Degradation of lipid droplet-associated proteins by chaperone-mediated autophagy facilitates lipolysis

Susmita Kaushik¹² and Ana Maria Cuervo¹²,³

Chaperone-mediated autophagy (CMA) selectively degrades a subset of cytosolic proteins in lysosomes. A potent physiological activator of CMA is nutrient deprivation, a condition in which intracellular triglyceride stores or lipid droplets (LDs) also undergo hydrolysis (lipolysis) to generate free fatty acids for energetic purposes. Here we report that the LD-associated proteins perilipin 2 (PLIN2) and perilipin 3 (PLIN3) are CMA substrates and their degradation through CMA precedes lipolysis. In vivo studies revealed that CMA degradation of PLIN2 and PLIN3 was enhanced during starvation, concurrent with elevated levels of cytosolic adipose triglyceride lipase (ATGL) and macroautophagy proteins on LDs. CMA blockage both in cultured cells and mouse liver or expression of CMA-resistant PLINs leads to reduced association of ATGL and macrolipophagy-related proteins with LDs and the subsequent decrease in lipid oxidation and accumulation of LDs. We propose a role for CMA in LD biology and in the maintenance of lipid homeostasis.

Autophagy maintains cellular homeostasis by degrading proteins, lipids and organelles in lysosomes.¹² Turnover of non-functional cellular components by autophagy ensures quality control and recycling of degraded products provides energy.³ In CMA, the heat shock cognate protein of 70 kDa (hsc70) recognizes proteins with a pentapeptide motif and delivers them to the lysosome surface for binding to lysosome-associated membrane protein 2A (LAMP-2A; L2A), the rate-limiting component of CMA (ref. 6). L2A organizes into a multimeric complex and in cooperation with lysosomal hsc70 (ref. 8) mediates lysosomal translocation of the unfolded substrates for degradation. CMA occurs in most mammalian cell types and is maximally activated following prolonged starvation, mild oxidative stress, hypoxia and lipogenic stressors. A decrease in the lysosomal stability of LAMP-2A results in reduced CMA activity in old organisms.

In addition to the well-characterized turnover of proteins by autophagy, lipids can also undergo degradation by macroautophagy, by sequestration into double-membrane vesicles (autophagosomes) that then fuse with lysosomes. Autophagy-mediated lipolysis (macrolipophagy) selectively targets LDs, intracellular lipid stores that serve as an energy source through hydrolysis of triglycerides into free fatty acids (FFAs). LDs are surrounded by structural proteins of the perilipin (PLIN) family, with PLIN1 being primarily an adipocyte protein and PLIN2 and PLIN3 expressed ubiquitously. Lipolysis can occur through cytosolic lipases, such as ATGL (ref. 21), or by lysosome luminal lipases when autophagosomes, formed through assembly of autophagy-related proteins (ATGs) on the LD surface, engulf portions of LDs and target them to lysosomes.

Although CMA can degrade only proteins and no lipids, mice with a constitutive blockage of CMA in the liver exhibit pronounced steatosis, even though non-selective macroautophagy was intact. The discrete increase in lipogenesis, due to reduced degradation of lipogenic enzymes through CMA (ref. 22), could not account alone for the massive hepatic lipid accumulation in these animals. This finding, combined with the facts that in tissues, such as the liver, lipid influx, lipolysis and CMA activation occur at maximal rates during starvation, lipogenic challenges initially upregulate macrolipophagy ATGs. Reduced CMA precludes recruitment of the upstream regulator of both macrolipophagy and cytosolic lipolysis.

1Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461, USA. 2Institute for Aging Studies, Albert Einstein College of Medicine, Bronx, New York 10461, USA.
3Correspondence should be addressed to A.M.C. (e-mail: ana-maria.cuervo@einstein.yu.edu)
RESULTS
LAMP-2A-deficient cells accumulate LDs
Using both, livers from mice conditionally knocked out for LAMP-2A (L2A) in hepatocytes22 (L2AKO) and mouse fibroblasts (NIH3T3 cells) knocked down for L2A (L2A(−)) to block CMA (ref. 25) we confirmed that, despite the lower dependence of fibroblasts on lipid metabolism when compared with hepatocytes, L2A-deficient fibroblasts accumulated significantly more triglycerides than control fibroblasts (Fig. 1a). These differences in triglyceride content were even higher when intracellular lipid usage was forced by reducing glucose in the media or after a lipogenic stimulus (oleate; OL; Fig. 1a).

As in the L2AKO mice22, we found only a slight trend towards higher triglyceride synthesis in L2A(−) cells compared with control cells (Fig. 1b). In contrast, L2A(−) cells showed significantly reduced β-oxidation rates, an event downstream of triglyceride hydrolysis, both under basal and lipogenic conditions (Fig. 1c), and failed to increase oxygen consumption rates (OCRs) on OL exposure (Fig. 1d; CTR cells: Δ20.9 ± 1.7 pmol min⁻¹; L2A(−) cells: Δ −1.3 ± 0.2 pmol min⁻¹ OCR change). Decreased β-oxidation rates in L2A(−) cells were not due to defective mitochondria per se, because the fraction of mitochondria with intact membrane potential (Supplementary Fig. 1a) and mitochondria turnover (detected with pH-sensitive MitoKeima reporter27; Supplementary Fig. 1b) were comparable to CTR cells. Differences in β-oxidation rates between control and L2A(−) cells were minimized in the presence of etomoxir (Fig. 1a).

LD proteins PLIN2 and PLIN3 are CMA substrates
As only proteins and not lipids can be CMA substrates, we next investigated a role for CMA in degradation of LD proteins, which could explain defective LD utilization in CMA-deficient cells. Immunoblotting (Fig. 2a and Supplementary Fig. 2a) and immunofluorescence (Fig. 2b) for the ubiquitously expressed PLIN2 and PLIN3 showed significantly higher levels under basal, OL treatment and post-OL treatment in L2A(−) cells. Consistent with the described PLIN2 turnover by the ubiquitin–proteasome pathway28,29, proteasomal inhibitors increased LD number (Supplementary Fig. 2b) and PLIN2 levels (Supplementary Fig. 2c) in control cells but not in CMA-defective cells, although proteasomal degradation rates of ubiquitylated proteins and of a degron–GFP reporter30 were preserved in these cells (Supplementary Fig. 2c–e). Lipid accumulation after proteasomal or lysosomal inhibition was comparable (Supplementary Fig. 2b), leaving open the possibility that PLINs could also be amenable to lysosomal degradation.

To examine the possible contribution of CMA to PLIN2 and PLIN3 degradation, we first investigated their association with lysosomes. In support of PLIN lysosomal turnover, inhibiting lysosomal degradation with ammonium chloride and leupeptin in control cells significantly increased the co-localization of PLIN2 or PLIN3 with the lysosomal marker (LAMP1), and more so on OL addition (Fig. 2c,d and Supplementary Fig. 2f,g). In contrast, L2A(−) cells showed decreased association of PLIN2 and PLIN3 with lysosomes and impaired flux with lysosomal inhibitors (Fig. 2c,d and Supplementary Fig. 2f,g). PLIN2 and PLIN3 were enriched in lysosomes active for CMA (CMA+, containing hsc70 and LAMP-2A; ref. 31) as compared with lysosomes with lower CMA activity (CMA−, containing LAMP-2A but deficient in hsc70; ref. 31; Fig. 2e, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a CMA substrate28, is shown as a positive control). Lysosomal levels of both PLINs were reduced in L2A(−) cells (Fig. 2f). We confirmed that lysosomal association of PLIN2 and PLIN3 led to their degradation in vivo, because blocking lysosomal proteolysis in starved rats with leupeptin for 2 h was sufficient to increase levels of these LD proteins in CMA-active lysosomes (Fig. 2g and Supplementary Fig. 2h). LD number (Supplementary Fig. 2b) increased the co-localization of PLIN2 or PLIN3 with each other on LDs, which increased on OL treatment post-OL treatment in L2A(−) cells (Fig. 1a). Electron microscopy confirmed that LD number, average size and occupied cellular area were significantly higher in CMA-deficient cells than in control cells in all conditions tested (Fig. 1f).

