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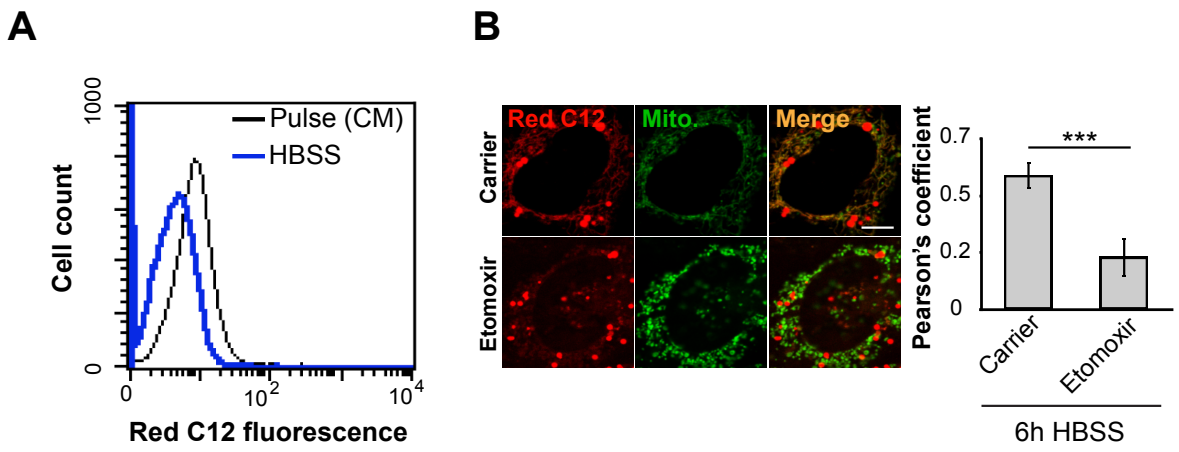

Figure S1. Validation of fluorescent FA pulse-chase assay, related to Figure 1.

(A) To determine cellular Red C12 metabolism, we assayed MEFs as described in Figure 1A and measured cellular Red C12 content in pulsed or 24 h starved MEFs by FACS analysis. (B) WT MEFs were assayed as described in Figure 1A and chased in HBSS for 6 h in the absence or presence etomoxir (300 nM). Mitochondria were labeled using MitoTracker Green. Scale bar, 10 μm. Red C12 overlap with mitochondria was determined by Pearson's coefficient analysis.

