (2). *FAF1*$^{-/-}$; *pUAS* SV589 cells were generated by stably transfecting *FAF1*$^{-/-}$ cells with pCMV-FAF1UAS-Myc, which encodes the UAS domain of FAF1 tagged by the Myc epitope. *FAF1*$^{-/-}$; *pEGFP-FAF1* SV589 cells were generated by stably transfecting *FAF1*$^{-/-}$ cells with pEGFP-C1-FAF1, which encodes FAF1 tagged at the N-terminus with EGFP. All SV589-based cells were maintained in Dulbecco's modified Eagle's medium with 1 g/l glucose, 100

IU/ml penicillin, 100 µg/ml streptomycin sulfate, and 5% fetal calf serum, in monolayers at 37˚C in 5% $CO_2$.

*FAF1*$^{-/-}$ Huh7 cells (human hepatoma cells) were generated using the CRISPR/Cas9 approach by infection of Huh7 cells with lentivirus containing LentiCRISPR v2 encoding gRNA targeting *FAF1* followed by selection and single cell cloning in medium containing 3 µg/ml puromycin. All Huh7-based cells were maintained in Dulbecco's modified Eagle's medium with 4.5 g/l glucose, 100 IU/ml penicillin, 100 µg/ml streptomycin sulfate, and 5% fetal calf serum, in monolayers at 37˚C in 5% $CO_2$.

To guard against potential genomic instability, an aliquot of each cell line was passaged for only 4 weeks before a fresh batch of cells was thawed and propagated for experimental use.

**Carbonylated protein assay.** On day 0, cells were seeded at 300,000 cells per 60 mm plate. On day 1, they were treated with 10 µM ω-alkynyl AA for 12 h or 10 µM ω-alkynyl AA for 12 h followed by co-treatment with 50 nM RSL3 for 4 h. Cells were lysed in PBS (pH 7.8) containing 1% SDS and heated at 95°C for 5 min. Biotin was attached to carbonylated proteins by incubating cell lysates with 1 mM $CuSO_4$, 100 µM Biotin-azide and 1 mM Tris (2-carboxyethyl) phosphine hydrochloride for 1 h at room temperature. Biotin-conjugated carbonylated proteins in the reaction mixture was detected by SDS-PAGE followed by blotting with streptavidin-HRP (1:10000).

**Lipid peroxidation probed by BODIPY 581/591 C11.** For flow cytometry analysis, on day 0, cells were seeded at 200,000 cells per 10 cm plate. On day 1, they were treated as described in legend to Fig. 1 and co-incubated with 2 µM BODIPY 581/591 C11 for 30 min before harvesting. Cells suspended in 200 µl PBS containing 0.1% BSA were subjected to

flow cytometry analysis with FL1 filter to detect green fluorescent cells with a BD Accuri C6 plus instrument. Data were analyzed by the Flow Jo V10 software. For fluorescent microscopy, on day 0, cells were seeded at 100,000 cells per 35-mm glass bottom dish (MatTek). On day 1, they were treated as described in the figure legend and co-incubated with 2 µM BODIPY 581/591 C11 and 1 µg/ml Hoechst 33342 for 30 min before fluorescent microscopy analysis with Confocal Zeiss LSM880 Airyscan equipped with 405 nm, 488 nm, and 560 nm lasers. Image analysis was performed with Image J software.

**BN-PAGE analysis**. On day 0, cells were seeded at 200,000 cells per 10 cm plate. On day 1, cells were switched to medium supplemented with 5% delipidated FCS and 1 µM A939572 for 24 h. On day 2, cells were treated with 10 µM indicated FAs as described in legend to Fig. 5 for 4 h. Cells were homogenized in NativePAGE™ Sample Buffer (Thermo Fisher Scientific) by passing through a needle multiple times as previously described (3) followed by centrifugation at 20,817×g for 10 min. It is important that this procedure was performed below 4 ºC and finished within 30 min. The supernatant was analyzed immediately by 3-12% blue native gel (Thermo Fisher Scientific) on ice.

**Live cell fluorescent microscopy.** On day 0, cells were seeded at 100,000 cells per 35-mm glass bottom dish (MatTek). On day 1, fluorescent images were acquired with EX 492/EM 535 nm filter of Deltavision RT microscope in a 37°C chamber before and after incubation for 310 sec with the indicated FAs (200 µM) in PBS.