PLINs interact with CMA chaperone hsc70
The first step in CMA is substrate interaction with hsc70 for subsequent lysosomal targeting. We found hsc70 in isolated rat liver LDs and its levels increased during starvation, when hepatic lipolysis is highly active, coinciding with a decrease in LD levels of PLIN2 and PLIN3 (Fig. 3a). Immunofluorescence confirmed hsc70 co-localization with each PLIN on LDs, which increased on OL challenge that induces lipolysis (Fig. 3b,c and Supplementary Fig. 3a). Forcing lipid mobilization by placing cells in serum-free media post-OL challenge reduced association of hsc70 with LDs (Supplementary Fig. 3b). Remarkably, L2A(−) cells exhibited...
higher hsc70 co-localization with PLIN2 or PLIN3 in LDs under all conditions (Fig. 3b,c and Supplementary Fig. 3a,b). A similar higher abundance of hsc70 was also observed in LDs isolated from livers of L2AKO mice compared with control littermates (Fig. 3d).

We found direct interaction of hsc70 with PLIN2 and with PLIN3 in cultured cells. Hsc70 was recovered in PLIN2 and 3 pulldowns (Fig. 3e,f) and both PLINs were also detected in hsc70 pulldowns (Supplementary Fig. 3c,d). For the same amount of PLINs pulled down, we consistently observed higher levels of bound hsc70 in L2A(-) cells (Fig. 3d). Increased binding to hsc70 is characteristic of CMA substrates in these cells where disruption of CMA occurs at the level of the lysosomal receptor (L2A) whereas substrate recognition of CMA substrates in these cells where disruption of CMA occurs at higher levels of bound hsc70 in L2A(-) cells (Fig. 3d). Increased binding to hsc70 is characteristic of CMA substrates in these cells where disruption of CMA occurs at the level of the lysosomal receptor (L2A) whereas substrate recognition of CMA substrates in these cells where disruption of CMA occurs at the level of the lysosomal receptor (L2A) whereas substrate recognition

Figure 1 LAMP-2A-deficient cells accumulate LDs. (a) Total triglycerides (TG) in control mouse fibroblasts (CTR) and in cells stably knocked down for LAMP-2A (L2A(-)) (inset) untreated or treated with OL, incubated with serum-supplemented regular media (OL > S) or low-glucose media (OL > S) after OL treatment. n = 4 (Low Glc), 6 (OL > S+) and 7 (all other conditions) independent experiments. (b) Triglyceride synthesis in cells as in a. n = 4 independent experiments. (c) Beta-oxidation rates in cells as in a. n = 6 independent experiments. (d) OCRs in CTR and L2A(-) cells with the indicated treatments. Eto: etomoxir. n = 5 time points from 8 independent experiments. (e) BODIPY493/503 staining in CTR and L2A(-) cells untreated or treated with OL, incubated with serum-supplemented medium (OL > S+) or serum-deprived medium (OL > S-) after OL treatment. (f) Electron microscopy of cells treated as in e. Graphs: area occupied by LD or average LD number per cell and LD size. n = 3 independent experiments with 5 micrographs per condition. In e and f insets depict higher magnification images of lipid droplets. (g) DPH staining in CTR and L2A(-) cells transfected with hL2A, untreated or treated with OL. Asterisks: transfected cells. Graph: average LD number per cell calculated from orthoviews. n = 5 independent experiments with 40 cells per condition in each experiment. Values are mean ± s.e.m. Differences are significant for *P < 0.05, **P < 0.01, ***P < 0.001 using Student’s t-test. Source data are available in Supplementary Table 1.
Figure 2 PLIN2 and PLIN3 are CMA substrates. (a) Immunoblot for indicated proteins of total cell lysates from CTR and L2A(−) cells untreated or treated with OL or incubated with serum-supplemented medium (OL > S+) or serum-deprived medium (OL > S−) after OL treatment. Graph: PLIN2 levels relative to untreated CTR cells. (b) Immunostaining for PLIN2 in CTR and L2A(−) cells treated as in a. Graph: average puncta per cell. n = 5 independent experiments with 40 cells per condition in each experiment. (c) Co-immunostaining for PLIN2 and LAMP1 in CTR and L2A(−) cells treated or not with OL followed by treatment with lysosomal inhibitors, ammonium chloride and leupeptin (NL). Top: co-localized pixels in white. Bottom: merged image of the outlined area at higher magnification. (d) Percentage of co-localization of PLIN2 with LAMP1 from c. n = 5 independent experiments with 40 cells per condition in each experiment. (e) Immunoblot for the indicated proteins of homogenates (HOM), lysosomes with high (CMA+) and low (CMA−) activity isolated from fed or starved (Stv) rat livers. Representative blots from 5 independent experiments. (f) Immunoblot for indicated proteins of isolated lysosome-enriched fractions from OL-treated control and L2A(−) cells. (g) Immunoblot for indicated proteins of HOM, CMA+ and CMA− lysosomes isolated from fed or starved (Stv) livers of rats untreated or treated with leupeptin (Leup). Graph: PLIN2 and PLIN3 levels in leupeptin-treated CMA+ lysosomes relative to untreated. Representative blots from 3 independent experiments (the other two are shown in Supplementary Fig. 2h). Values are mean ± s.e.m. Differences are significant for *P < 0.05, **P < 0.01, ***P < 0.001 using Student’s t-test. Unprocessed original scans of blots are shown in Supplementary Fig. 8. Source data are available in Supplementary Table 1.

by hsc70 is intact (Supplementary Fig. 3e shows the same effect in two other CMA substrates). This enhanced binding of hsc70 for multiple substrates explains why higher levels of PLINs (a single substrate) are not observed in hsc70 pulldowns (Supplementary Fig. 3c,d). On treatment of L2A(−) cells with increasing OL concentrations previously shown to activate CMA (ref. 15), levels of hsc70 bound to PLIN2 or PLIN3 further increased whereas in control cells they remained constant, supporting continuous turnover of LD proteins.
by CMA (Fig. 3c–f and Supplementary Fig. 3f). As, contrary to the almost exclusive LD location of PLIN2, PLIN3 can be present in other cellular compartments, we isolated mouse liver LDs and confirmed that interaction of hsc70 with PLINs occurs in LDs (Fig. 3g). These results are compatible with PLIN2 and PLIN3 being recognized by the CMA chaperone hsc70 at the LD surface before undergoing CMA-mediated degradation.

**Modifications of CMA-targeted PLINs**

To further confirm the involvement of CMA in PLIN turnover, we activated CMA in OL-challenged cells using an atypical retinoic acid derivative\(^3\). Chemical activation of CMA significantly reduced PLIN2 levels (Fig. 4a) and LD content (Fig. 4b) in control cells but not in L2A(−) cells. In fact, restoration of L2A levels in L2A(−) cells through transient transfection was sufficient to bring PLIN2 levels close to control cell values (Fig. 4b).

To identify possible post-translational modifications that trigger PLIN2 for CMA degradation, we performed bidimensional electrophoresis and immunoblotting and found two very prominent forms of PLIN2 with isoelectric points of 5.6 and 5.9 and a less abundant isoform with an isoelectric point of 5.3. When lipolysis and subsequent PLIN2 degradation was induced with OL, the less positively charged isoform was no longer observed in control cells but it persisted in L2A(−) cells (Fig. 4c). Phosphatase treatment confirmed that PLIN2 was phosphorylated during lipolysis in CTR cells but not in L2A(−) cells that accumulated a form of PLIN2 unchanged by the phosphatase (Fig. 4c). Analysis of LDs by electrophoresis using Phos-tag (for resolution of phosphorylated variants) confirmed the presence of a phosphorylated form of PLIN2 in control cells almost completely absent in L2A(−) cells (Fig. 4d). Although further studies are needed to elucidate the reasons for reduced levels of phospho-PLIN2 in the absence of functional CMA, our findings suggest that phosphorylation of PLIN2 may be required to trigger its CMA degradation.

**CMA-resistant PLIN2 causes steatosis**

Hsc70 binds CMA substrates through a pentapeptide motif\(^5\). PLIN3 bears a canonical CMA motif (\(^{100}\)LDRLQ) and PLIN2 a putative...
motif (SLKVQ). To analyse the consequences of preventing CMA degradation of PLIN2, independently from other changes that may occur during CMA blockage, we mutated the PLIN2 CMA-targeting motif to SLKAA. Expression of wild-type (WT) and CMA-mutant (MT) PLIN2-GFP in control cells led to higher levels of MT PLIN2 compared with WT PLIN2-expressing cells under basal conditions (Fig. 5b) and on OL challenge (Fig. 5c).

