SUPPLEMENTAL MATERIAL

Supplemental Figures

Suppl. Fig. S1. Human Atg2A localizes to cytoplasmic LDs. A) Stable GFP-Atg2A/U2OS cells were treated with control medium (CM), rapamycin (RM) or wortmannin (WM) for 3h, followed by live-cell microscopy (CM n=2; RM, WM n=1). Still images are displayed and corresponding movies provided (**Suppl. Videos V1 – V3**). **B)** U2OS cells transiently expressing *myc*-Atg2A for 24 h were treated with control medium (CM), rapamycin (RM) or wortmannin (WM) for 3 h. Cells were immunostained with anti-*myc*/IgG-conjugated Alexa 546 antibodies (red) and TOPRO3 (blue), and imaged by confocal LSM (CM n=2; RM, WM n=3). **C)** U2OS cells stably expressing GFP-Atg2A were transfected with mCherry-tubulin for 24 h, subjected to starvation treatment (nutrient-free medium) and imaged by live-cell microscopy. 11.4 mM Vitamin C was added to reduce phototoxicity and photobleaching. Images were acquired every 5 sec over a period of 2 min 35 sec, 5 h 11 min after starvation stimulation and 2 h 11 min after vitamin C addition. The boxed area corresponds to merged still images of **Fig. 1C** and **Suppl. Video V4**. **D)** Representative confocal LSM of *myc*-Atg2A transiently expressed in rapamycin-treated HeLa cells (n=3). Cells were immunostained with anti-*myc*/IgG-conjugated Alexa 546 antibodies to detect *myc*-Atg2A (red) and TOPRO3 to mark the nucleus (blue). The cell boundary is marked by a dotted line and the boxed area is highlighted by magnification. **E)** U2OS cells transiently expressing *myc*-Atg2A were treated with rapamycin for 3h and immunostained using anti-*myc*/Alexa 546 antibodies (red). LDs were stained with LipidTOX Green (green) and colocalization (yellow) was demonstrated by confocal LSM. Scale bars 20 μm.

Suppl. Fig. S2. Oleic acid treatment stimulates LD formation. A) G361 cells were treated with or without 400 µM oleic acid (OA), followed by 3 h tretaments with nutrient-free medium (NF). Cells were fixed and stained with DAPI (blue) and LipidTOX Green and analyzed using a high content imaging platform (In Cell Analyzer 1000). **B)** G361 cells were incubated with oleic acid for 24 h and subjected to electron microscopy. LDs are marked in red. **C)** Qualitative freeze fracture anti-GFP immuno-EM of stable GFP-Atg2A/U2OS cells (>50 fractures) revealed that Atg2A is not detected as a stable membrane protein of the LD monolayer.

Suppl. Fig. S3. GFP-Atg2A colocalizes with endogenous ADRP. A) GFP-Atg2A/U2OS cells were immunostained with anti-ADRP/IgG Alexa 546 antibodies (red) and TOPRO3 (blue), analyzed by confocal LSM (upper panels) (n=3). Fluorescence intensity profiles (lower panels) demonstrate colocalization of GFP-Atg2A and ADRP. **B)** G361 cells transiently expressing GFP-Atg2A were incubated with or without oleic acid (OA) for 24 h, followed by treatments with nutrient-free medium (NF) for 3h. Upon fixation cells were immunostained with anti-ADRP/IgG Alexa 546 antibodies (red) and TOPRO3 (blue) and subsequently analysed by confocal LSM (upper panels) (n=3). Magnified cytoplasmic areas are presented. White arrows indicate the positioning of the corresponding fluorescence intensity profiles (lower panels).

Suppl. Fig. S4. Quantification of vesicular GFP-Atg14L and *myc*-Atg2A. U2OS cells transiently coexpressing GFP-Atg14L and *myc*-Atg2A for 24 h were treated with control medium (CM), nutrient-free medium (NF) or with wortmannin (WM) for 3 h,

followed by indirect immunofluorescence analysis using anti-*myc*/IgG-conjugated Alexa 546 antibodies and confocal LSM. **A)** Fluorescence area of *myc*-Atg2A as percentage of cell area. **B)** Colocalization analysis of *myc*-Atg2A and GFP-Atg14L, and **C)** GFP-Atg14L and *myc*-Atg2A in U2OS. Quantified image sets correspond to **Fig. 5C**, (CM n = 19, NF n = 21, WM n = 14 cells, from 3 experiments). Mean ± SEM. P-values: n.s. (not significant) $p \geq 0.05$, ** $p \leq 0.01$.