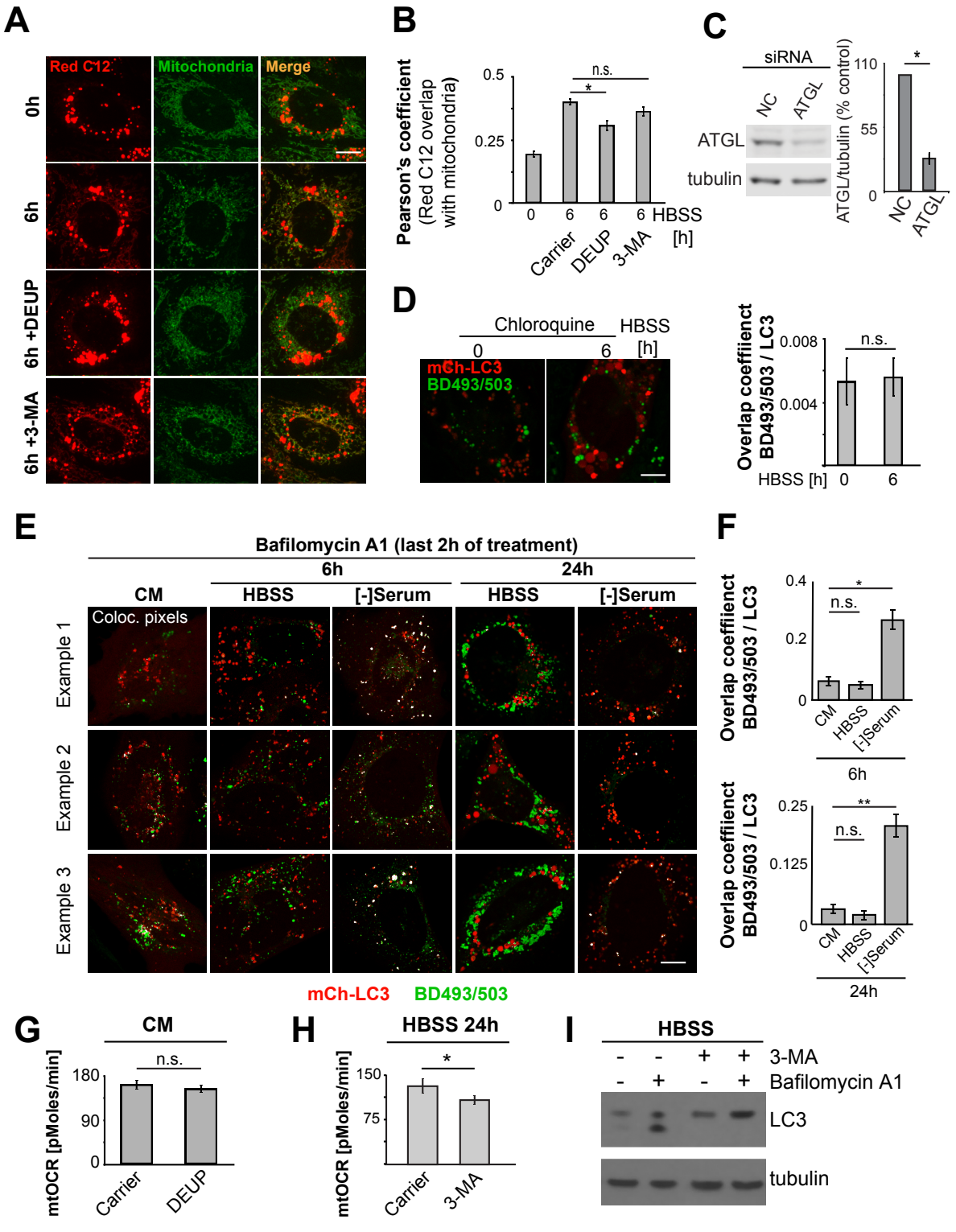

Figure S2. Lipase activity, not lipophagy, drives LD-mitochondria FA transfer, related to Figure 2. (A, B) WT MEFs were assayed as described in Figure 1A, starved for 6 h in HBSS

and images of live cells were captured. (A) During the 6 h chase with HBSS cells were incubated in the absence or presence of the lipase inhibitor DEUP (100 μM) or the autophagy inhibitor 3-MA (10 mM). Scale bar, 10 μm. (B) Red C12 transfer to mitochondria was measured using Pearson's coefficient analysis. (C) Representative Western blot image of siRNA mediated knock-down of ATGL in WT MEFs and quantification, normalized to tubulin. (D) Lipophagy was determined in 6 h starved mCherry-LC3 transfected WT MEFs. For the last 2 h of starvation 1 μM chloroquine was added to inhibit autophagosomal degradation and accumulate cargo-loaded autophagosomes. Live images were acquired and lipophagy levels were determined by quantification of the signal correlation between BODIPY 493/503 and mCherry-LC3. (E, F) Lipophagy was determined in mCherry-LC3 transfected WT MEFs starved in HBSS or serum-deficient medium for 6 or 24 h. For the last 2 h of starvation 250 nM bafilomycin A1 was added to inhibit autophagosomal degradation and accumulate cargo-loaded autophagosomes. (E) Live images were acquired and (F) lipophagy levels were determined by quantification of the signal correlation between BODIPY 493/503 and mCherry-LC3. (G) Mitochondrial respiratory activity of WT MEFs grown in CM was measured in the absence or presence of DEUP (100 μM). (E) Mitochondrial respiratory activity of 24 h starved WT MEFs. For the last 2 h of starvation 10 mM 3-MA was included to inhibit autophagy. (I) Representative Western blot image of 3-MA responsive autophagy induction of 24 h HBSS-starved MEFs. Scale bars, 10 μm.

