Supplemental information

LC3B is lipidated to large lipid droplets during prolonged starvation for noncanonical autophagy

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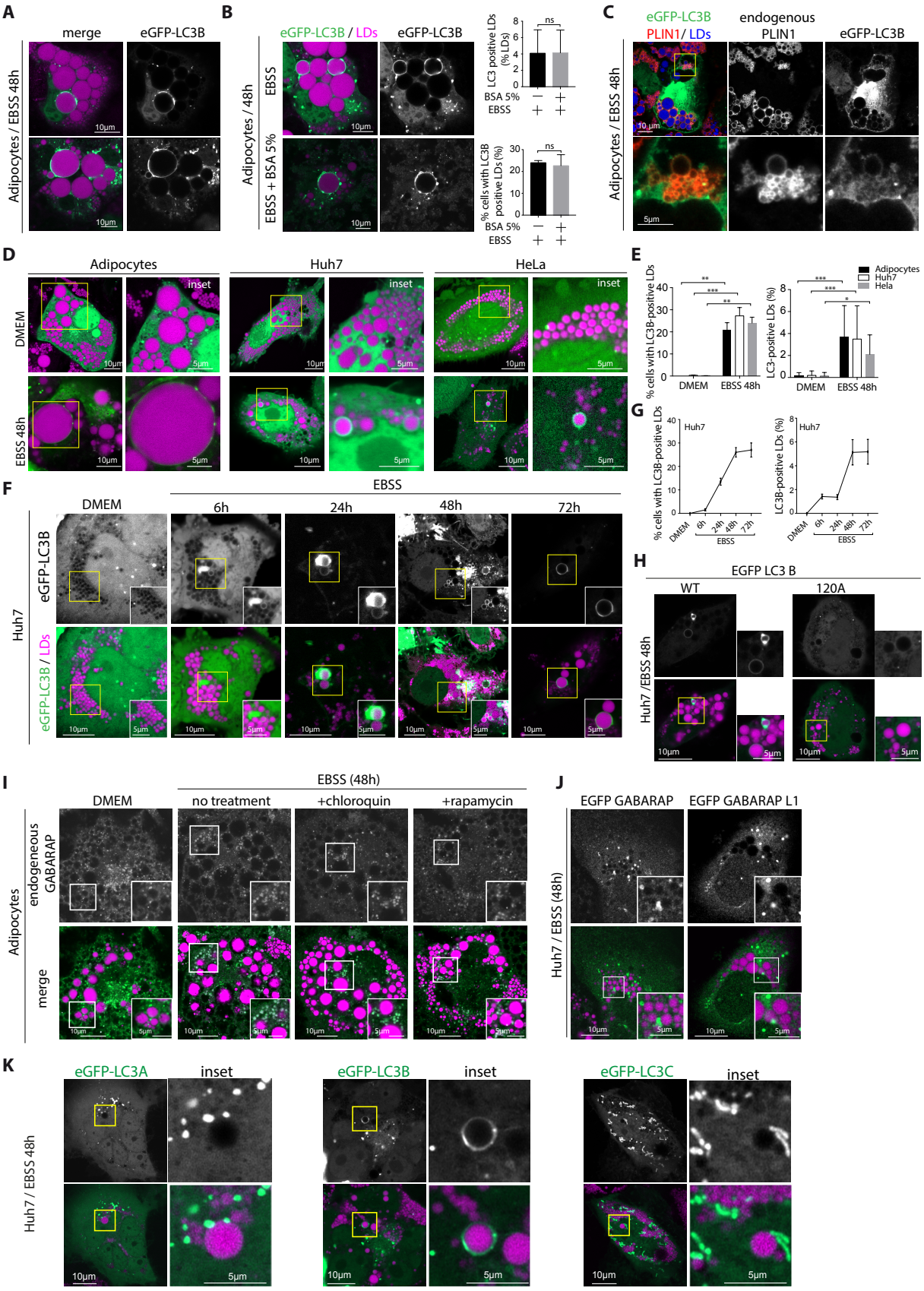

Figure S1. LC3B is recruited to LDs during long-term nutrient deprivation, Related to Figure 1.