MT PLIN2-GFP accumulation was mainly due to its reduced lysosomal degradation, as treatment with lysosomal inhibitors (NL) increased WT but not MT PLIN2-GFP levels (Fig. 5c) and, unlike for WT PLIN2, failed to increase co-localization of MT PLIN2 with the lysosomal marker LAMP1 (Fig. 5d).

We confirmed that disruption of the CMA-targeting motif in PLIN2 almost completely abrogates its binding to hsc70 (Fig. 5e), explaining the reduced co-localization of hsc70 with in total cell lysates from CTR and L2A(−) cells untreated, treated with OL, or maintained in OL > S+ media. Bottom inset shows samples treated with lambda phosphatase (λPPase). Arrows: estimated isoelectric points (pI).

Representative blots of n = 3 (OL > S+) and 6 (None and OL) independent experiments. (d) PLIN2 immunoblots of total cell lysates from CTR and L2A(−) cells subjected to the indicated treatments run with Phos-tag to resolve phosphorylated proteins. Immunoblots from two independent experiments are shown. Arrows: orange (unphosphorylated PLIN2), blue (phosphorylated PLIN2). Values are mean ± s.e.m. Differences are significant for """"P < 0.001 using Student’s t-test. Unprocessed original scans of blots are shown in Supplementary Fig. 8. Source data are available in Supplementary Table 1.
lipid accumulation observed in CMA-deficient cells (Figs 1 and 2) and to reduce LD/lysosome interactions.

**Defective CMA of PLINs blocks lipolysis**

Triglycerides in LDs are broken down into FFA either by cytosolic lipases or by lysosomal lipases that gain access to these cytosolic stores through macroautophagy. To assess which lipolytic pathway failed to mobilize LDs in L2A(−) cells (Fig. 1), we treated cells with a lipase inhibitor, diethylumbelliferyl phosphate, and with lysosomal inhibitors (NL) and found that β-oxidation decreased in control cells but L2A(−) cells remained unresponsive (Fig. 6a). Overall these findings suggested that blocking CMA results in impaired lipolysis by cytosolic lipases and macroautophagy.

The rate-limiting cytosolic lipase in most cell types, ATGL (ref. 21), co-localizes with LDs (lipid- or protein-labelled) during lipogenic stimulus (OL) and in OL > S− or OL > low-glucose S+ conditions to induce lipolysis (Fig. 6c and Supplementary Fig. 5a,b). Similarly, chemical activation of CMA increased co-localization of ATGL with LDs (Fig. 6d). In contrast, CMA-defective cells exhibited significantly reduced association of ATGL with LDs in all conditions (Fig. 6c and Supplementary Fig. 5a,b) despite total ATGL levels remaining unchanged (Fig. 6e), pointing towards defective localization of this lipase on LDs. Reduced lipolytic activity of ATGL in CMA-deficient cells could not be attributed to changes in its association with endogenous modulators because, on the contrary, pulldown for ATGL in L2A(−) cells revealed reduced binding to its inhibitor GαG1 switch gene 2 (G0S2) and enhanced association with its co-activator comparative gene identification 58 (CGI-58; Fig. 6f,g), probably reflecting cellular compensatory efforts to sustain lipolysis. Similar changes occur in vivo as association of ATGL with rat liver LDs was enhanced on starvation, which activates both lipolysis and CMA (Fig. 6h), but LDs isolated from livers of starved L2AKO mice exhibited a discrete but consistent reduction in ATGL levels and a more pronounced decrease in levels of its co-activator CGI-58 when compared with LDs isolated from control mice (Fig. 6i and Supplementary Fig. 5c). Furthermore, expression of the CMA-resistant PLIN2 mutant was sufficient to significantly reduce the amount of LC3, ATG5 and Beclin1 associated with LDs (Fig. 7i and Supplementary Fig. 7a,b and Supplementary Video 4 and 3D reconstructions of Z-sections shown in Supplementary Fig. 7c and Supplementary Video 5), confirming that inability to degrade LD-associated PLINs through CMA inhibited the association of the macroautophagy machinery with LDs. We propose that failure to anchor ATGs at the LD surface prevents initiation of macroautophagy. Ultrastructural analysis confirmed that the membranous structures observed in large LDs in control cells on induction of lipolysis (by OL challenge or starvation) and previously proposed as a signature of macroautophagy of portions of LDs (ref. 18) were almost completely absent in LDs of L2A(−) cells (Supplementary Fig. 7d). Collectively, these results indicate that removal of PLINs through CMA precedes macroautophagy.

 Altogether, we propose that degradation of the LD proteins PLIN2 and PLIN3 by CMA is required for the LD association of both the cytosolic lipase and the macroautophagy effector proteins, and for subsequent lipolysis, explaining the intracellular lipid accumulation observed on CMA compromise (Fig. 8).

**DISCUSSION**

In this study, we demonstrate the role of CMA in regulating mobilization of intracellular lipid stores by degrading the LD proteins PLIN2 and PLIN3. Removal of these LD proteins is a prerequisite for subsequent lipolysis to occur, because failure to remove these proteins through CMA results in decreased LD association of components of both arms of the lipolytic machinery—cytosolic lipases and macroautophagy (Fig. 8).

Triglycerides in LDs are broken down by cytosolic lipases to generate FFAs that undergo β-oxidation in mitochondria and by lysosomal lipases on sequestration of complete or fractions of LDs by macroautophagy. In cultured fibroblasts on OL challenge (Supplementary Fig. 6a,b). Co-immunostaining in L2A(−) cells revealed a significant decrease in co-localization with LDs of components of the macroautophagy initiation complex (Beclin1, ATG14, Vps15 and Vps34), proteins involved in autophagosome elongation (ATG5) and the structural autophagosome component (LC3; Fig. 7c and Supplementary Fig. 6c,d). We also analysed two cargo-recognition proteins and found that LDs in L2A(−) cells show lower co-localization with NBR1 (Fig. 7e,f), the one that normally increases on LDs during lipolysis (Fig. 7d and Supplementary Fig. 6a,b), whereas co-localization with p62, which does not change during lipolysis, remained also unchanged in L2A(−) cells (Supplementary Fig. 6e). Co-localization between LDs and ATG7, the ligase that mediates elongation, remained unchanged whereas other ATG proteins such as ULK1, ATG9 and UVRAG did not co-localize with LDs either in control or L2A(−) cells (Supplementary Fig. 6f). Co-localization of Rab7 with LDs, required for lysosome-mediated lipolysis, was also significantly reduced in L2A(−) cells (Fig. 7g). In vivo analysis using isolated liver LDs confirmed that levels of LC3, ATG5, Beclin1 and NBR1 were reduced in L2A-defective mice as compared with control mice (Fig. 7h). Furthermore, even in the context of functional CMA, expression of the CMA-resistant PLIN2 mutant was sufficient to significantly reduce the amount of LC3, ATG5 and Beclin1 associated with LDs (Fig. 7i and Supplementary Fig. 7a,b and Supplementary Video 4) and previously proposed as a signature of macroautophagy of portions of LDs (ref. 18) were almost completely absent in LDs of L2A(−) cells (Supplementary Fig. 7d). Collectively, these results indicate that removal of PLINs through CMA precedes macroautophagy.

 Altogether, we propose that degradation of the LD proteins PLIN2 and PLIN3 by CMA is required for the LD association of both the cytosolic lipase and the macroautophagy effector proteins, and for subsequent lipolysis, explaining the intracellular lipid accumulation observed on CMA compromise (Fig. 8).
membrane forms in situ on the LD surface. In fact, recent studies have shown that LDs are a site for autophagosome biogenesis\(^{36}\). We have uncovered that CMA is required before the selective sequestration of extracts to GST-hsc70 immobilized on agarose beads. Representative blots of \(n=3\) independent experiments. (f) Immunostaining for hsc70 in OL-treated cells expressing WT or MT PLIN2–GFP. Co-localized pixels are in white. Insets: higher magnification. 3D reconstruction shown in Supplementary Fig. 4a. (g) Percentage of co-localization of PLIN2–GFP with hsc70. \(n=5\) independent experiments with 40 cells per condition in each experiment. (h) Live-cell imaging of cells co-transfected with WT or MT PLIN2–GFP and LAMP1–RFP and treated with OL. Sequential frames at 60 s intervals are shown. Arrows: LD. (i, j) LD kiss-run events per cell (i) and count per cell (j), tracking velocity, displacement and displacement rate calculated from images as in h. Small and large LD clusters defined as <1 \(\mu\text{m}^2\) and >1 \(\mu\text{m}^2\), respectively. i: \(n=24\) cells from 3 independent experiments. j: \(n=103\) (WT) and 85 (MT) LD clusters from 6 (WT) and 8 (MT) videos with >8 cells per video from 2 independent experiments. Values are mean ± s.e.m. Differences are significant for \(\ast P<0.05\), \(\ast\ast P<0.01\), \(\ast\ast\ast P<0.001\) using Student’s \(t\)-test. Unprocessed original scans of blots are shown in Supplementary Fig. 8. Source data are available in Supplementary Table 1.