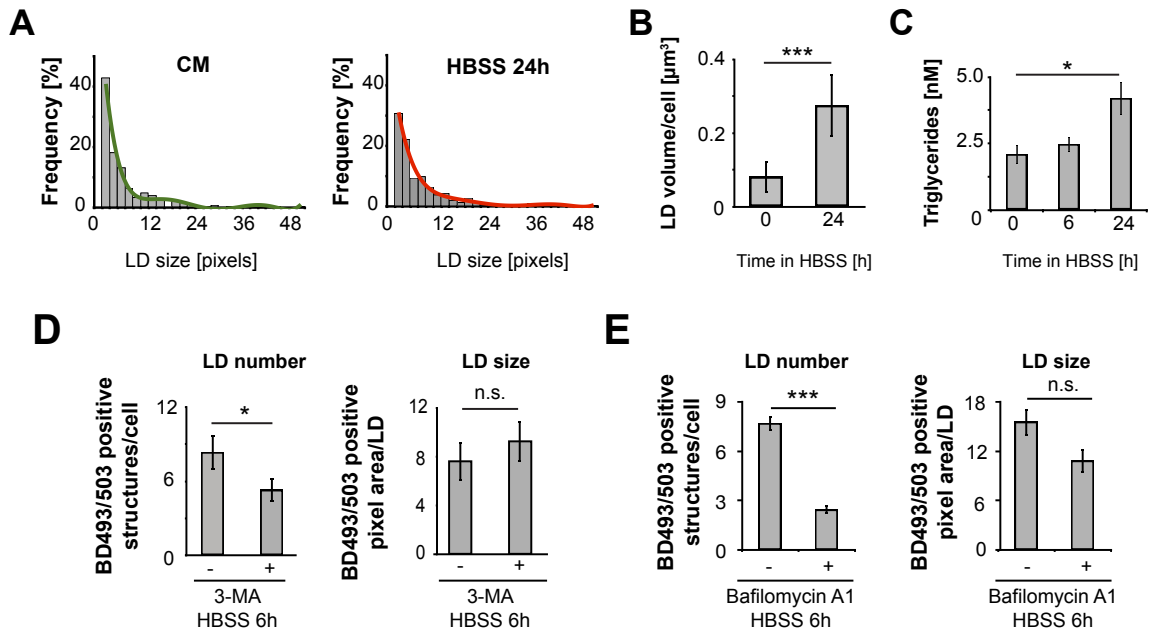

Figure S3. LDs grow in an autophagy dependent manner during starvation, related to Figure 3. (A, B) LDs in WT MEFs were visualized by BODIPY 493/503 and (A) LD size frequency and (B) LD volume were determined in cells grown in CM or HBSS. (C) Enzymatic measurement of triglyceride concentration in unstarved (0 h HBSS) or starved (HBSS) WT MEFs, at the time points indicated. (D, E) 6 h starved WT cells were incubated with (D) 10 mM 3-MA or (E) 250 nM bafilomycin A1 and LD size and number quantified.