A. Confocal imaging of eGFP-LC3B, and LDs in differentiated 3T3-L1 adipocytes virally transfected with eGFP-LC3B. Cells are incubated in EBSS for 48hs after transfection. Scale bar, 10 μm.
B. Confocal imaging of eGFP-LC3B, and LDs in differentiated 3T3-L1 adipocytes virally transfected with eGFP-LC3B. Cells are incubated in EBSS alone or EBSS containing 5% of BSA FFA for 48hs after transfection. Scale bar, 10 μm. Right bar graphs show the percentage of cells with eGFP-LC3B-positive LDs and the percentage of eGFP-LC3B-positive LDs per cell. Data are from three independent experiments. Student's unpaired t-test is used (ns P>0,05).
C. Immunofluorescence staining of PLIN1 (red) and LDs (blue) in differentiated 3T3-L1 adipocytes, virally transfected with eGFP-LC3B and incubated in EBSS for 48hs. Scale bar,10 μm (5 μm in insets).
D. Confocal imaging of eGFP-LC3B and LDs in differentiated 3T3-L1 adipocytes, Hela and Huh7 cells. HeLa and Huh7 cells were treated with OA to induce LD formation for 24hs and then they were incubated in EBSS for 48hs after transfection. Scale bar,10 μm (5 μm in insets).
E. Left panel: Percentage of cells with eGFP-LC3B-positive LDs, Right panel: percentage of eGFP-LC3B-positive LDs per cell. Data are from three independent experiments done as described in D. Student's unpaired t-test is used (*** P<0,0001), (** P<0,001).
F. Confocal imaging of eGFP-LC3B and LDs in Huh7 cells virally transfected with eGFP-LC3B, treated with OA to induce LDs, and then incubated in EBSS for the indicated time. Scale bar,10 μm (5 μm in insets).
G. Quantification of the experiment presented in F: Left panel: Percentage of cells with eGFP-LC3B-positive LDs, Right panel: percentage of eGFP-LC3B-positive LDs per cell.
H. Confocal imaging of Huh7 cells transfected with eGFP-LC3B or G120A-mutant eGFP-LC3B treated with OA to induce LDs and then incubated in EBSS for 48hs. (Scale bar,10 μm (5 μm in insets).
I. Immunofluorescence staining of GABARAP+GABARAPL1+GABARAPL2 and LDs in differentiated 3T3-L1 adipocytes incubated in EBSS for 48hs. Scale bar,10 μm (5 μm in insets).
J. Confocal imaging of Huh7 cells transfected with eGFP-GABARAP or eGFP-GABARAPL1 and treated with oleic acid for 24hs and then incubated in EBSS for 48hs. Scale bar,10 μm (5 μm in insets).
K. Confocal imaging of Huh7 cells transfected with eGFP-LC3A, eGFP-LC3B or eGFP-LC3C and treated with oleic acid for 24hs and then incubated in EBSS for 48hs. Scale bar,10 μm (5 μm in insets).

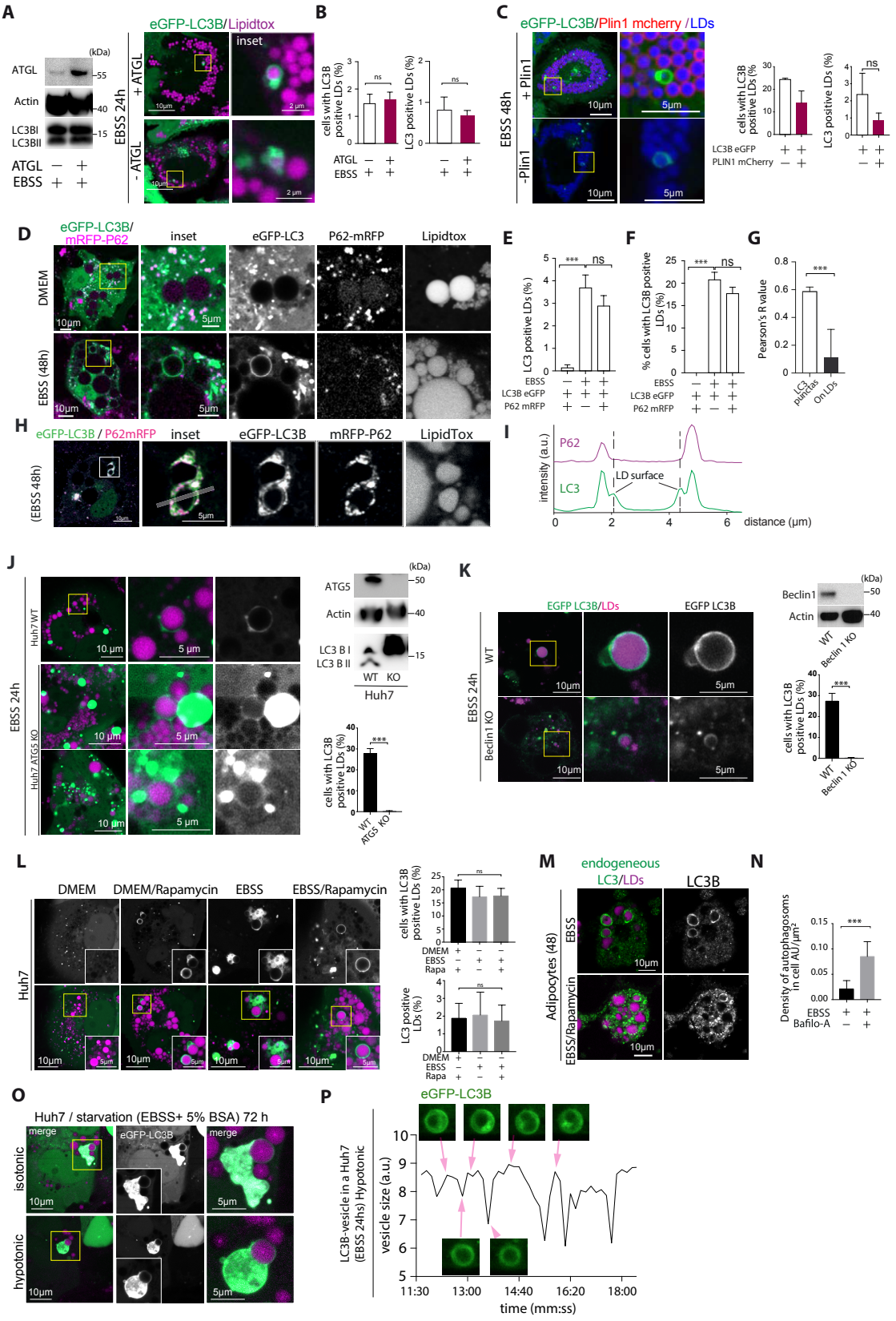

Figure S2. ATGL, Plin1, and P62 are not responsible for LC3 recruitment to LDs, Related to Figure 2.