**Immunoblot analysis.** Cells lysed in buffer A supplemented with 1% NP40 were analyzed by SDS-PAGE followed by immunoblot analysis with the indicated antibodies (1:5,000 dilution for anti-FAF1, 1:5,000 dilution for anti-ACSL4, 1:1000 dilution for anti-Myc, and 1:5,000 dilution for anti-actin). Mouse livers lysed in buffer C (50 mM Tris-HCl,

150 mM NaCl, 1% NP40, 0.1 % SDS, 0.5% sodium deoxycholate, 1 mM EDTA, proteinase inhibitor cocktail, pH=7.5) were analyzed by SDS-PAGE followed by immunoblot analysis with the indicated antibodies (1:20,000 dilution for anti-FAF1, 1:10,000 dilution for anti-calnexin). Bound antibodies were visualized with a peroxidase-conjugated secondary antibody using the Super Signal ECL-HRP substrate system (Pierce).

**Measurement of HETEs**

HETEs were measured through eicosanoid analysis, which was performed at the UCSD Lipidomics Core (http://www.ucsd-lipidmaps.org/home). All solvents were of chromatography purity. Eicosanoids used for primary standards in standard curves as well as their deuterated analogs were from Cayman Chemicals (Ann Arbor, MI) and Biomol (Enzo Life Science, Framingdale, NY). For extraction, cells were homogenized in PBS containing 10% methanol, supplemented with a cocktail consisting of 26 deuterated internal standards and purified by solid phase extraction on Strata-X columns (Phenomenex, Torrance, CA). Samples were eluted with 1 ml of 100% methanol, the eluent was dried under vacuum and dissolved in 50 µl of buffer A consisting of water/acetonitrile/acetic acid (60/40/0.02, v/v/v) and was immediately separated by reverse phase chromatography using a 1.7uM 2.1x100 mm BEH Shield Column (Waters, Milford, MA) and an Acquity UPLC system (Waters, Milford, MA). The column was equilibrated with buffer A and 10 µl of sample were injected via the autosampler. Samples were eluted with a step gradient to 100% buffer B consisting of acetonitrile/isopropanol (50:50, v/v). The liquid chromatography effluent was interfaced with a mass spectrometer and mass spectral analysis was performed on an AB SCIEX 6500 QTrap mass spectrometer equipped with an IonDrive Turbo V source (AB SCIEX, Framingham, MA). Eicosanoids were measured using multiple reaction monitoring (MRM) pairs with the

instrument operating in the negative ion mode. Collisional activation of the eicosanoid precursor ions was achieved with nitrogen as the collision gas, and the eicosanoids were identified by matching their MRM signal and chromatographic retention time with those of pure identical standards. Eicosanoids were quantified by the stable isotope dilution method. Briefly, identical amounts of deuterated internal standards were added to each sample and to all the primary standards used to generate standard curves. To calculate the amount of eicosanoids in a sample, ratios of peak areas between endogenous eicosanoids and matching deuterated internal eicosanoids were calculated. Ratios were converted to absolute amounts by linear regression analysis of standard curves generated under identical conditions. Currently, we can quantify over 150 eicosanoids at sub-fmol levels.

**LC-MS/MS analysis of PE-HpETE and PE-HETE.** All solvents were either HPLC or LC/MS grade and purchased from Sigma-Aldrich. All lipid extractions were performed in 16×100 mm glass tubes with PTFE-lined caps (Fisher Scientific). Glass Pasteur pipettes and solvent-resistant plasticware pipette tips (Mettler-Toledo) were used to minimize leaching of polymers and plasticizers. Suspension of 200,000 cells were dried under $N_2$ and extracted by the Bligh/Dyer reagent (4). The organic phase was dried under $N_2$ and resuspended in hexane for measurement of phospholipids by LC-MS/MS using a SCIEX QTRAP 6500+ equipped with a Shimadzu LC-30AD HPLC system and a 150×2.1 mm, 5 µm Supelco Ascentis silica column at 25 ºC. Samples were injected at a flow rate of 0.3 ml/min at 2.5% solvent B (methyl tert-butyl ether) and 97.5% solvent A (hexane). Solvent B was increased to 5% over 3 min and then to 60% over 6 min. Solvent B was decreased to 0% over 0.5 min while solvent C (90:10 (v/v) Isopropanol-water) was increased to 20%. During the following 11 min, solvent C was increased to 40%. Solvent C was further increased to 44% over 6 min and