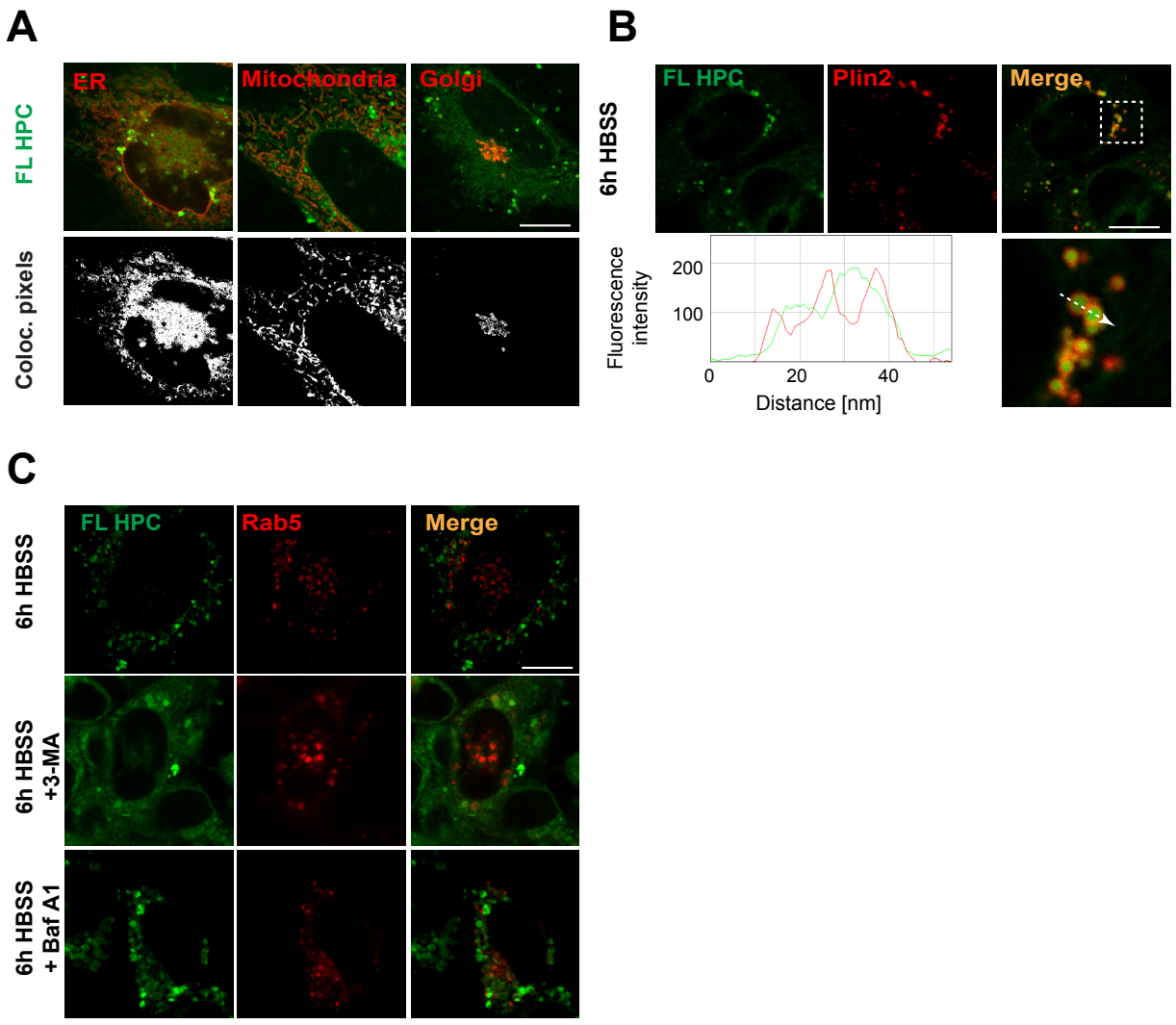

Figure S4. Distribution of FL HPC in cellular membranes, related to Figure 4. (A) WT MEFs expressing fluorescent proteins targeted to the ER (RFP-KDEL), mitochondria (mito-RFP), or Golgi (GM130-mCherry) were pulsed with FL HPC overnight, washed, and incubated with CM for 1 h in order to allow the FL HPC to incorporate into cellular membranes. Cells were then imaged and the subcellular localization of FL HPC was determined. (B) WT MEFs expressing mCherry-Plin2 were assayed as described in Figure 4A. The linescan reveals that FL HPC signal is in the core of the LDs rather than on the LD surface, suggesting hydrolysis of the FA moiety and incorporation into neutral lipid. (C) WT MEFs expressing TagRFP-Rab5 were assayed as described in Figure 4A.