A. Western blot and confocal imaging of Huh7 cells co-transfected with eGFP-LC3B and untagged ATGL, treated with OA to induce LDs, and then incubated in EBSS for 48hs. Scale bar, 10 μm (5μm in insets).
B. Percentage of cells with eGFP-LC3B-positive LDs (left) and eGFP-LC3B-positive LDs per cell (Right). Quantifications are from three independent experiments. Student's unpaired t-test is used (P>0,05)
C. Huh7 cells co-transfected with eGFP-LC3B and PLIN1-mCherry, treated with OA to induce LDs, and incubated in EBSS for 48hs. The bottom image panel shows a cell that is transfected by eGFP-LC3 alone in the same cell batch of the upper panel. Scale bar, 10 μm, (5μm in insets). Right, the percentage of eGFP-LC3B-positive LDs per cell and the percentage of cells with eGFP-LC3B-positive LDs. Quantifications are from three independent experiments. Student's unpaired t-test is used (* P<0,05, ns P>0,05).
D. Confocal imaging of differentiated 3T3-L1 adipocytes co-transfected with eGFP-LC3B, P62-mRFP. Cells are incubated in DMEM or EBSS for 48hs after transfection. Scale bar,10 μm (5 μm in insets).
E. Percentage of eGFP-LC3B-positive LDs per cell. Cells are co-transfected or not with P62-mRFP and incubated or not in EBSS. Quantifications are from three independent experiments. Student's unpaired t-test is used (ns P>0,05), (*** P<0,0001).
F. Percentage of cells with eGFP-LC3B-positive LDs. Quantifications are from three independent experiments. Student's unpaired t-test is used (ns P>0,05), (*** P<0,0001).
G. Pearson's R-value between eGFP-LC3B and P62-mRFP in LC3 puncta and around LDs. Quantifications are from three independent experiments. Student's unpaired t-test is used (*** P<0,0001).
H. Confocal imaging of differentiated 3T3-L1 adipocytes. An example is shown of colocalization between eGFP and P62 near an LC3-positive LD. Scale bar,10 μm (5 μm in insets).
I. Intensity profile of eGFP-LC3B, P62-mRFP of the line drawn in the inset image of H.
J. Confocal imaging of Huh7 ATG5 KO and control cells. Cells were virally transfected with eGFP-LC3B, incubated with OA to induce LDs, and then placed in EBSS for 24h. Scale bar, 10 μm, (5μm in insets). Right, Western blot of ATG5 KO and control cells and quantification of cells with eGFP-LC3B positive LDs from three independent experiments. Student's unpaired t-test is used (*** P<0,0001).
K. Confocal imaging of Huh7 Beclin1 KO and control cells. Cells were virally transfected with eGFP-LC3B, incubated with OA to induce LDs, and then placed in EBSS for 24h. Scale bar, 10 μm, (5μm in insets). Right, Western blot of Beclin1 KO and control cells and quantification of cells with eGFP-LC3B positive LDs from three independent experiments. Student's unpaired t-test is used (*** P<0,0001).
L. Confocal imaging of Huh7 cells transfected with eGFP-LC3B, treated with OA, and then incubated in DMEM, DMEM rapamycin, EBSS, or EBSS rapamycin for 24h. Right: quantification of the percentage of eGFP-LC3B-positive LDs per cell and percentage of cells with eGFP-LC3B-positive LDs from three independent experiments. An ordinary one-way ANOVA test was used (ns P> 0,05).
M. Immunofluorescence staining of LC3B and LDs in differentiated 3T3-L1 adipocytes incubated in EBSS or EBSS with rapamycin for 48hs.
N. Quantification of eGFP-LC3B puncta's intensity (autophagosomes) of 10 cells treated as described in Figure 2I from three independent experiments. Student's unpaired t-test is used (*** P<0,0001).
O. Confocal imaging of Huh7 cell, transfected with eGFP-LC3B, treated with OA to induce LDs, and then incubated in EBSS + 5% BSA for 72h. The cells were imaged before and after incubation with the hypotonic medium. Scale bar,10 μm (5 μm in insets).

P. Time course of an LC3B-positive vesicle in an Huh7 cell submitted to a hypotonic medium. The vesicle size shows cycles of expansion and shrinkage likely due to burst events, as known for artificial vesicles submitted to a hypotonic shock.