then to 60% over 50 sec. The system was held at 60% of solvent C for 1 min before re-equilibration by 2.5% of solvent B and 97.5% solvent A for 5 min at a flow rate of 1.2 ml/min. Solvent D (95:5 (v/v) Acetonitrile-water with 10 mM Ammonium acetate) was infused post-column at 0.03 ml/min. Electrospray ionization source parameters were: GS1 40, Cur 20, temperature 150 °C, declustering potential 60, and collision energy 50. GS1 and 2 were zero-grade air while Cur and CAD gas was nitrogen. Data were acquired in negative ionization mode using multiple reaction monitoring (MRM) of the *m*/*z* transitions 783.6→319.3 for PE(18:0_HETE), and 799.6→317.3 for PE(18:0_HpETE). These transitions were previously selected based on the product ions of 1-Stearoyl-2-15(S)-HETE-sn-glycero-3-PE (PE(18:0_HETE)), and 1-Stearoyl-2-15(S)-HpETE-sn-glycero-3-PE (PE(18:0_HpETE)) standards (Cayman). The LC-MS/MS data were analyzed using MultiQuant software (SCIEX).

**GC-MS analysis of AA.** To measure AA incorporated into neutral lipids and phospholipids, cells were extracted by a three-phase lipid extraction approach through which neutral lipids and phospholipids can be separated into different phases (5). Following hydrolysis of acyl chains from these lipids, the amount of free AA in each fraction was quantified through GC-MS analysis. Briefly, 1 ml hexanes, 1 ml methyl acetate, 0.75 ml acetonitrile, and 1 ml $H_2O$ was added to glass tubes containing 250k cells. The mixture was vortexed for 5 sec and then centrifuged at 2671×*g* for 5 min, resulting in separation of three distinct liquid phases. The upper phase, which contained neutral lipids, and the middle phase, which contained phospholipids, was collected into separate glass tubes and dried under $N_2$. The dried extracts were resuspended in 1 ml 0.5 M KOH dissolved in methanol, spiked with 100 µl of fatty acid standards (FA(16:0{$^2H_{31}$}), FA(20:4ω6{$^2H_8$}) and FA(22:6ω3{$^2H_5$})

(Cayman) at 0.5 µg/ml, and hydrolyzed at 80°C for 60 min. Hydrolyzed fatty acids were extracted by adding 1 ml dichloromethane and 1 ml water into the hydrolysis solution. The mixture was vortexed and centrifuged at 2671×*g* for 5 min, and the organic phase was collected to a fresh glass tube and dried under $N_2$.

For the analyses of free AA, 0.5 ml brine solution (saturated solution of NaCl), 0.5 ml 50 mM HCL dissolved in methanol, 1 ml isooctane, and 100 µl FA standards (FA(16:0{$^2H_{31}$}, FA(20:4ω6{$^2H_8$} and FA(22:6ω3{$^2H_5$} at 0.5 µg/ml) were added to glass tubes containing 250k cells or solutions containing the entire in vitro reactions. The tubes were vortexed for 5 min and then centrifuged at 2671×*g* for 5 min, resulting in two distinct liquid phases. This process was repeated twice, and the organic phases (upper layer) were pooled in a fresh glass tube and dried under $N_2$.

For GC-MS profiling of AA, dried FA extracts were resuspended in 50 µl 1% triethylamine dissolved in acetone, derivatized with 50 µl 1% pentafluorobenzyl bromide (PFBBr) dissolved in acetone at room temperature for 25 min in capped glass tubes, dried under $N_2$, and resuspended in isooctane for GC-MS analysis using an Agilent 7890/5975C (Santa Clara) by electron capture negative ionization (ECNI) equipped with a DB-5MS column (40 m×0.18 mm with 0.18 µm film thickness, Agilent) as previously described (6). Hydrogen (carrier gas) flow rate was at 1.6 ml/min and injection port temperature was set at 300°C. Sample injection volume was 1 µl. Initial oven temperature was set at 150°C, and then increased to 200°C at a rate of 25°C/min, followed by an increase to 300°C at a rate of 8°C/min, and was maintained at this temperature for 2.2 min. The total run time was 16.7 min. AA was analyzed in selected ion monitoring (SIM) mode, and was normalized to the internal standards. Data was processed using Mass-Hunter software (Agilent).