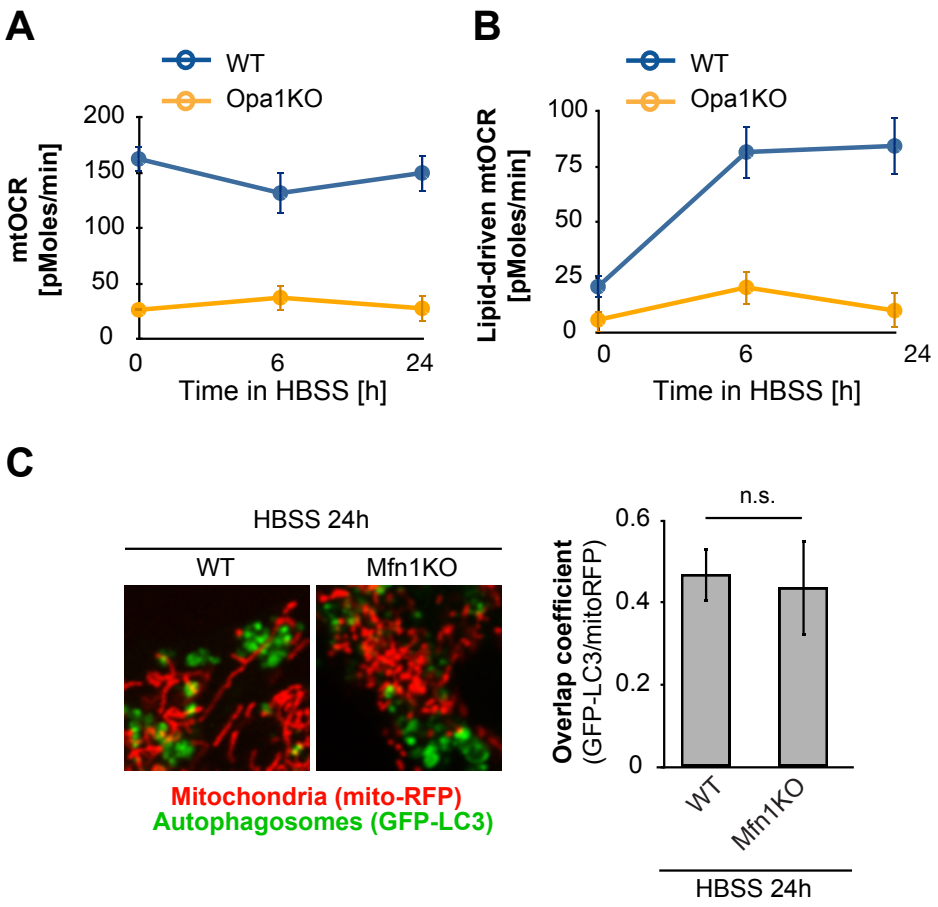

Figure S5. Opa1 KO cells are respiratory deficient, related to Figure 5. (A) WT and Opa1KO cells were assayed as described in Figure 5, and mitochondrial oxygen consumption rates (mtOCR) and lipid-driven mtOCR were determined at the time points indicated. (B) WT or Mfn1KO MEFs expressing mito-RFP and GFP-LC3 were incubated in HBSS for 24 h, live images were acquired and mitophagy levels were measured by overlap coefficient.