Suppl. Fig. S5. Full cell presentation magnifications used for Figure 6. A) U2OS cells were transiently transfected with GFP-Atg14L, *myc*-Atg2A and HA-WIPI-1. Cells were starved (nutrient-free medium, NF) for 3 h and immunostained with anti-*myc*, anti-WIPI-1, anti-mouse Alexa 546 and anti-rabbit Alexa 633 antibodies. **B)** U2OS cells transiently expressing GFP-Atg14L, *myc*-Atg2A and HA-WIPI-1 were treated with wortmannin for 3 h and subjected to confocal LSM. **C)** U2OS cells transiently transfected with GFP-Atg2A, *myc*-DFCP-1 and HA-WIPI-1 were treated with nutrient-free medium for 3 h, fixed and immunostained. Representative images were acquired by confocal LSM. Scale bars 20 µm.

Suppl Fig. S6. LD size analysis upon Atg2A or Atg14L downregulation. A, B) HeLa or G361 cells were treated with siControl, siAtg2A or siAtg14L for 48 h and downregulation of Atg2A and Atg14L mRNA was verified by quantitative PCR. **C, D)** siRNA treated HeLa or G361 cells were cultured for 3 h in control medium (CM) or nutrient-free medium (NF), fixed, stained with DAPI and LipidTOX Green and cellular LD size was quantified in individual cells by high content analysis (n=3-4). This analysis corresponds to **Fig. 8B**.

Suppl Fig. S7. Phylogenetic analysis of the Atg2 protein family. A) The human Atg2A protein sequence was used for BLAST search-based CLANS cluster analyses, and phylogenetic inference based on the neighbor-joining approach. Atg2 and Vps13 family sequences were found to share common sequence features. Top left: Sequence similarity clustering of all blast hits returned for the Homo sapiens Atg2A protein. Dots represent sequences and the lines connecting dots represent their pairwise similarity. The darker the line the more similar the sequences. Two main groupings of similar sequences are apparent: one combining VPS13-family sequences and the second combining Atg2-family sequences. Bottom left: Cluster-map for the Atg2-family sequences only. The various sequence similarity clusters identifiable in the map correspond to major subdivisions/clades in the taxonomy of eukaryotic organisms. Right: Neighbor-joining phylogenetic tree based on the protostome, deuterostome and outgroup sequences identified in the Atg2 cluster map. **B)** Histograms of sequence regions for the human Atg2A, Atg2B and *Dictyostelium discoideum* Vps13 proteins revealed consistent N- and C-terminal similarities of Atg2 and Vps13. Histogram of which sequence regions (X-axis) generated BLAST HSP's to other sequences (Y-axis, higher=more hits), for the Human Atg2A, Atg2B and *Dictyostelium discoideum* VPS13 proteins. Regions highlighted in green denote those regions for which BLAST could detect similarities between VPS13 and Atg2 proteins. **C)** In human Atg2, this region is here referred to as AVH1 for N-terminal and AVH2 for C-terminal *A*tg2 *V*ps13 *H*omology region.

Supplemental Videos

Suppl. Videos V1 – V3. Stable GFP-Atg2A U2OS cells were treated with control medium (CM, **Suppl. Video V1**), rapamycin (RM, **Suppl. Video V2**) or wortmannin

(WM, **Suppl. Video V3**) for 3 h, followed by live-cell microscopy. These videos correspond to the still images presented in **Suppl. Fig. S1A**.

Suppl. Video V4. Corresponding video to **Fig. 1C** and **Suppl. Fig. 1C**.

Suppl. Video V5. Confocal LSM-based 3D visualization of *myc*-Atg2A localization at the cytoplasmic face of LipidTOX Green-stained LDs.

Suppl. Video V6. Confocal LSM-based 3D visualization of GFP-Atg14L localization at the cytoplasmic face of LipidTOX Red-stained LDs.