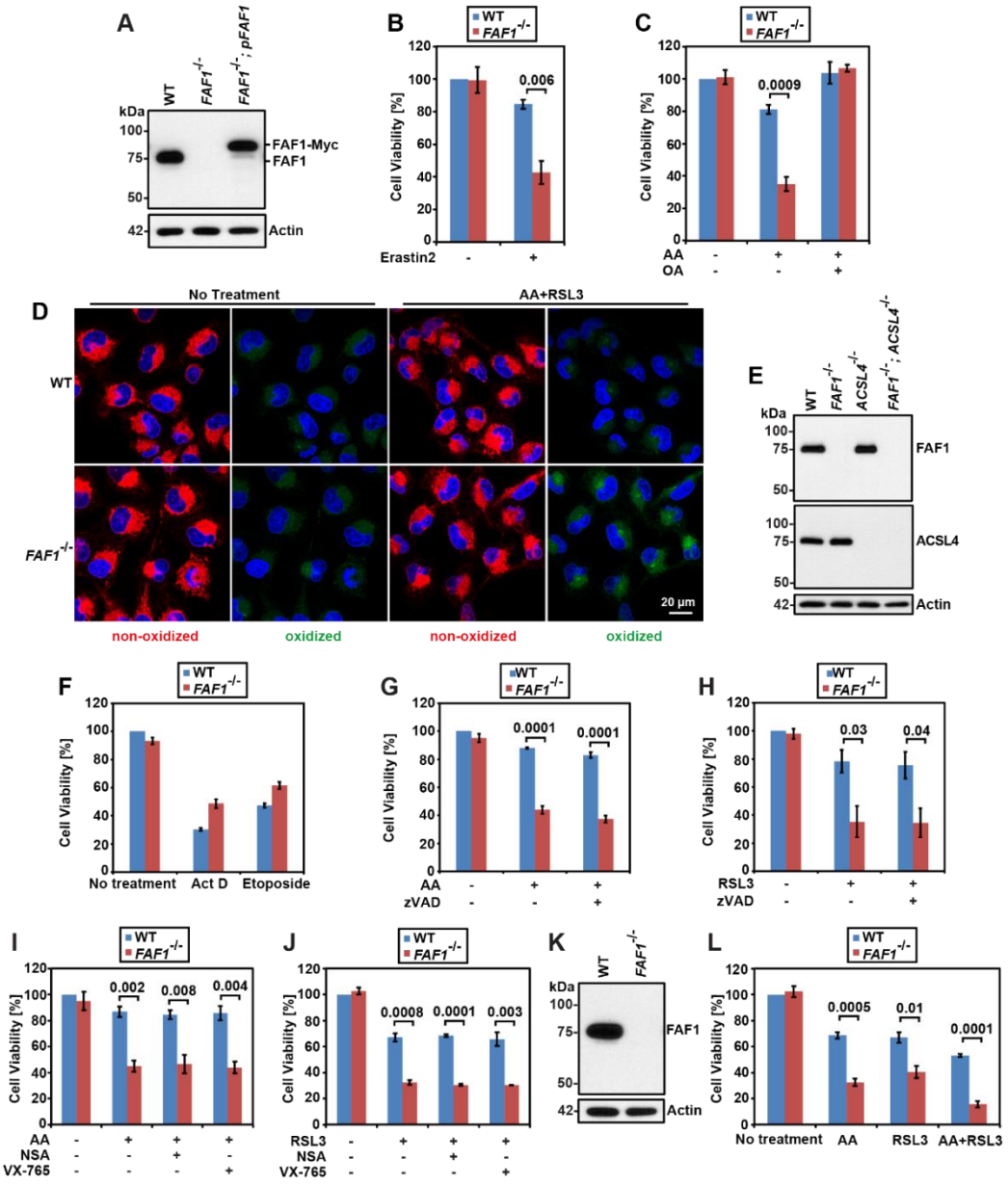


**Fig. S1.** FAF1 protects cultured cells from ferroptosis. (A, E and K) Immunoblot analysis of FAF1 in the indicated SV589 (A and E) or Huh7 cells (K). (B, C, and F-J) Viability of indicated SV589 cells treated with Erastin2 (60nM), indicated FAs (10µM), RSL3 (10nM), actinomycin D (Act D, 10µM), etoposide (10µM), Z-VAD-FMK (zVAD, 0.1µM), necrosulfonamide (NSA, 1µM), VX-765 (10µM) for 24h was measured as described in Fig. 1A. (D) BODIPY 581/591 C11 fluorescent microscopy images of the cells treated as described in Fig. 1F. (L) Viability of indicated Huh7 cells treated with 50µM AA, 30nM RSL3, or 20 µM AA together with 20nM RSL3 for 24h was measured as described in Fig. 1A. (B, C, F-J and L)Results are reported as mean ± S.E.M. from three independent experiments. The statistical significance was calculated with unpaired, two-tailed t-test.