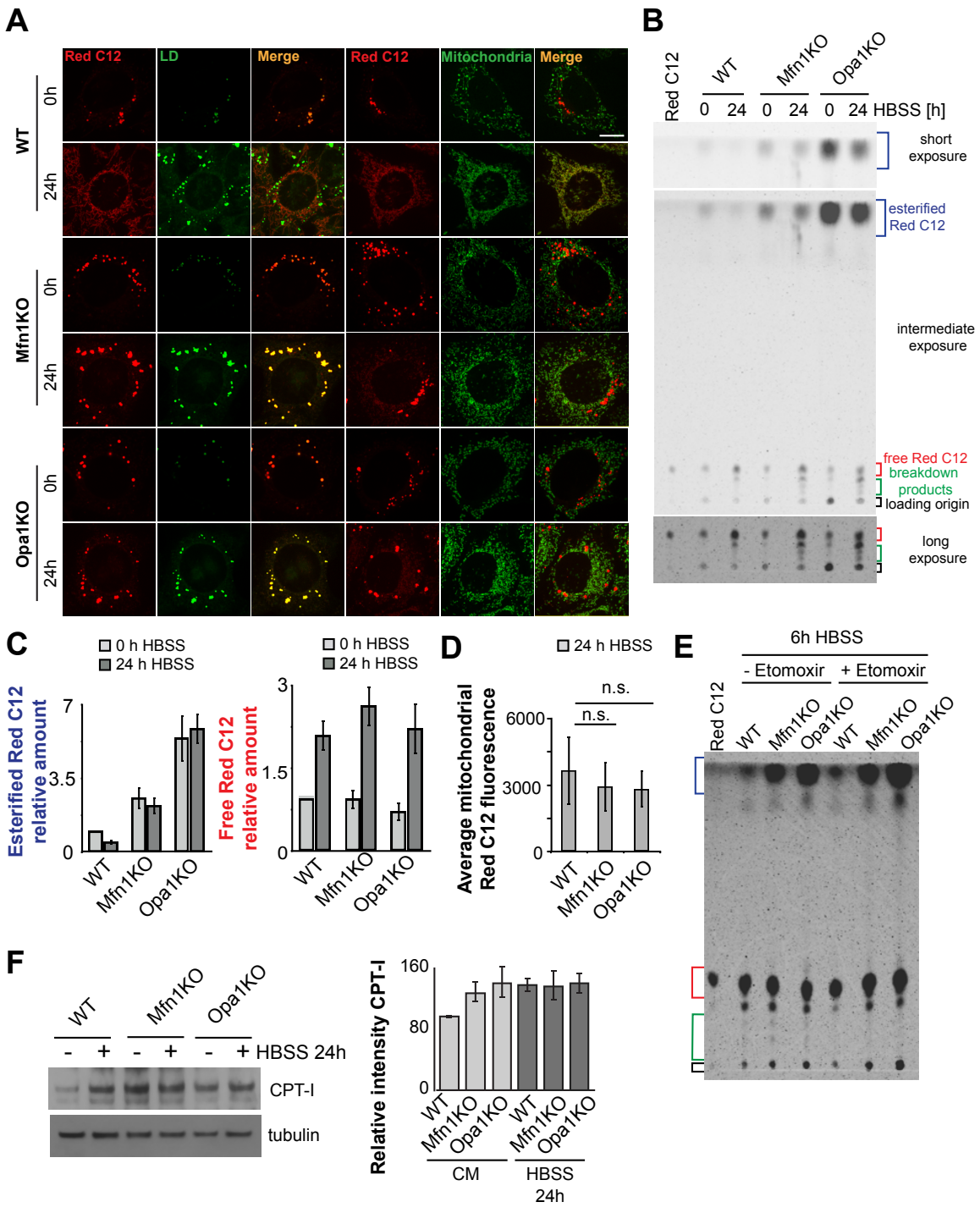

Figure S6. Mitochondrial fusion deficiency is dispensable for lipolysis or mitochondrial FA import, related to Figure 6.