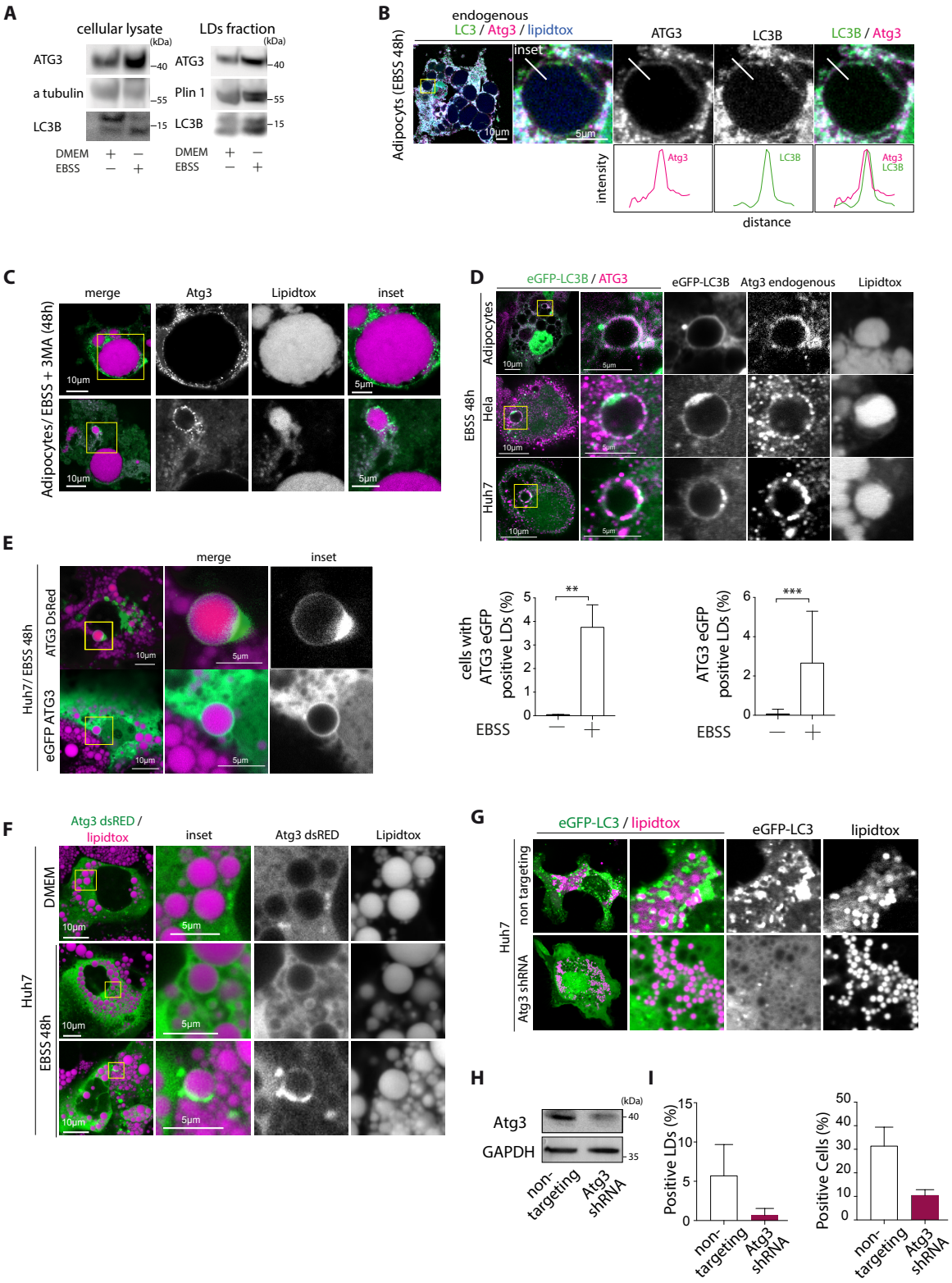

Figure S3. ATG3 is recruited to the lipid droplet surface during long-term nutrient starvation and is required for LC3B localization to LDs, Related to Figure 3.

A. Western blot of lysate and LD fractions of differentiated 3T3-L1 adipocytes incubated in EBSS or DMEM for 48hs.
B. Immunofluorescence staining of LC3B and ATG3 in differentiated 3T3-L1 adipocytes incubated in EBSS for 48hs. Below each panel is represented the intensity line profile of each protein signal. Scale bar, 10 μm (5 μm in insets).
C. Immunofluorescence staining of ATG3 in differentiated 3T3-L1 adipocytes incubated in EBSS containing 3MA for 48hs.
D. Immunofluorescence staining of ATG3 and LDs in differentiated 3T3-L1 adipocytes, Huh7, and Hela cells transfected with eGFP-LC3B and incubated in EBSS for 48hs.
E. Confocal imaging of Huh7 cells transfected with eGFP-ATG3 (green) or ATG3-dsRED (green). Cells are treated with OA to induce LDs (magenta) and then incubated in EBSS for 48hs. Bar graphs present the percentage of cells with eGFP-ATG3-positive LDs (right) and eGFP-ATG3-positive LDs per cell (left). Quantifications are from three independent experiments. Student's unpaired t-test is used (** P<0,001), (*** P<0,0001).
F. Confocal imaging of Huh7 cells transfected with ATG3-dsRED. Cells are treated with OA to induce LDs formation and then incubated in DMEM or EBSS for 48hs.
G. Confocal imaging of eGFP-LC3B and LDs in Huh7 cells stably transfected with an ATG3 shRNA or the non-targeting shRNA. Cells are treated with OA to induce LDs and then incubated in DMEM or EBSS for 72h.
H. Western blot of Huh7 cells stably transfected with the ATG3 shRNA or the non-targeting shRNA and treated as described in G.
I. Percentage of eGFP-LC3B-positive LDs per cell and percentage of cells with eGFP-LC3B-positive LDs respectively. Quantifications are from three independent experiments. Student's unpaired t-test is used (*** P<0,0001)