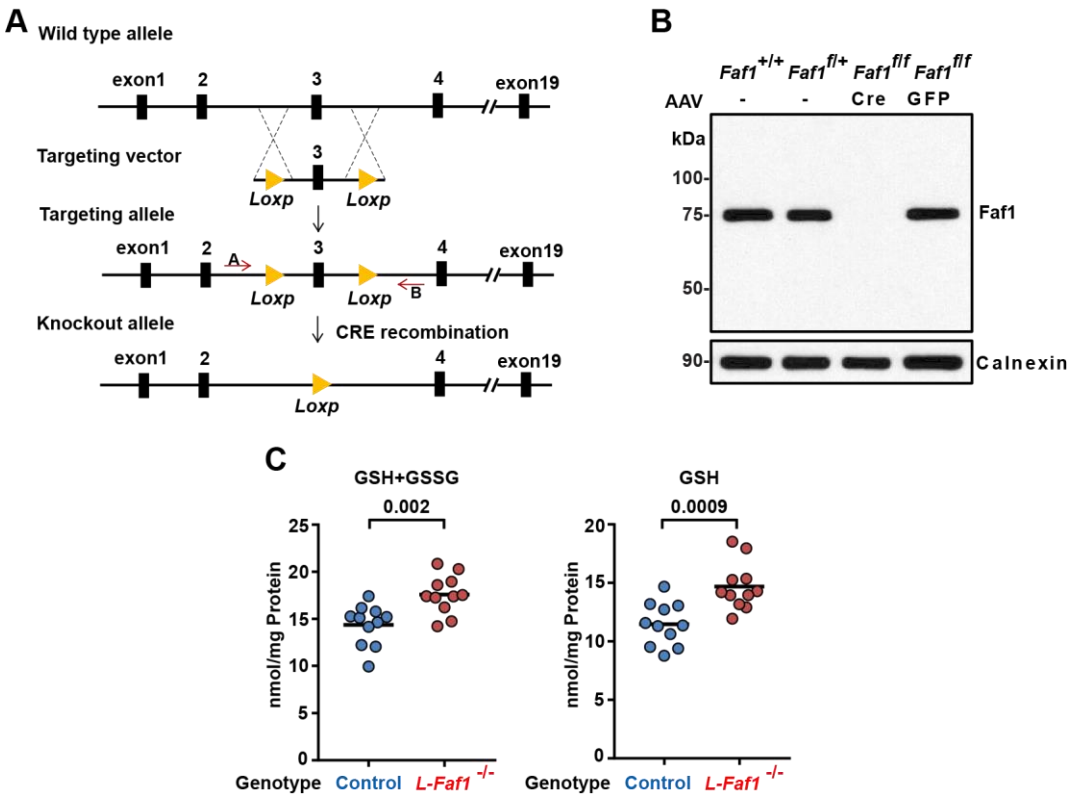


**Fig. S2.** FAF1 inhibits AA peroxidation in mouse livers. (A) Graphic illustration for generation of *Faf1*$^{-/-}$ mice. Primers used for genotyping (see Materials and Methods) are highlighted by red arrows. (B) Immunoblot analysis of FAF1 in livers of mice injected with the indicated AAV. (C) The amounts of total glutathione (GSH+GSSG) and reduced glutathione (GSH) in livers of indicated mice fed with an AA-enriched diet shown in Fig. 2A. The statistical significance was calculated by unpaired, two-tailed t-test.