Figure 5 Cells expressing CMA-mutant PLIN2 accumulate LDs. (a) Immunoblot for the indicated proteins of total cell lysates from untransfected cells (--) or cells transfected with wild-type (WT) or CMA-mutant (MT) PLIN2–GFP. o, overexpressed protein; e, endogenous protein. Representative blots of \(n=3\) independent experiments. (b) Cells expressing WT or MT PLIN2–GFP. Inset: higher magnification of the outlined area. Graphs: average LD number or LD size. \(n=5\) (LD number) and 3 (LD size) independent experiments with 40 cells per condition in each experiment. (c) Cells expressing WT or MT PLIN2–GFP treated with OL with or without lysosomal inhibitors ammonium chloride and leupeptin (NL). Inset: higher magnification of the outlined area. Graphs: average LD number or LD size. \(n=5\) (LD number) and 3 (LD size) independent experiments with 40 cells per condition in each experiment. (d) Immunostaining for LAMP1 in cells treated as in c. Top: merged images. Bottom: co-localized pixels in white. Insets: higher magnification of the outlined area. Graphs: average LD number or LD size. (e) Binding of WT and MT PLIN2–GFP from cellular extracts to GST–hsc70 immobilized on agarose beads. Representative blots of \(n=3\) independent experiments. (f) Immunostaining for hsc70 in OL-treated cells expressing WT or MT PLIN2–GFP. Co-localized pixels are in white. Insets: higher magnification. 3D reconstruction shown in Supplementary Fig. 4a. (g) Percentage of co-localization of PLIN2–GFP with hsc70. \(n=5\) independent experiments with 40 cells per condition in each experiment. (h) Live-cell imaging of cells co-transfected with WT or MT PLIN2–GFP and LAMP1–RFP and treated with OL. Sequential frames at 60 s intervals are shown. Arrows: LD. (i, j) LD kiss-run events per cell (i) and count per cell (j), tracking velocity, displacement and displacement rate calculated from images as in h. Small and large LD clusters defined as <1 \(\mu\text{m}^2\) and >1 \(\mu\text{m}^2\), respectively. i: \(n=24\) cells from 3 independent experiments. j: \(n=103\) (WT) and 85 (MT) LD clusters from 6 (WT) and 8 (MT) videos with >8 cells per video from 2 independent experiments. Values are mean ± s.e.m. Differences are significant for \(\ast P<0.05\), \(\ast\ast P<0.01\), \(\ast\ast\ast P<0.001\) using Student’s \(t\)-test. Unprocessed original scans of blots are shown in Supplementary Fig. 8. Source data are available in Supplementary Table 1.
lipolysis observed in CMA-deficient cells could be attributable to in-bulk macroautophagy, shown to be upregulated in these cells\(^{25}\), and where cargo recognition is not required.

Cytosolic lipolysis is regulated by complex interactions between PLINs and lipases\(^{39}\). ATGL associates with LDs under maximal lipolytic conditions\(^{39}\) and PLINs at the LD surface exert a 'shielding
Figure 7 Failure to remove PLINs by CMA blocks macrolipophagy. (a) BODIPY493/503 staining in CTR and L2A(−) cells untreated or treated with OL, or treated with 3-methyladenine (3MA) or lysosomal inhibitors ammonium chloride and leupeptin (NL). Full fields shown in Supplementary Fig. 5d. Graph: average LD number per cell. n = 9 fields with 1,350 cells per condition from 3 independent experiments. (b) Cells stained as in a treated or not with rapamycin (Rapa). Insets: higher magnification. (c) Average LD number per cell. n = 9 fields with 1,350 cells per condition from 3 independent experiments. (d) Immunoblot for the indicated proteins in homogenates (HOM) and lipid droplets (LD) isolated from livers for fed (F) and starved (S) rats. Representative blots of n = 5 independent experiments. (e) Co-staining for the indicated ATG proteins and BODIPY493/503 in CTR and L2A(−) cells treated with OL. Insets: higher magnification of the outlined areas with co-localized pixels in white. (f) Percentage of co-localization with BODIPY. n = 6 independent experiments with 40 cells per condition in each experiment. (g) Co-staining for Rab7 in the same cells as in e. Graph: percentage of co-localization with BODIPY. n = 4 independent experiments with 40 cells per condition in each experiment. Inset: higher magnification of the outlined area with co-localized pixels in white. 3D reconstruction shown in Supplementary Fig. 7c. Graph: percentage co-localization of LC3 with PLIN2–GFP. n = 4 independent experiments with 40 cells per condition in each experiment. Values are mean ± s.e.m. Differences are significant for **P < 0.01, ***P < 0.001 using Student’s t-test. Unprocessed original scans of blots are shown in Supplementary Fig. 8. Source data are available in Supplementary Table 1.

...that modulates the lipase’s accessibility to triglycerides. In this regard, overexpression of PLIN2 reduces LD association of ATGL and hence, lipolysis39,40. We propose that CMA facilitates lipolysis by removing PLINs from LDs because we found decreased association of ATGL on LDs in cells with elevated PLIN2 levels, either due to impaired CMA or due to expression of the CMA mutant. In addition,
PLIN2 removal could also facilitate binding of the co-activator to ATGL already on LDs, because CMA blockage reduced levels of CGI-58 in LDs.

Although PLIN2 has been reported to be a proteasome substrate, both lysosomes and proteasomes can degrade the same protein depending on the cellular requirement or the cell type, as reported, for example, for PLIN1 (refs 41–43). In our study, we observed both lysosomal and proteasomal degradation of PLIN2, but interestingly, CMA blockage also prevented PLIN2 proteasomal degradation. Although it is plausible that CMA might indirectly facilitate proteasomal degradation of PLIN2, our data disfavor this option because: PLIN2 directly interacts with hsc70; we detect PLIN2 in CMA-active lysosomes and its degradation in this compartment; and eliminating the CMA-targeting motif retains PLIN2 in LDs.

We propose that PLIN2 and PLIN3 as the first lipid-anchored proteins to be CMA substrates. The presence of hsc70 on LDs, also described before, and its persistence at the LD surface when CMA is blocked, together with the altered LD and lysosomal dynamics in these conditions, suggests the presence of a microenvironment at the LD surface, wherein lysosomes and LDs are in close proximity. Dynamic and transient LD interactions, similar to the ones we propose here with lysosomes, have been described between LDs and other organelles, such as mitochondria and peroxisomes, to channel FFA from lipolysis to sites of oxidation. Our studies with live-cell microscopy support a marked reduction in kiss-and-run events between LDs and lysosomes in conditions that compromise PLIN degradation by CMA such as mutations in the CMA-targeting motif of PLIN2 that prevent hsc70 interaction with this protein.

We thus propose that degradation of PLINs through CMA occurs in discrete areas on LDs to facilitate punctual removal of PLINs from the LD surface for ATGL and ATGs to access the stored triglycerides. ATGL rapidly cycles on and off of LDs to catalyse lipolysis and, similarly, association of LC3 with LDs is polarized and occurs in discrete areas on LDs to facilitate punctual removal of PLINs from LDs (ref. 51).