(A) Images of the Red C12 pulse-chase assay performed in WT, Mfn1KO and Opa1KO MEFs. Scale bar, 10 μm. (B) Thin layer chromatography (TLC) resolving Red C12 isolated from WT, Mfn1 KO or Opa1 KO MEFs assayed as described in Figure 1A and chased for 24 h with HBSS.

(C) Relative amounts of esterified and free Red C12 levels were quantified from images of TLC plates in (B), normalized to Red C12 levels of WT cells at 0 h time point. (D) Absolute amounts of mitochondrial Red C12 were quantified from Red C12 assay images, as presented in (B). (E) TLC resolving Red C12 isolated from WT, Mfn1 KO or Opa1 KO MEFs assayed as described in Figure 1A and chased for 6 h with HBSS. Mitochondrial Red C12 breakdown products were determined by inhibiting mitochondrial FA import with etomoxir. Red, unesterified Red C12; blue, Red C12 incorporated into neutral lipids; green, breakdown products of mitochondrial β-oxidation. (F) Western blot analysis of cellular CPT-I levels in 24 h starved WT, Mfn1KO and Opa1KO cells. β-tubulin staining was used a loading control.

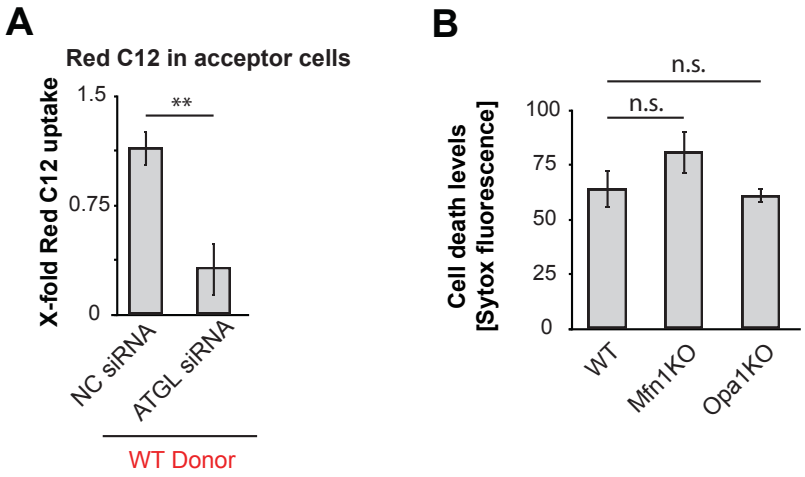

Figure S7. Lipase activity is necessary for cellular export of FAs, related to Figure 7. (A) Co-culture assay was performed as described in Figure 7 with WT donor cells transfected with non-coding or ATGL siRNA, and the transfer of Red C12 into WT acceptor cells was measured by flow cytometry analysis. (B) Cell death quantification, based on cellular staining of fed and 24 h starved WT and KO cells with live-dye Sytox green. Mean Sytox-green fluorescence was determined by FACS analysis.