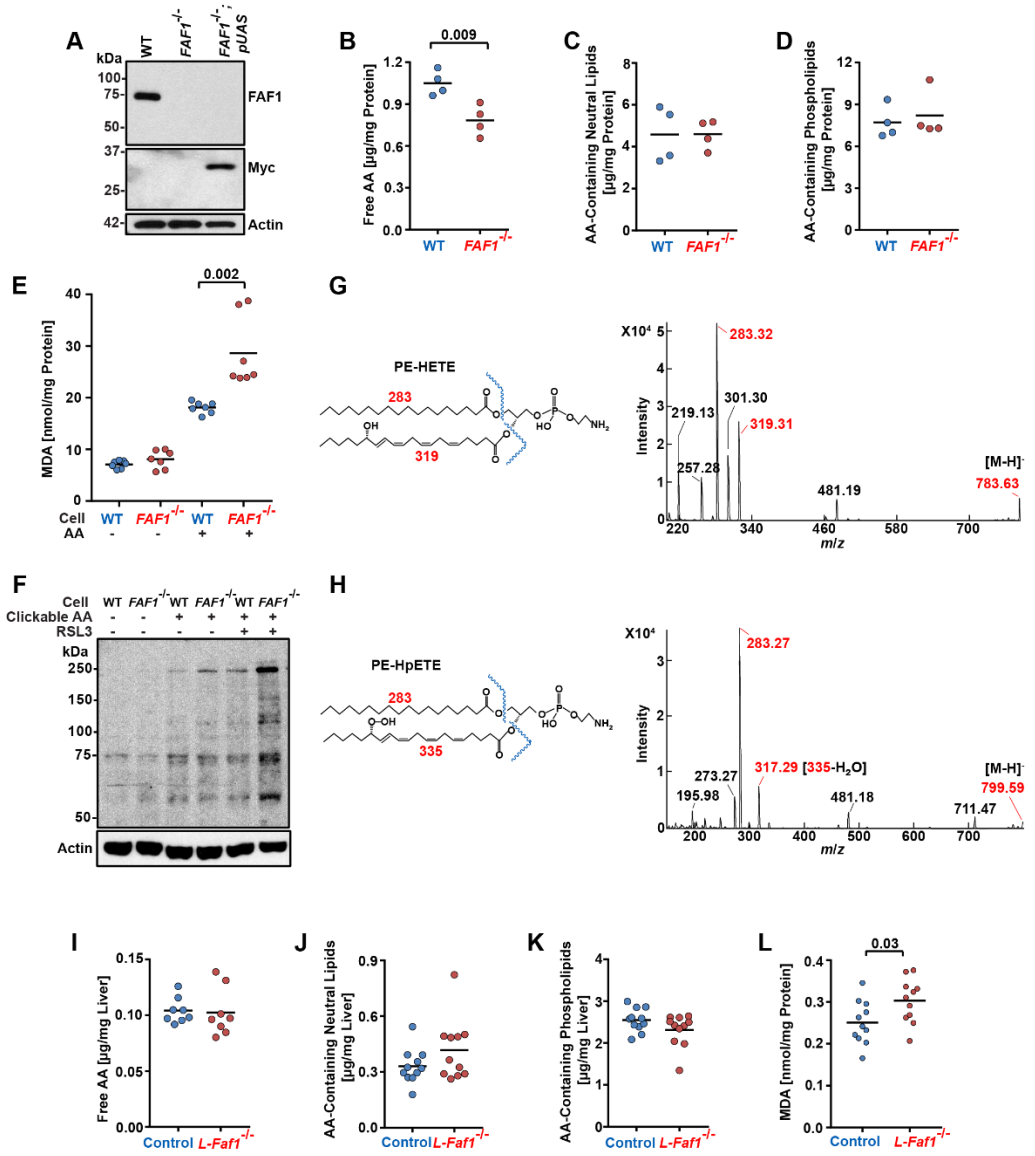


**Fig. S3.** Effect of FAF1 on AA metabolism. (A) Immunoblot analysis of FAF1 and Myc-tagged UAS domain of FAF1 in the indicated cells. The antibody against FAF1 does not recognize the USA domain of the protein. (B-D) The amount of free AA (B) and that incorporated into neutral lipids (C) and phospholipids (D) in indicated cells treated with 10μM AA for 12h. Data were derived from the same experiments shown in Table 1. (E) MDA produced in indicated cells treated with 10μM AA for 18h. (F) Streptavidin blot of carbonylated proteins labeled by biotin generated in indicated cells subjected to indicated treatments were performed as described in Supporting Materials and Methods. (G and H) The spectrum of PE(18:0_HETE) (G) and PE(18:0_HpETE) (H) standards with the fragmentation position and the molecular weight of the fragments highlighted in blue and red, respectively. (I-L) The amount of free AA (I), AA incorporated into neutral lipids (J) and phospholipids (K), and MDA (L) in livers of indicated mice fed with AA in experiments shown in Fig. 2A. The statistical significance was calculated by unpaired, two-tailed t-test.