We propose that the pronounced hepatic lipid accumulation observed in L2AKO mice results from a combination of: increased levels of lipogenic enzymes degraded through CMA (ref. 22), reduced VLDL secretion, and reduced lipolysis due to the inability of cytosolic lipases and ATGs to bind to LDs when PLINs are not degraded by CMA. We have previously shown that chronic lipid loading inhibits CMA activity. In light of our current findings of regulation of lipid
stores by CMA, it is conceivable that a compromise in CMA would set up a vicious cycle of lipid overloading in cells. CMA activity decreases with age\textsuperscript{16}, and it is plausible that changes in CMA would impact cellular lipohomeostasis and the steatosis observed during ageing, which in turn would further suppress CMA activity. Manipulating CMA could thus be a viable therapeutic option against excessive lipid accumulation.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**ACKNOWLEDGEMENTS**

This work was supported by grants from the National Institutes of Health AG021904, AG031782, AG038072, DK094808 and the generous support of Robert and Renée Belfer. We thank B. Patel for performing the electron microscopy, R. Singh for assistance in biochemical lipid assays and the Analytical Imaging Facility for support with live-cell imaging.

**AUTHOR CONTRIBUTIONS**

S.K. designed and performed the experiments, analysed and interpreted the data, and contributed to writing the manuscript; A.M.C. coordinated the study, contributed to designing and interpretation of the experiments and to writing the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Published online at http://dx.doi.org/10.1038/ncb3166

Reprints and permissions information is available online at www.nature.com/reprints

Note: Supplementary Information is available in the online version of the paper.

**REFERENCES**

1. Mizushima, N. & Komatsu, M. Autophagy: renovation of cells and tissues. Cell 147, 728–741 (2011).
2. Yang, Z. & Klionsky, D. J. Mammalian autophagy: core molecular machinery and multi-enzyme complex mediates the catabolism of cellular fat stores. *Proc. Natl Acad. Sci. USA* 103, 5805–5810 (2006).
3. Kalayaya, H., Kogure, T., Mizushima, N., Yoshimori, T. & Mwawari, A. A sensitive and quantitative technique for detecting autophagic events based on lysosomal delivery. *Chem. Biol.* 18, 1042–1052 (2011).
4. Ranalli, M. V., Gabrielli, B. G. & Gonda, T. J. High-content imaging of neutral lipid droplets with 1,6-diphenylhexatriene. *Biotechniques* 51, 35–42 (2011).
5. Masuda, Y. et al. ADRP/adipophin is degraded through the proteasome-dependent pathway during regression of lipid-storing cells. *J. Lysos. Res.* 47, 87–98 (2006).
6. Xu, G. et al. Post-translational regulation of adipocyte differentiation-related protein by the ubiquitin/proteasome pathway. *J. Biol. Chem.* 280, 42841–42847 (2005).
7. Dohi, E. et al. Identification and characterization of associated with lipid droplet biogenesis. *J. Cell Biol.* 175, 27447–27456 (2000).
8. Wang, H. et al. Unique regulation of adipose triglyceride lipase (ATGL) by perilipin 5, a lipid droplet-associated protein. *J. Biol. Chem.* 286, 10463–10470 (1993).
9. Koga, H., Kaushik, S. & Cervo, A. M. Altered lipid content inhibitors autophagic vesicular fusion. *FASEB J.* 24, 3052–3065 (2010).
10. Anguiano, J. et al. Chemical modulation of chaperone-mediated autophagy by retinoids. *Chem. Biol.* 19, 374–382 (2012).
11. Bostrom, P. et al. Cytoplasmic lipid droplets increase in size by microtubule-dependent complex formation. *Arterioscler. Thromb. Vasc. Biol.* 25, 1945–1951 (2005).
12. Targett-Adams, P. et al. Live cell analysis and targeting of the lipid droplet-binding adipocyte differentiation-related protein. *J. Biol. Chem.* 278, 15998–16007 (2003).
13. Llano, A., San, K. T. & Lee, Y. H. β-adrenergic receptor-stimulated lipolysis requires the RAB7-mediated autolysosomal lipid degradation. *Autophagy* 9, 1228–1243 (2013).
14. Dupont, N. et al. Neutral lipid stores and lipase PNPLA5 contribute to adipocyte dysfunction and adipose tissue biogenesis. *Cell Metab.* 24, 609–620 (2014).
15. Wang, H. et al. Regulation of adipocyte triglyceride lipase (ATGL) by perilipin 5, a lipid droplet-associated protein. *J. Biol. Chem.* 286, 15707–15715 (2011).
16. Listemberger, L. L., Ostermeyer-Fay, A. G., Goldberg, E. B., Brown, W. J. & Brown, D. Neutral lipid stores and lipase PNPLA5 contribute to adipocyte dysfunction and adipose tissue biogenesis. *Cell Metab.* 24, 609–620 (2014).
17. Wang, H. et al. Unique regulation of adipose triglyceride lipase (ATGL) by perilipin 5, a lipid droplet-associated protein. *J. Biol. Chem.* 286, 15707–15715 (2011).
18. Listemberger, L. L., Ostermeyer-Fay, A. G., Goldberg, E. B., Brown, W. J. & Brown, D. Adipocyte differentiation-related protein reduces the lipid droplet association of adipocyte triglyceride lipase and slows triacylglycerol turnover. *J. Lipid Res.* 48, 2751–2761 (2007).
19. Kovsan, J., Ben-Romano, R., Souza, S. C., Greenberg, A. S. & Rudich, A. Regulation of adipocyte lipolysis by degradation of the perilipin protein: nelfinavir enhances lysosome-mediated perilipin proteolysis. *J. Biol. Chem.* 282, 21704–21711 (2007).
20. Ogasawara, J. et al. Oligonol-induced degradation of perilipin 1 is regulated through lysosomal degradation machinery. *Nat. Prod. Commun.* 7, 1193–1196 (2012).
21. Xu, G., Szalayd, C. & Londos, C. Degradation of perilipin is mediated through ubiquitination-proteasome pathway. *Biochim. Biophys. Acta* 1761, 83–90 (2006).
22. Cermelli, S., Guo, Y., Gross, S. P. & Weile, M. A. The lipid-droplet proteome reveals that droplets are a protein-storage depot. *Curr. Biol.* 21, 1783–1796 (2006).
23. Fujimoto, T. & Osaki, Y. Cytoplasmic lipid droplets: rediscovery of an old structure as a unique platform. *Ann. N.Y. Acad. Sci.* 1036, 104–115 (2006).
24. Parent, R., Qu, X., Petit, M. A. & Beretta, L. The heat shock cognate protein 70 is implicated in protein translocation. *Traffic* 7, 1254–1269 (2006).
25. Binns, D. et al. An intimate collaboration between peroxisomes and lipid bodies. *J. Cell Biol.* 173, 719–731 (2006).
26. Blanchette-Mackie, E. J. & Scow, R. O. Movement of lipolytic products to mitochondria in brown adipose tissue of young rats: an electron microscope study. *J. Lipid Res.* 24, 229–244 (1983).
27. Wang, H. et al. Perilipin 5, a lipid droplet-associated protein, provides physical and biochemical linkage to mitochondria. *J. Lipid Res.* 52, 2159–2168 (2011).
28. Bartz, R. et al. Dynamic activity of lipid droplets: protein phosphorylation and GTP-mediated protein translocation. *J. Proteome Res.* 6, 3256–3265 (2007).
METHODS

Cell culture and treatments. NIH3T3 mouse fibroblasts (American Type Culture Collection) were cultured in complete Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich), 10% heat-inactivated newborn calf serum (HyClone), 1% penicillin/streptomycin (Invitrogen) and maintained at 37 °C/5% CO2. NIH3T3 clones stably infected for LAMP-2A (L2A(−)) were generated as described previously25. All of the cell lines were tested for mycoplasma contamination using a DNA staining protocol with Hoechst 33258 dye. Oleic acid (Sigma-Aldrich) was conjugated to albumin as described previously26, and cells were treated with 0.06 mM oleate (OL) for 24 h unless otherwise stated. Where indicated, OL was washed off with Hank's balanced salt solution (Invitrogen) and replaced by DMEM or low-glucose DMEM with or without serum (OL- > S-, OL+ > S+, OL- > S- respectively) for 16 h. Cells were treated with lysosomal inhibitors ammonium chloride (20 mM; American Bioanalyticals) and leupeptin (100 μM; Fisher Scientific), macroautophagy activator rapamycin (100 nM; Sigma-Aldrich), and mitochondrial depolarizer FCCP (2 μM; Sigma-Aldrich) for 16 h unless indicated otherwise. For imaging experiments, cells were treated with eotoxin (10 μM; Sigma-Aldrich) for 6 h.