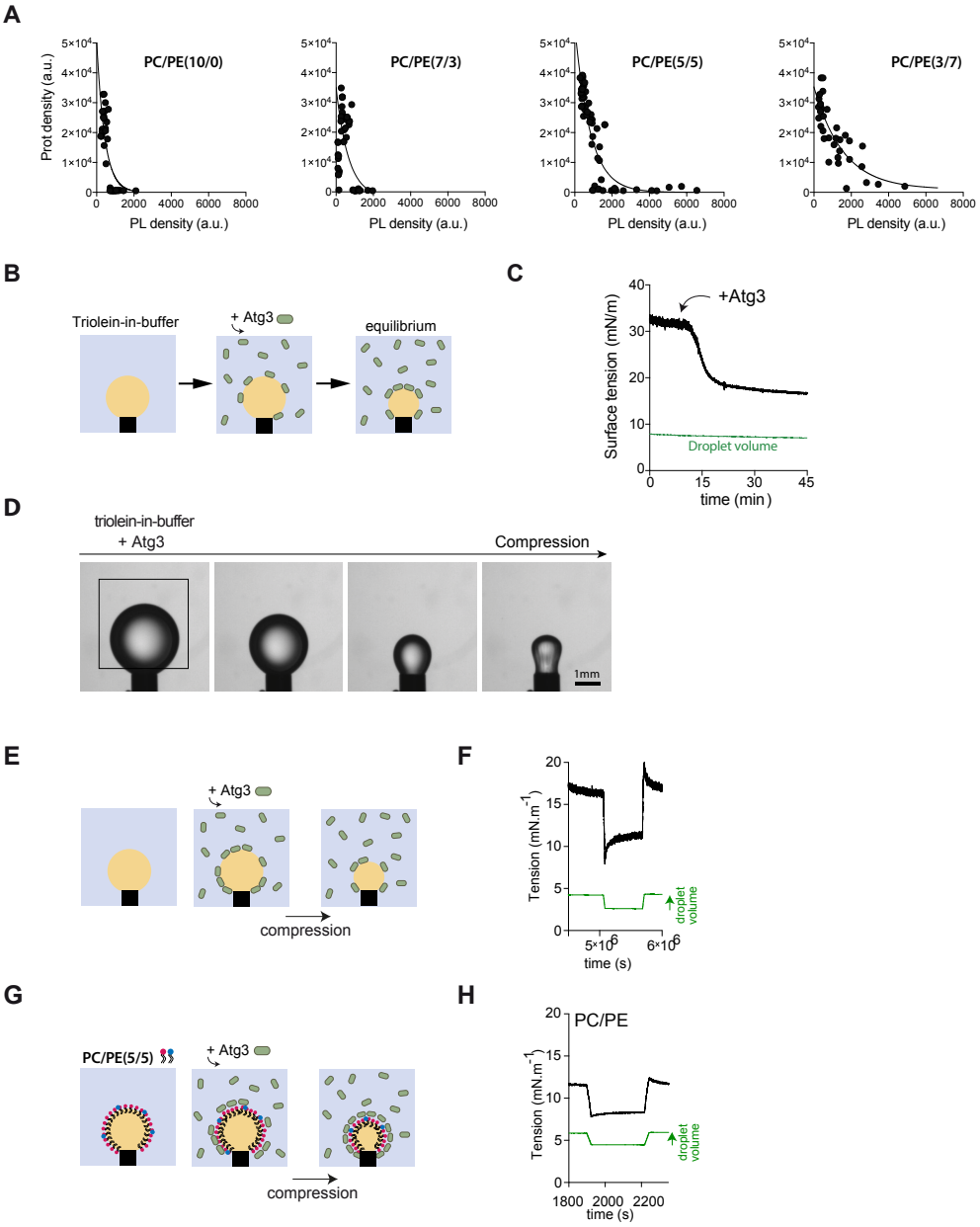

Figure S4. Characterization of Atg3 binding, Related to Figure 4.

A. ATG3-YFP recruitment to triolein-in-buffer droplets as a function of the phospholipid density, reported by Rho-PE, for the different PC/PE ratios, 10/0, 7/3, 5/5, and 3/7.

B. Schematic representation of the oil droplet tensiometer. An oil droplet, whose volume can be adjusted, is formed at the end of a J-tube in an aqueous buffer. The addition of protein that binds the droplet results in a reduction in surface tension to an equilibrium value.

C. The interfacial tension of the triolein/water interface decreases over time after ATG3 addition, dropping from 32mN/m to an equilibrium value of around 18m/Nm.

D. Brightfield imaging of the droplet in presence of ATG3 during surface compression. Scale bare (1mm). Ripples appear at the end of the compression and are a signature of a high density of proteins at the interface.

E. Schematic representation of the effect of oil droplet shrinkage which reduces its surface area.

F. Stable equilibrium following injection of ATG3 is first reached. Then, the droplet area (size) is rapidly reduced to produce a surface compression and re-expanded after a few minutes. The graph shows changes in surface tension overtime during this procedure.
G. Schematic representation illustrates the shrinking of an oil droplet decorated by phospholipids.
H. Triolein/buffer surface, decorated with PC/PE (3/7) was generated. Stable equilibrium following injection of ATG3 is first reached. Then, the droplet area (size) is rapidly reduced to produce a surface compression and re-expanded after a few minutes. The graph shows changes in surface tension overtime during this procedure. The fall off of the protein following compression is important as in F.