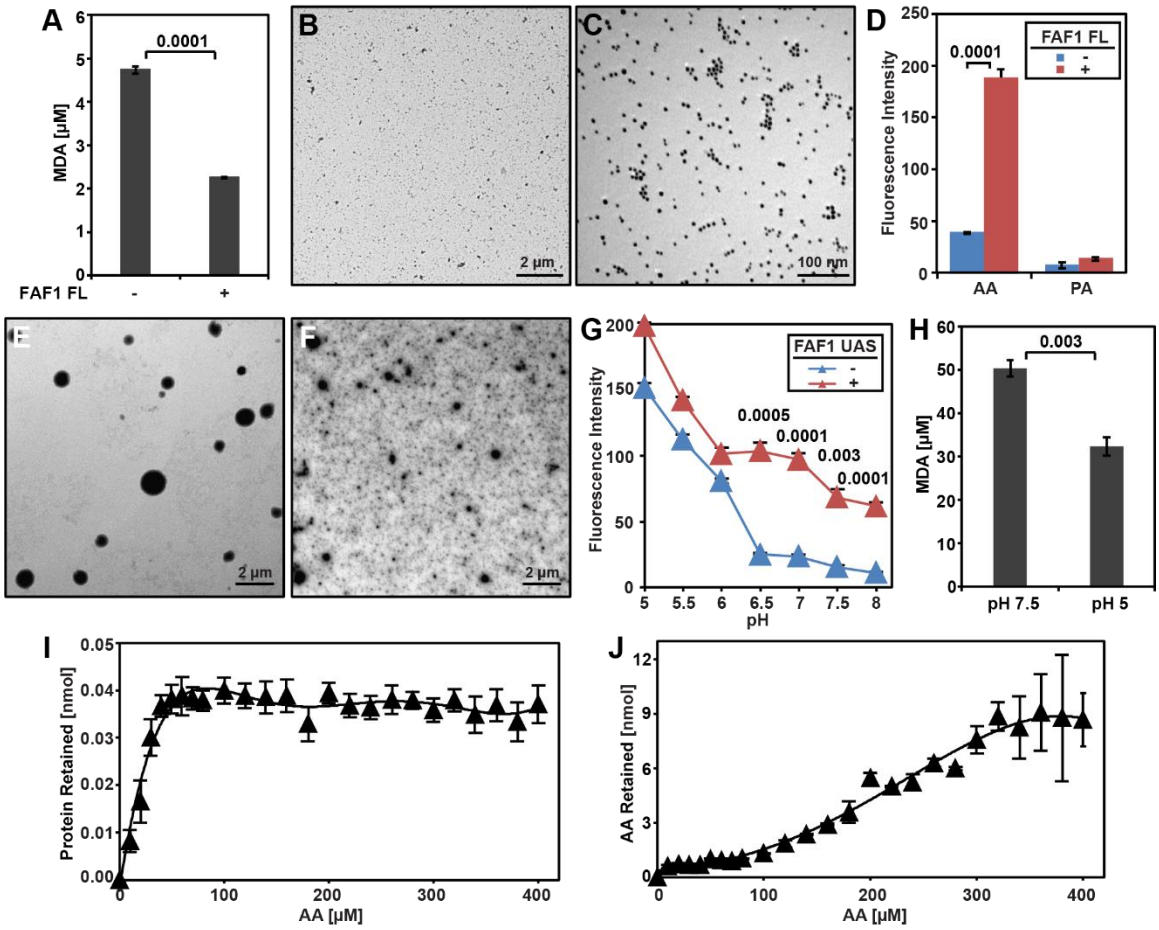


**Fig. S4.** FAF1 and acidic pH may stimulate formation of AA particles. (A) Amount of MDA produced in reactions containing 30μM AA, 50μM $H_2O_2$ and 100μM $FeSO_4$ with or without 0.9 mg/ml purified full length FAF1 (FAF1 FL) for 5 min. (B and C) Negative staining EM images of 30μM PA mixed with the 0.1mg/ml UAS domain (B) or gold-labeled UAS domain (C). (D) Nile Red fluorescence of solutions containing 30μM AA or PA with or without 0.3 mg/ml purified FAF1 FL. (E and F) Negative staining EM images of 100μM AA in a buffer with pH=5 (E), and in a buffer with pH=7.5 in the presence of 0.1mg/ml UAS domain of FAF1 (F). (G) Nile Red fluorescence of solutions containing 30μM AA and 0.1mg/ml purified UAS domain of FAF1 under indicated pH. (H) Amount of MDA produced in reactions containing 200μM AA and 1mM $FeSO_4$ under indicated pH for 10 min. (I and J) Amounts of the protein (I) and AA (J) retained by the filtration of solutions containing 50μg/ml purified UAS domain with indicated concentration of AA. (A, D, G, H, I and J) Results are reported as mean ± S.E.M. from three independent experiments. The statistical significance was calculated by unpaired, two-tailed t-test.