Plasmids and reagents. Mouse PLIN2-GFP and G6S-GFP plasmids were from Origene, rat LAMP1-RFP plasmid was from the Addgene plasmid repository, human L2A (PGK-L2A, plasmid includes the GFP transgene, to facilitate identification of transfected cells) was generated in our laboratory30. Mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Agilent Biotechnologies). Cells were transfected with 1 μg DNA plasmid (or 0.5 μg of each plasmid in the case of co-transfection) using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Sources of chemicals were as previously described12,20,25.

Antibodies. The dilutions and sources of antibodies used for immunoblot (IB) and immunofluorescence (IF) in this study are: PLIN2 (1:2,000 (IB); 1:100 (IF); Progen Biotechnik; GP40), PLIN3 (1:2,000 (IB); 1:100 (IF); ProSci Inc; 3881), L2A (1:5,000 (IB); Life Technologies; 512200), mouse LAMP1 (1:3,000 (IB); 1:100 (IF); (Developmental Studies Hybridoma Bank; ID4B), rat LAMP1 (1:3,000 (IB); Enzo Life Sciences; ly1C6), human LAMPP2 (1:3,000 (IB); 1:100 (IF); Developmental Studies Hybridoma Bank; HAB4), LAMP2B (1:5,000 (IB); generated in our laboratory), Hsc70 (1:5,000 (IB); 1:100 (IF); Novus Biologicals; 133D3), GAPDH (1:3,000 (IB); Abcam; 6C5), hexokinase (1:1,000 (IB); Cell Signalling; C35C4), IgGF (1:2,000 (IB); Origene;2H8), ATGL (1:100 (IB); 1:100 (IF); Cell Signaling; 30A4), CG58 (1:3,000 (IB); ProSci Inc; 45186), ATG5 (1:2,000 (IB); 1:100 (IF); Novus Biologicals; 53818), Beclin1 (1:2,000 (IB); 1:100 (IF); Novus Biologicals; 500249), LC3 (1:1,000 (IB); 1:100 (IF); Cell Signaling; 2775), Atg14 (1:100 (IF); MBL; PD0026), Atg7 (1:100 (IF); Cell Signaling; 2631), Atg5 (1:100 (IF); Novus Biologicals; NB310463), ps13 (1,200 (IB); 1:100 (IF); Life Technologies; 382100), p62 (1:2,000 (IB); Enzo Life Sciences; 9860), NBR1 (1:3,000 (IB); 1:100 (IF); Abnova; A01), Rab7 (1:100 (IF); Abcam; EPR75838), ULK1-P (1:100 (IF); Cell Signaling; 5869), Atg9 (1:100 (IF); Novus Biologicals; 56893), UVRAG (1:100 (IF); Abcam; 174550), K48-specific ubiquitin (1:2,000 (IB); Millipore; Apu2), JkB (1:5,000 (IB); Santa Cruz Biotechnology; C21), and actin (1:10,000 (IB); Abcam; AC15).

Animals and treatments. Adult Wistar male rats (25) and C57BL/6 male mice (12) (Charles River Laboratories) were used. L2A knockout mice were generated as described previously25. Where indicated, rats or mice were starved for 48 h or 24 h before organellar isolation by removing food but maintaining water ad libitum. Animals were injected intraperitoneally with leupeptin (2 mg per 100 g body weight) 2 h before organellar isolation. All animal studies were approved by the Institutional Animal Care and Use Committee. Randomization between control and L2AKO mice was not possible as animal group is based on genotype. In the case of starvation and by respiratory measurements. Briefly, the rate of carbon dioxide production resulting from the oxidation of [14C]oleate was measured. Cells treated with or without OL (0.06 mM) were treated with [14C]oleate–BSA (0.8 μCi, 4 h). Where indicated, the inhibitors ammonium chloride (20 mM) and leupeptin (100 μM), and diethylumbelliferyl phosphate (Deep) (100 μM), were added with the radiolabelled oleate for 4 h. The released [14C]carbon dioxide was trapped at 37 °C for 1 h onto filter paper pre-soaked in 100 mM sodium hydroxide. The rate of beta-oxidation was calculated as the amount of trapped [14C]carbon dioxide in relative units produced per microgram protein per hour. Equal numbers of cells were plated in XF96 plates (Seahorse Bioscience) in low-glucose DMEM (GIBCO) supplemented with serum. Cells were treated with OL (0.12 mM) for 16 h, following which cell respiration was assayed by time-resolved measurements of oxygen consumption rate (OCR) in a respirometer (Seahorse Bioscience). Fatty acid beta-oxidation was calculated as specified by the manufacturer by injecting eotoxin (50 μM) in the buffered assay medium.

Cell imaging. For immunofluorescence microscopy, cells grown on coverslips were fixed with 4% paraformaldehyde (or pre-chilled methanol in the case of staining for Hsc70, to highlight the membrane-associated protein) for 30 min, permeabilized and blocked with 1% BSA, 0.01% Triton X-100 in phosphate buffer saline (PBS). Incubation with primary and secondary antibody conjugated to Alexa Fluor 488 or Cy5 (Invitrogen) in 0.1% BSA in PBS was performed at room temperature for 1 h each. For LD staining, cells were incubated with BODIPY 493/503 (Invitrogen, 20 μg/mL) or 1,6-diphenyl-1,3,5-hexatriene (DPH, Sigma-Aldrich, 5 μM; pseudo-coloured to red or green) for 20 min at 37 °C before fixation. For mitochondria staining, cells were stained with MitoTracker (50 mM) and MitoTracker CMXRos (50 mM; Invitrogen) for 20 min 37 °C before fixation. Coverslips were mounted in DAPI–Fluoromount-G (Southern Biotechnological) or Drag5 (Invitrogen; pseudo-coloured to blue) to stain the nucleus. All images were acquired with an Axiovert 200 fluorescence microscope (Carl Zeiss Microscopy) with a ×63 objective and 1.4 numerical aperture, mounted with an ApoTom.2 slider, and prepared using Adobe Photoshop CS3 (Adobe Systems) and ImageJ (NIH). Where indicated, cells plated in glass-bottom 96-well plates were treated, stained with BODIPY 493/503, fixed and images were acquired using a high-content microscope (Operetta, Perkin Elmer). Images of 9 different fields per well were captured, resulting in an average of 1,500–2,000 cells. Nuclei and puncta were identified using the manufacturer's software. The number of particles/puncta per cell was quantified using the 'analyse particles' function of ImageJ after thresholding in non-saturated images. The percentage of co-localization was determined by the 'JACoP' plugin in ImageJ after thresholding of individual frames. Where indicated, the co-localized pixels were determined with the 'overlay plugin' of ImageJ. Three-dimensional reconstruction images were modelled from Z-sections as mixed renderings using the Inside4D module for AxioVision Rel. 4.8. after applying the Nyquist–Shannon sampling theorem. Immunofluorescence and direct fluorescence quantifications of the number of lipid droplets, number of puncta and percentage co-localization were performed blinded.

Live-cell imaging was performed in cells grown in glass-bottom dishes (MatTek) 24 h post-transfection (with 8 h OL treatment) at 37 °C in a CO2-enriched atmosphere in recording medium (phenol red-free DMEM (GIBCO), 3.7 mg ml−1 NaHCO3, and 25 mM HEPES, pH 7.4) using a 4D spinning-disc confocal microscope (PerkinElmer, TE2000S) with a ×60 NA 1.4 oil objective and ORCA-ERA camera (Hamamatsu). Images were acquired as a stack of 40–50 Z optical planes (0.4 μm) encompassing the entire cell width at a fixed maximum speed for ~20 min. Image sequences were processed with Velocity and ImageJ. Using Velocity, small and large LD clusters were identified as <1 μm3 and >1 μm3, respectively, and were tracked using the software to calculate the displacement (straight line distance between the start and the end point of the track), displacement rate (displacement/time of last time point in the track–time of the first time point of the track) and tracking velocity (average speed over the whole track).