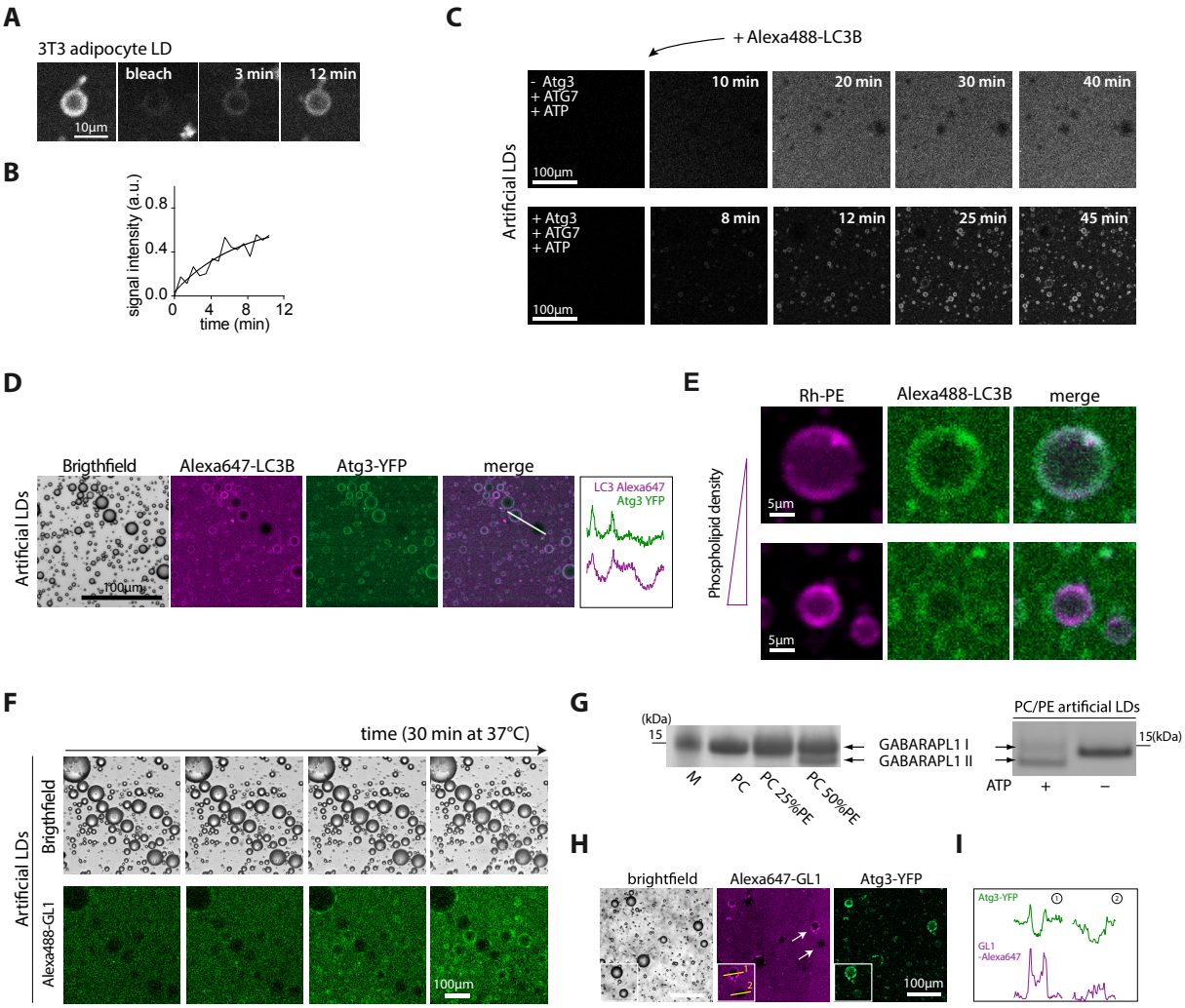

Figure S5. Atg3 lipidates LC3 to LDs, Related to Figure 5.

A. FRAP analysis of LC3B-Alexa488 being lipidated to purified adipocyte LD, from the experience described in Figure 5A. The LD is completely bleached and images at 3min and 12 minutes post-bleach are shown. Scale bar 10 µm.

B. Recover kinetics of the experiment in A.

C. Artificial LDs prepared as described in Figure 5C are incubated with LC3B-Alexa488, ATP, and ATG7, in the absence (top) or presence (bottom) of ATG3. LC3B-Alexa488 is lipidated to the droplets only in ATG3 presence.

D. Confocal imaging of triolein-in-buffer droplets from the experiment described in Figure 5E. The droplets are decorated by PC/PE (7/3) at different monolayer phospholipid densities varied from 0.005% to 0.2% (w/w to triolein) and incubated with LC3B-Alexa647 and Atg3/Atg3-YFP (80/20), ATP and ATG7. Line profiles of the protein signal on some droplets are shown on right.

E. Example showing the lipidation of LC3B-Alexa488 depending on the phospholipid density, reported by Rhodamine-PE.

F. Confocal images of artificial LDs (PC/PE 50/50) incubated with GABARABL1-Alexa488, ATG7, ATG3, and ATP. GABARABL1 is lipidated to the droplets.

G. Left. Coomassie blue stained SDS–PAGE gel of triolein droplets decorated with PC/PE (100/0, 75/25, 5050) and incubated with GABARABL1, ATG7, ATG3, and ATP. Right.

Coomassie blue stained SDS–PAGE gel of PC/PE triolein droplets incubated with GABARABL1, ATG7, and ATG3 in the presence or absence of ATP.

H. Confocal image of triolein droplets prepared as described in Figure 5C, incubated with GABARABL1-Alexa647, ATG3-YFP, ATG7, and ATP.