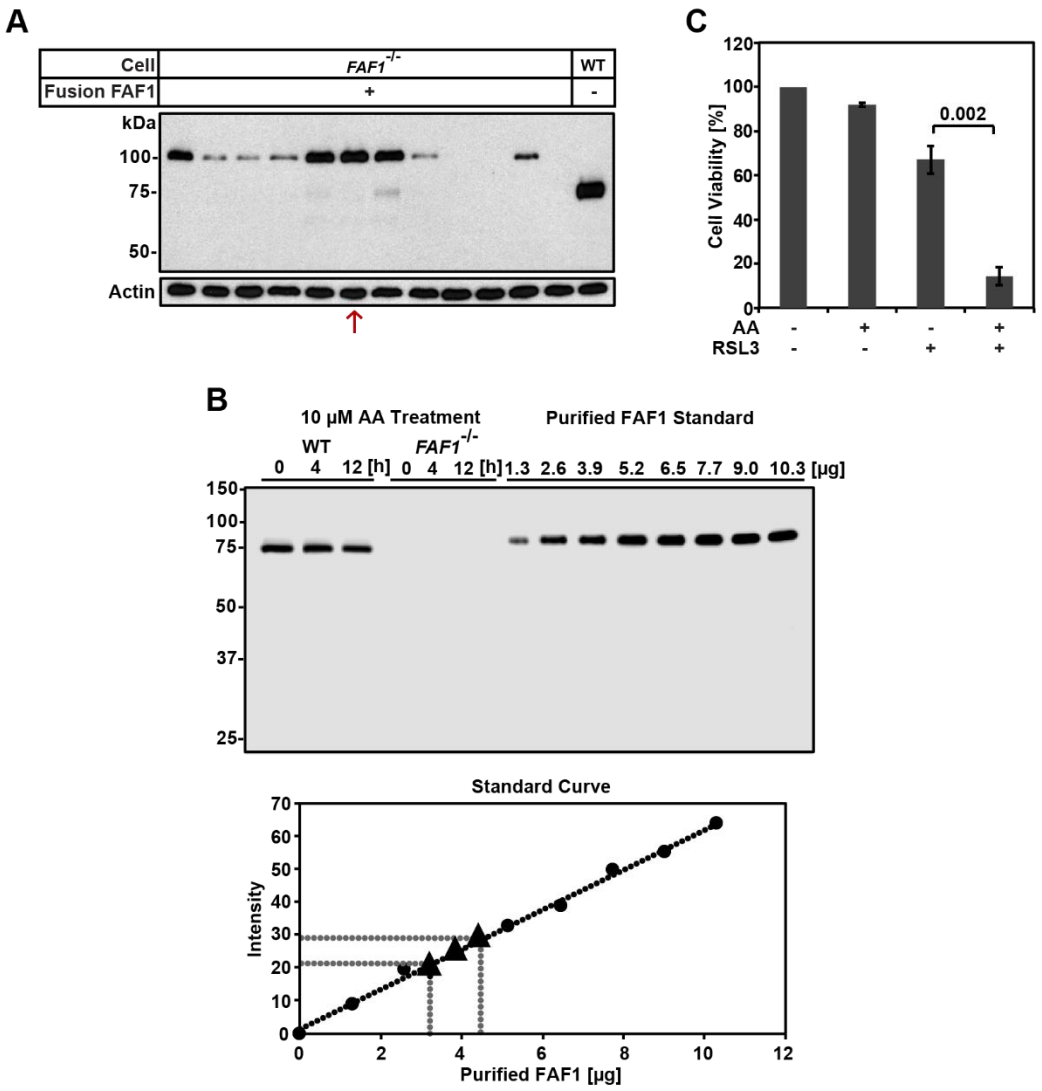


**Fig. S5.** Physiological relevance of the FAF1-AA complex. (A) Immunoblot analysis of FAF1 in cells stably transfected with a plasmid encoding FAF1 fusion protein. The single clone of the cells expressing GFP-tagged FAF1 used in Figs. 5G-J is highlighted by red arrow. (B) The amount of FAF1 in indicated cells subject to indicated treatments was determined by immunoblot analysis calibrated by known amounts of purified FAF1 (upper panel) followed by quantificatoin of the immunoblot analysis (lower panel). (C) Viability of WT cells treated with or without 10μM AA and/or 10nM RSL3 for 24h was measured as described in Fig. 1A. Results are reported as mean ± S.E.M. from three independent experiments. The statistical significance was calculated by unpaired, two-tailed t-test.

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