For electron microscopy, cells were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.4 at 4 °C for 3 h. Post-fixation with 1% osmium tetroxide was followed by dehydration with graded series of ethanol, infiltration and embedding in EMBed 812 (Electron Microscopy Sciences). Sections (80 nm) were cut with an ultramicrotome (Leica), stained with uranyl acetate followed by lead citrate and viewed in a JEM-1200EX transmission electron microscope (JEOL) at 80 kV. Morphometric analysis was done using ImageJ with LDs identified as electron translucent structures lacking surrounding membrane. Micrographs were independently annotated by two people and the average of their scoring was used for each micrograph.
**Cellular subfractionation.** Rat liver lysosomes were isolated from a light mitochondrial–lysosomal fraction by centrifugation in a discontinuous metrizamide density gradient\(^ {57}\). Rat and mouse liver LDs were isolated as described previously\(^ {18}\). Livers homogenized in 0.25 M sucrose were centrifuged at 6,800 \( g \) for 5 min at 4°C. The supernatant and the fatty layer were centrifuged at 17,000 \( g \) for 10 min at 4°C to pellet lysosomes. The supernatant was adjusted to 20% sucrose (LD-enriched fraction) and centrifuged in a discontinuous sucrose density gradient at 28,000 \( g \) for 60 min at 4°C. The LD fraction collected was delipidated, solubilized in SDS, and an equal protein amount was analysed by immunoblot.

**Co-immunoprecipitation.** Co-immunoprecipitation was performed in total cell lysates or LD-enriched fractions prepared in 25 mM Tris (pH 7.2), 150 mM NaCl, 5 mM MgCl\(_2\), 0.5% NP-40, 1 mM dithiothreitol, 5% glycerol and protease inhibitors using standard procedures. An equal protein amount of each fraction was incubated with primary antibody (1 \( \mu \)g) overnight followed by incubation with protein A-conjugated Sepharose for 1 h, centrifugation and washes.

**General methods.** Total cell lysates were prepared in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% NaDoc, 0.1% SDS, 50 mM Tris pH 8) containing protease and phosphatase inhibitors. Protein concentration was measured using bovine serum albumin as a standard\(^ {58}\). Isoelectric focusing (IEF) was done using the Protean IEF Cell (Bio-Rad) at 20°C with rapid ramping to voltage 10,000 \( V \) at a current limit of 50 \( \mu \)A using IPG Strips with 4–7 pH range (Bio-Rad). Where indicated, total cellular fractions were treated with \( \lambda \)-phosphatase according to the manufacturer’s instructions (New England BioLabs) before IEF. Phos-tag PAGE was performed according to the manufacturer’s instructions (Wako) using 100 \( \mu \)M Phos-tag. Samples were subjected to SDS–PAGE, transferred to nitrocellulose membrane, blocked with low-fat milk and incubated with primary antibody overnight. The proteins were visualized by using peroxidase-conjugated secondary antibodies and chemiluminescent reagent (PerkinElmer) in a LAS-3000 Imaging System (Fujifilm). Densitometric quantification was performed on unsaturated images using ImageJ.

**Sample size, randomization and statistical analyses.** In the studies performed in cell lines in culture, all experiments were repeated at least 3 times and often with duplicate blots in the same experiment. All numerical results are reported as mean \( \pm \) standard error of the mean (s.e.m.). Statistical significance of the difference between experimental groups was analysed by two-tailed unpaired Student’s \( t \)-test using MS Excel. Differences were considered statistically significant for \( P < 0.05 \). For those experiments in which an estimate of variation was possible based on previous studies or published work by others, we have confirmed that the variance is similar between the control and experimental groups compared. For those instances in which previous information was not available, we have made a comparison with other conditions in which changes in cellular components resulted in changes in CMA.