I. Line profiles of GABARABL1-Alexa647, and ATG3-YFP for the yellow line profiles in the inset in H.

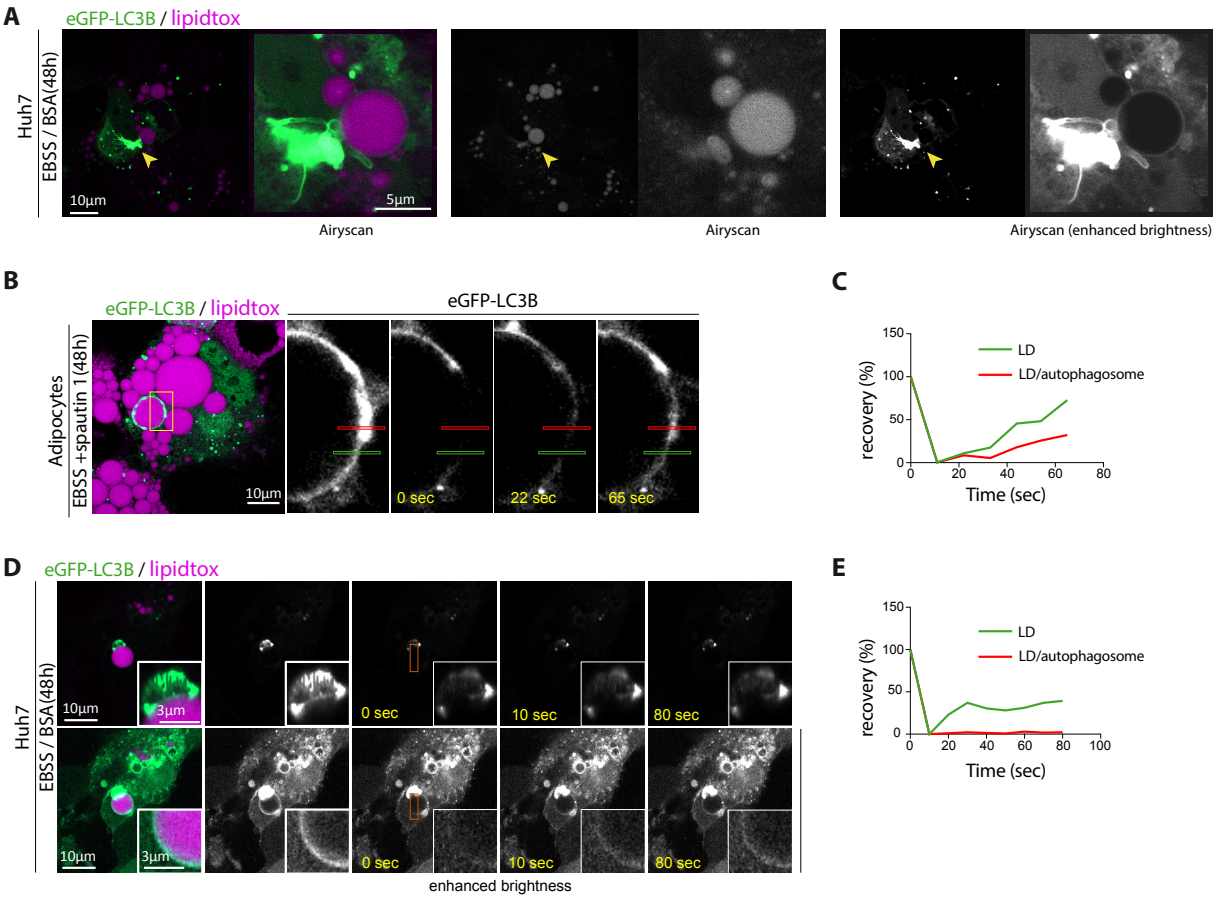

Figure S6. LC3B localizes around LDs in DU145 cells during long-term nutrient starvation, Related to Figure 6.

A. Confocal imaging of eGFP-LC3B and LDs in Huh7 cells virally transfected with eGFP-LC3B, treated with OA to induce LDs formation, and then incubated in EBSS containing BSA for the indicated time. Scale bar,10 µm (5 µm in insets).

B. FRAP analysis of eGFP-LC3B in differentiated 3T3-L1 adipocytes LDs virally transfected with eGFP-LC3B and incubated in EBSS containing spautin 1 for 48hs. The green and red lines mark the bleached regions (red for the LC3B LD-membrane interacting area and green for the LD surface region). Scale bar,10 µm.

C. Recover kinetics of the experiment in B

D. Top, FRAP analysis of eGFP-LC3B in Huh7 cells virally transfected with eGFP-LC3B and treated with OA to induce LDs formation, and then incubated in EBSS containing BSA for the indicated time. The red rectangle indicates the bleaching area, insets show the membrane-LD contact area. Scale bar,10 µm (3 µm in insets). The bottom panel exhibits the same images as the upper panel, but the eGFP-LC3B brightness is enhanced to display the signal on the LD surface, and the insets represent the LD surface not interacting.