Supplemental Experimental Procedures

Cell culture, transfection, plasmids, inhibitors and antibodies

Mouse embryonic fibroblasts (MEFs) were maintained in DMEM with 10% FBS and glutamine (complete medium, CM). Opa1KO MEFs from e10.5 embryos were obtained from the American Type Culture Collection (CRL-2995). Mfn1KO MEFs from e10.5 embryos and Atg5 KO MEFS from e13.5 embryos were obtained from D. C. Chan and N. Mizushima, respectively (Chen et al., 2005; Kuma et al., 2004). Cells were cultured in 10µg/ml fibronectin-coated LabTek Chambered Coverglass (Thermo Scientific). Images were acquired on 3i Intelligent Imaging Innovations spinning disk or Zeiss 710 confocal microscopes using 63x or 100x oil objectives, at 37 °C and 5% CO2 or 15 mM HEPES-buffered medium. For starvation experiments Hank's buffered salt solution (HBSS, 14025-092) was purchased from Invitrogen. Cells were transfected with GFP-LC3, mCherry-LC3, LAMP1-mCherry, RFP-KDEL, mito-RFP, GM130-mCherry, mCherry-Plin2, or TagRFP-Rab5 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. RNA interference was performed using 100 nM ON-TARGETplus Mouse Pnpla2 (66853) siRNA SMARTpool or ON-TARGETplus non-targeting pool (Thermo Scientific) and DharmaFECT 1 (Thermo Scientific) according to the manufacturer's instructions. Knock-down was performed twice, 48 h apart.
The following chemicals, dyes and antibodies were used: 100µM diethylubelliferyl phosphate (DEUP, Sigma Aldrich), 300 nM etomoxir (Sigma Aldrich), oligomycin A1 (0.5 µg/ml Sigma Aldrich), 10 mM 3-methyladenine (3-MA, Sigma Aldrich), 100 nM rotenone (Sigma Aldrich), 1 µM antimycin (Sigma Aldrich), 100nM MitoTracker Green FM (Life Technologies), 200ng/ml BODIPY493/503 (Life Technologies), 1 µM BODIPY 558/568 C12 (Life Technologies), 2 µM FL HPC (Life Technologies), 10µg/ml fibronectin (Sigma Aldrich), anti-CPTI (MitoSciences Abcam), anti-ATGL (Cell Signaling Technology #2138).

All inhibitor concentrations used present sub-lethal doses. Further experiments were performed for 24h starvation where this was well tolerated by the cells. However, we limited starvation to 6h for conditions where drug treatments during 24h starvation resulted in significant cell death (eg. etomoxir, inhibition of autophagy with 3-MA or in Atg5KO cells).

Determination of lipophagy levels

WT MEFs were transfected with mCherry-LC3 and 200 ng/ml BODIPY493/503 was added 6h prior to imaging to visualize autophagosomes and LDs, respectively. Cells were incubated either

in CM or HBSS for 6h and 250nM bafilomycin A1 (Sigma Aldrich) was added for the last 2h of starvation to inhibit autophagosomal degradation and enhance the number of cargo-loaded autophagosomes. Lipophagy levels were determined from binary images subjected to overlap coefficient analysis using ImageJ 'JACoP' plugin (National Institutes of Health).

TG measurement

MEFs ($4x10^6$) were lysed in 0.1 ml lysis buffer (5% NP40 in H2O) and assayed for triglyceride using a Triglyceride Quantification Colorimetric Kit (Biovision Inc.) according to the manufacturer's instructions. Briefly, samples were twice heated to 90°C for 2 minutes and cooled to room temperature, then centrifuged to remove any insoluble material, and diluted two-fold in Triglyceride Assay Buffer. Samples were then incubated with lipase for 20 minutes, followed by a 30-minute incubation with Triglyceride Reaction Mix. The OD 570 nm was measured and triglyceride amount was calculated using a standard curve.

SDS-PAGE and Western blot

Cells were lysed in ice cold lysis buffer (20 mM Tris pH 7.4, 150mM NaCl, 2mM EDTA, 1% Triton X-100), 10µg protein lysate was separated on 4-12% gradient NuPAGE gels (Invitrogen) and transferred to PVDF membranes. Membranes were incubated with the following primary antibodies: anti-CPT-I (MitoSciences/Abcam) and anti-ATGL (Cell Signaling Technology). Western blot for β-tubulin was used as loading control.

Cell death assay

MEFs were plated and incubated in CM or HBSS for the time points indicated. For the last 30 minutes of the treatment, 100 nM of Sytox green (LifeTechnologies) was added to the cells, accumulating in dying cells. Mean cellular Sytox green levels were determined by Flow Cytometry.

Supplemental References

Chen, H., Chomyn, A., and Chan, D.C. (2005). Disruption of fusion results in mitochondrial heterogeneity and dysfunction. The Journal of biological chemistry *280*, 26185-26192.

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