52. Goldstein, J. L., Basu, S. K. & Brown, M. S. Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. Methods Enzymol. 98, 241–260 (1983).
53. Boland, B. et al. Autophagy induction and autophagosome clearance in neurons: relationship to autophagic pathology in Alzheimer’s disease. J. Neurosci. 28, 6926–6937 (2008).
54. Massey, A. C., Follenzi, A., Kiffin, R., Zhang, C. & Cuervo, A. M. Early cellular changes after blockage of chaperone-mediated autophagy. Autophagy 4, 442–456 (2008).
55. Kaushik, S., Massey, A. C. & Cuervo, A. M. Lysosome membrane lipid microdomains: novel regulators of chaperone-mediated autophagy. EMBO J. 25, 3921–3933 (2006).
56. Bolte, S. & Cordelieres, F. P. A guided tour into subcellular colocalization analysis in light microscopy. J. Microsc. 224, 213–232 (2006).
57. Kaushik, S. & Cuervo, A. M. Methods to monitor chaperone-mediated autophagy. Methods Enzymol. 452, 297–324 (2009).
58. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275 (1951).
**Supplementary Figure 1** LAMP-2A-deficient cells accumulate LD. (a) MitoTracker and MitoTracker CMXRos staining in CTR and L2A(-) cells untreated or treated with OL. Graph: average percentage colocalization between the two fluorophores. n=6 independent experiments with 40 cells per condition in each experiment. (b) CTR and L2A(-) expressing mtKeima untreated or treated with OL. FCCP is shown as a positive control. Graph: average number/cell. n=9 fields with 1350 cells per condition from 3 independent experiments. (c) Oil Red O staining in WT and L2AKO liver sections. Graphs: LD number, average LD size and percentage of cytosolic area occupied by LD. n=6 micrographs from 3 animals in each group. (d) LD size for the cells shown in Fig. 1e. n=7 independent experiments with 40 cells per condition in each experiment. (e, f) DPH staining in CTR and L2A(-) cells untreated or treated with OL, or incubated with serum-supplemented medium (OL>S+) or serum-deprived medium (OL>S-) after OL treatment. Graph: average LD number/cell. n=5 independent experiments with 40 cells per condition in each experiment. (g) BODIPY 493/503 staining in OL-treated CTR and L2A(-) cells untreated or treated with etomoxir. Graph: average LD number/cell. n=5 independent experiments with 40 cells per condition in each experiment. (h) BODIPY 493/603 staining in CTR and L2A(-) cells maintained in the indicated conditions. Graph: average LD number/cell. n=18 fields with 2700 cells per condition from 3 independent experiments. (i) Immunoblot for hLAMP2 in CTR and L2A(-) cells transfected with hL2A. (j) Immunostaining for hLAMP2 in CTR and L2A(-) cells transfected with hL2A and treated with OL. Values are mean ± SEM. Differences are significant for *P<0.05, **P<0.01, ***P<0.001 using Student’s t-test. Uncropped images of blots are shown in Supplementary Figure B. Source data is available in Supplementary Table 1.
Supplementary Figure 2 PLIN2 and PLIN3 are CMA substrates. (a) Immunoblot for indicated proteins in total cell lysates from CTR and L2A(-) cells. Two sets of cells are shown. (b) BODIPY 493/503 staining in control cells untreated or treated with ammonium chloride and leupeptin (NL), lactacystin (Lacta) or MG132. Graph: average LD number/cell. n=5 independent experiments with 40 cells per condition in each experiment. (c) Immunoblot for indicated proteins in total cell lysates from CTR and L2A(-) cells treated or not with OL and lactacystin (Lacta). n=2 independent experiments. (d, e) CTR and L2A(-) cells expressing DGN or DGN-FS treated or not with OL and Lacta. Graph: average fluorescence intensity/cell. n=10 fields with 1500 cells per condition from 3 independent experiments. (f, g) Coimmunostaining for PLIN3 and LAMP1 in CTR and L2A(-) cells treated or not with OL, followed by treatment with lysosomal inhibitors (NL). Top: Colocalized pixels in white. Bottom: Merged image of the boxed area at higher magnification. Graph: percentage colocalization of PLIN3 with LAMP1. n=5 (L2A(-)) and 6 (CTR) independent experiments with 40 cells per condition in each experiment. (h) Immunoblot for indicated proteins of HOM, CMA+ and CMA- lysosomes isolated from fed or starved (Stv) livers of rat untreated or treated with leupeptin (Leup). These blots contributed to the quantification shown in Fig. 2g. All values are mean ± SEM. Differences are significant for *P<0.05, **P<0.01, ***P<0.001 using Student’s t-test. Uncropped images of blots are shown in Supplementary Figure 8. Source data is available in Supplementary Table 1.
Supplementary Figure 3 PLIN2 and PLIN3 associate with hsc70. (a) Coimmunostaining for PLIN3 and hsc70 in CTR and L2A(-) cells treated or not with OL. Colocalized pixels are in white. Boxed areas are shown at higher magnification. Bottom: graph: percentage colocalization of PLIN3 (bottom) with hsc70. n=5 independent experiments with 40 cells per condition in each experiment. (b) Coimmunostaining for PLIN2 and hsc70 in CTR and L2A(-) cells treated as indicated. Colocalized pixels are in white. Graph: percentage colocalization. n=4 independent experiments with 40 cells per condition in each experiment. (c, d) Immunoblot for indicated proteins of immunoprecipitates (IP) of hsc70 from total extracts of CTR and L2A(-) cells treated or not with OL. Representative blots of n=3 independent experiments. (e) Immunoblot for indicated proteins of IP of GAPDH (top) and IkB (bottom) from total extracts of CTR and L2A(-) cells. (f) Immunoblot for indicated proteins of IP of PLIN2 (top) and PLIN3 (bottom) from total extracts of CTR and L2A(-) cells treated with the indicated increasing concentrations of OL. These blots are an extension of those shown in Figure 3e and f to show the dose-dependence effect. Representative blots of n=3 (PLIN3) and 4 (PLIN2) independent experiments. Values are mean ± SEM. Differences are significant for *P<0.05, **P<0.01, ***P<0.001 using Student’s t-test. Uncropped images of blots are shown in Supplementary Figure 8. Source data is available in Supplementary Table 1.
Supplementary Figure 4 Inhibition of PLIN2 degradation by CMA leads to lipid droplet accumulation. (a) Immunostaining for hsc70 in OL-treated cells expressing WT or MT PLIN2-GFP. Right: 3D-reconstruction of the marked regions of the images shown on left. See also Supplementary Video 1. (b) Right: Serial Z-sections of the marked region of CTR cells expressing MT PLIN2-GFP shown on left.
Supplementary Figure 5  Altered cytosolic and lysosomal lipolysis in CMA-incompetent cells. (a) Coimmunostaining for PLIN2 and ATGL in CTR and L2A(-) cells untreated or treated with OL or incubated with serum-supplemented (OL>S+) or serum-deprived (OL>S-) medium after OL treatment. Insets: Higher magnification areas. Right: Images of the same fields with colocalized pixels shown in white. Graph: percentage colocalization of PLIN2 with ATGL. n=5 independent experiments with 40 cells per condition in each experiment. (b) Costaining for BODIPY493/503 and ATGL in CTR and L2A(-) cells incubated or not in OL>S+ regular or low glucose media. Bottom: Higher magnification areas. Graph: percentage colocalization of BODIPY with ATGL. n=5 independent experiments with 40 cells per condition in each experiment. (c) Immunoblot for ATGL of homogenates (HOM) and lipid droplets (LD) isolated from starved wild-type (+) or L2A knockout (-) mice livers (these 3 additional blots support the reproducibility of the blot shown in Fig. 6i). (d) BODIPY493/503 staining in CTR and L2A(-) cells untreated or treated with OL, or treated with 3-methyladenine (3MA) or lysosomal inhibitors ammonium chloride and leupeptin (NL). Higher magnification insets and quantification are shown in Fig. 7a. (e) Immunoblot for LC3 of total cell lysates from CTR and L2A(-) cells treated with OL in the presence or absence of 3-methyladenine (3MA). n=3 independent experiments. (f) Immunostaining for LC3 in OL-treated CTR and L2A(-) cells. Graph: average number of LC3 puncta/cell. n=6 independent experiments with 30 cells per condition in each experiment. Values are mean ± SEM. Differences are significant for **P<0.01, ***P<0.001 using Student’s t-test. Uncropped images of blots are shown in Supplementary Figure 8. Source data is available in Supplementary Table 1.
**Supplementary Figure 6** Failure to remove PLINs by CMA alters association of macroautophagy proteins with LD. (a, b) Costaining for BODIPY 493/503 and the indicated macroautophagy proteins in CTR cells treated or not with OL. Colocalized pixels are in white. Graph: Percentage colocalization with BODIPY. n=3 independent experiments with 40 cells per condition in each experiment. (c-f) Costaining for BODIPY493/503 and indicated macroautophagy proteins in CTR and L2A(-) cells treated with OL. Right in c: Colocalized pixels of the boxed area at higher magnification. e,f: Colocalized pixels are shown. Graphs: percentage colocalization with BODIPY. n=6 (d), 7 (e), 3(f) independent experiments with 30 (d) 40 (e), 10 (f) cells per condition in each experiment. Values are mean ± SEM. Differences are significant for *P<0.05, **P<0.01, ***P<0.001 using Student’s t-test. Source data is available in Supplementary Table 1.
Supplementary Figure 7 Failure to remove PLINs by CMA blocks macrolipophagy. (a) Live-cell imaging of cells cotransfected with wild-type (WT) or CMA-mutant (MT) PLIN2-GFP and dsRed-LC3 and treated with OL. Sequential frames at 30s intervals are shown. Arrows: LD. See also Supplementary Video 4. (b) Immunostaining for ATG5 (top) or Beclin1 (bottom) in cells expressing WT or MT PLIN2-GFP treated with OL. Higher magnification show colocalized pixels in white. Graph: percentage colocalization PLIN2-GFP. n=5 independent experiments with 40 cells per condition in each experiment. (c) Immunostaining for LC3 in OL-treated cells expressing WT or MT PLIN2-GFP. Right: 3D-reconstruction of the images shown on left. See also Supplementary Video 5. (d) Electron microscopy of CTR or L2A(-) cells maintained in serum-deprived media with or without OL. Several fields are shown. Insets on the right show details of different morphological features previously attributed to macrolipophagy and that include: LD (blue arrows; no limiting membrane); where a limiting membrane appears to be forming on the LD surface (yellow arrows); vesicles (putative autolysosomes) containing cargo compatible with lipids (red arrows; limiting membrane) and LD with membranes that originate from their surface toward the core of the LD (green arrows), previously described to correspond to LC3-positive limiting membranes. Micrographs originate from 3 independent experiments. Values are mean ± SEM. Differences are significant for ***P<0.001 using Student’s t-test. Source data is available in Supplementary Table 1.
Supplementary Figure 8 Images of the uncropped immunoblots with molecular weight markers of the blot data shown in the main and the supplementary figures. Boxes indicate cropped regions. Molecular weight markers are color coded according to the key shown on the bottom right.
Supplementary Figure 8 continued
Supplementary Table Legends

Supplementary Table 1  Statistics source data for main and supplementary figures. Values from individual experiments used to perform the statistic analysis presented in main and supplementary figures. Gray boxes denote figure number and panel. # indicates independent experiment number. Units are indicated on top of each subset of data.

Supplementary Videos Legends

Supplementary Video 1  3D-reconstruction of WT or MT PLIN2 and hsc70. 3D-reconstruction of fluorescence stacks of NIH3T3 cells transfected with wild type (WT) or CMA-motif mutated (MT) PLIN2-GFP and immunostained for hsc70. Left: progression from bottom to top. Right: planar rotation.

Supplementary Video 2  Dynamics of WT or MT PLIN2 in NIH3T3 cells. Time-lapse microscopy of NIH3T3 cells transfected with wild type (WT) or CMA-motif mutated (MT) PLIN2-GFP. Green channel shown. Arrows point to lipid droplets.

Supplementary Video 3  Dynamics of WT or MT PLIN2 and LAMP1 in NIH3T3 cells. Time-lapse microscopy of NIH3T3 cells co-transfected with wild type (WT) or CMA-motif mutated (MT) PLIN2-GFP and with LAMP1-RFP. Merged channels are shown. Right shows details at higher magnification. Arrows point to lipid droplets.

Supplementary Video 4  Dynamics of WT or MT PLIN2 and LC3 in NIH3T3 cells. Time-lapse microscopy of NIH3T3 cells co-transfected with wild type (WT) or CMA-motif mutated (MT) PLIN2-GFP and with dsRed-LC3. Merged channels are shown. Right shows details at higher magnification.

Supplementary Video 5  3D-reconstruction of WT or MT PLIN2 and LC3. 3D-reconstruction of fluorescence stacks of NIH3T3 cells transfected with wild type (WT) or CMA-motif mutated (MT) PLIN2-GFP and immunostained for LC3. Left: progression from bottom to top. Right: planar rotation.