E. Recover the kinetics of the experiment in D.

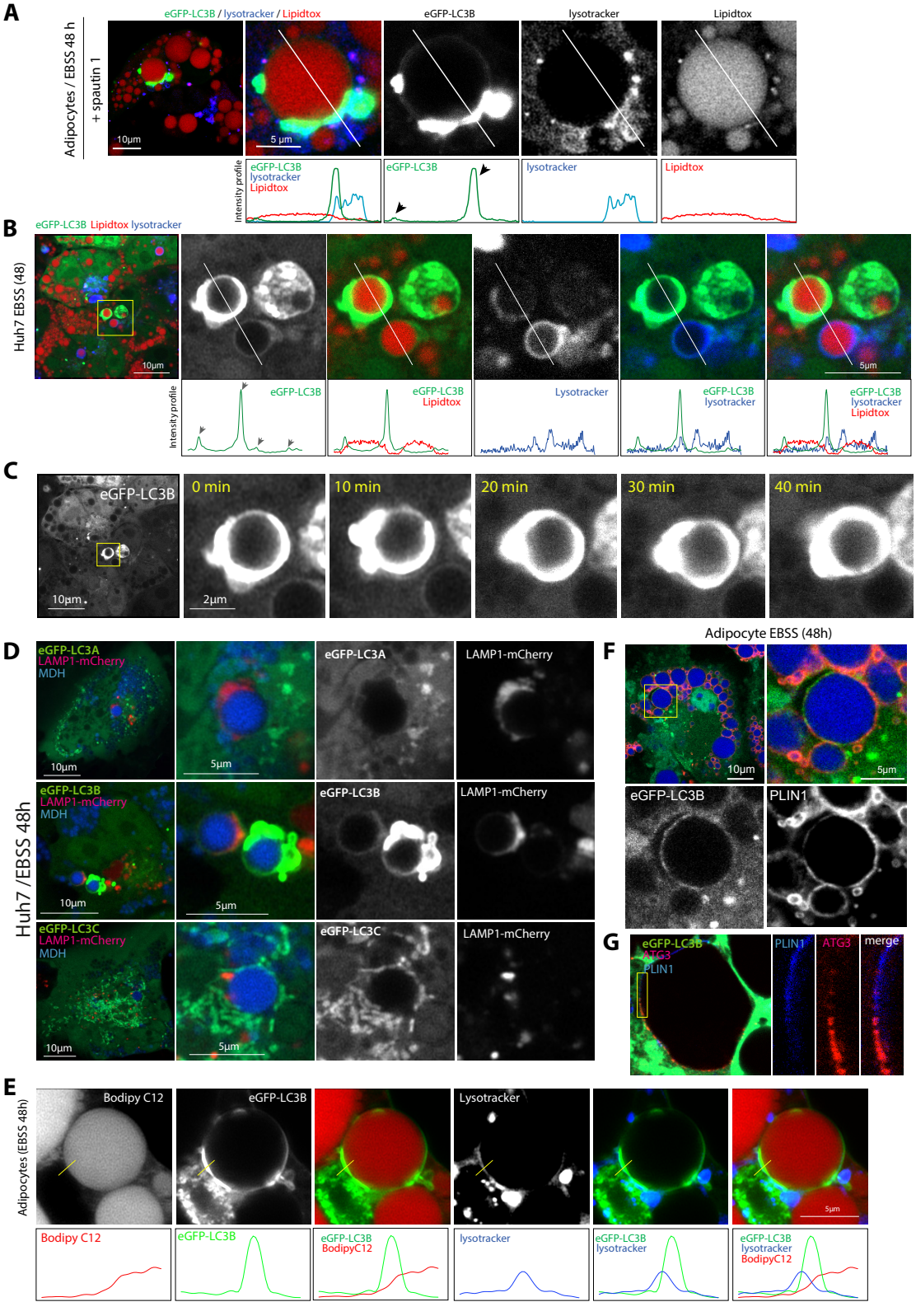

Figure S7: Interaction of LC3-positive LDs with LC3-positive membranes, Related to Figure 7.

A. Confocal imaging of eGFP-LC3B, lysotracker (blue) and LDs (red) in differentiated 3T3-L1 adipocytes virally transfected with eGFP-LC3B and then incubated in EBSS containing Spautin 1 for 48hs. Scale bar,10 µm (5 µm in insets). The intensity line profiles are shown underneath each channel.

B. Confocal imaging of eGFP-LC3B, lysotracker (blue), and LDs in Huh7 loaded with oleic acid for 24hs and virally transfected with eGFP-LC3B. Cells are then incubated in EBSS for 48hs after transfection. Scale bar,10 μm (5 μm in insets). The bottom panels are intensity profiles of the line drawn in each image.
C. Time-lapse of the eGFP-LC3B-positive LD interacting with LC3B-positive membranes in B. Scale bar,10 μm (2 μm in insets).
D. Confocal imaging of Huh7 co-transfected with LAMP1-mCherry and eGFP-LC3A or eGFP-LC3B or eGFP-LC3C, loaded with oleic acid for 24hs and then incubated in EBSS for 48hs. Scale bar,10 μm (5 μm in insets).
E. Confocal imaging of eGFP-LC3B, LDs (BODIPY 558/568 C12), and lysotracker in differentiated 3T3-L1 adipocytes virally transfected with eGFP-LC3B. cells were incubated in the last 48h of differentiation with DMEM containing OA/BODIPY 558/568 C12, transfected, and then placed in EBSS for 48hs. Scale bar,10 μm. The bottom panels are intensity graphs of the line drawn in each image.
F. Immunofluorescence staining of PLIN1 in differentiated 3T3-L1 adipocytes transfected with eGFP-LC3B and incubated in EBSS for 48hs.
G. Immunofluorescence staining of ATG3 and PLIN1 in differentiated 3T3-L1 adipocytes transfected with eGFP-LC3B and incubated in EBSS for 48hs.