Novel steps in the autophagic-lysosomal fluxes of phagophores and cytosolic cargo

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3MA, 3-methyladenine; LC3, microtubule-associated protein 1 light chain 3; LDH, lactate dehydrogenase; TG, thapsigargin

Running title
Multiple novel, early autophagic-lysosomal steps

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Autophagy is the process by which portions of cytoplasm are enclosed by membranous organelles, phagophores, which deliver the sequestered cytoplasm to degradative autophagic vacuoles. Genes and proteins involved in phagophore manufacture have been extensively studied, but little is known about how mature phagophores proceed through the subsequent steps of expansion, closure and fusion. Here we have addressed these issues by combining our unique autophagic cargo sequestration assay (using the cytosolic enzyme LDH as a cargo marker) with quantitative measurements of the lipidation-dependent anchorage and turnover of the phagophore-associated protein LC3. In isolated rat hepatocytes, amino acid-starved to induce maximal autophagic activity, the two unrelated, reversible autophagy inhibitors 3-methyladenine (3MA) and thapsigargin (TG) both blocked cargo sequestration completely. However, whereas 3MA inhibited LC3 lipidation, TG did not, thus apparently acting at a post-lipidation step to prevent phagophore closure. Intriguingly, the resumption of cargo sequestration seen upon release from a reversible TG block was completely suppressed by 3MA, revealing that 3MA not only inhibits LC3 lipidation, but also (like TG) blocks phagophore closure at a post-lipidation step. 3MA did not, however, prevent the resumption of lysosomal LC3 degradation, indicating that phagophores could fuse directly with degradative autophagic vacuoles without carrying cytosolic cargo. This fusion step was clearly blocked by TG. Furthermore, density gradient centrifugation revealed that a fraction of the LC3-marked phagophores retained by TG could be density-shifted by the acidotropic drug propylamine along with the lysosomal marker, cathepsin B, suggesting physical association of some phagophores with lysosomes prior to cargo sequestration.
Introduction

Autophagy was originally defined as the engulfment and transfer of cell fragments to lysosomes for degradation [1]. Such bulk sequestration of cytoplasm is now increasingly referred to as macroautophagy [2] to distinguish this process from the many examples of selective autophagy of cellular organelles and inclusions described more recently [3]. Macroautophagic activity can be measured quantitatively by the use of biochemical cargo sequestration assays [4], and in the present study we have used the cytosolic enzyme lactate dehydrogenase (LDH) as a macroautophagic cargo marker, this protein being sequestered non-selectively in the isolated rat hepatocytes that we are investigating [5]. However, since we also consider autophagic mechanisms that are independent of bulk cargo sequestration, we will henceforth mostly be using the more general term “autophagy”.

Ultrastructural, genetic and biochemical studies have defined a number of distinct steps in the autophagic-lysosomal pathway [3]. The organelles responsible for cargo sequestration, the phagophores [6], are assembled at various phagophore assembly sites or pre-autophagosomal structures (PAS) [3,7]. Here, membrane vesicles are assembled into flattened cisterns by the aid of numerous autophagy-related (Atg) proteins and equipped with receptors and presumably other proteins required for phagophore function. The final phase in phagophore assembly seems to be the conjugation of various members of the LC3 and GABARAP families to phosphatidylethanolamine in the phagophoric membrane (lipidation) [7].

In mammalian cells, the Atg8 family member LC3 has been shown to be involved in many selective autophagic processes [8], but it is also generally considered to be essential for macroautophagy. Its lipidation (converting the cytosolic form LC3-I into the phagophore-conjugated form LC3-II) as well as its transfer from a diffusely cytosolic to a punctate distribution (detectable by immunofluorescence microscopy) have, accordingly been widely used as indicators of autophagic activity in general [9]. However, we recently found that autophagic sequestration of cytosolic cargo in isolated rat hepatocytes could proceed very well without any apparent LC3 involvement [10], indicating that this receptor plays an auxiliary role, perhaps by adding some selectivity to an otherwise nonselective bulk sequestration [11]. Nevertheless, when anchored to phagophores, LC3 may serve as an informative marker of autophagic-lysosomal phagophore flux and help to clarify its relationship to autophagic-lysosomal cargo flux. We have, therefore, developed an LC3 immunoblotting strategy that allows quantitative measurements of both LC3-I and LC3-II [10]. Furthermore, we have used the protein synthesis inhibitor, cycloheximide, to block the synthesis of new LC3 molecules, thus simplifying the analysis of LC3 dynamics.

The mature phagophore envelops a portion of cytoplasm, eventually measurable as cargo sequestration upon phagophore closure to form a sealed vacuole, the autophagosome [12]. The autophagosome can fuse with an endosome to form an amphisome, a central autophagic vacuole that can receive repeated autophagosomal deliveries [13-15]. Whether
autophagosomes can also fuse directly with lysosomes is unclear. Some denaturation and contents processing can take place in amphisomes, but complete degradation of sequestered macromolecules requires fusion of amphisomes with lysosomes [15]. Various inhibitors have been found to inhibit different steps in this autophagic-lysosomal pathway: for example, 3-methyladenine (3MA) [16] and thapsigargin (TG) [17,18] suppress cargo sequestration, thus acting at, or prior to, the phagophore closure step; vinblastine and other microtubule poisons inhibits the fusion of autophagosomes with other autophagic/endocytic vacuoles [13,19]; asparagine [13] and leupeptin [14,20] suppress the fusion of amphisomes with lysosomes, and NH$_4$Cl, propylamine and other acidotropic amines [21] as well as bafilomycin A [22] inhibit intralysosomal degradation. However, it is likely that a more detailed investigation, e.g., into the possible sequential inhibitor effects on early steps, would provide more detailed information. In the present study, we have used inhibitors like 3MA, TG and NH$_4$Cl to perform block-release experiments with isolated rat hepatocytes. Although these inhibitors have multiple targets and effects in cells, their rapid, strong and, most importantly, reversible suppression of autophagic-lysosomal pathway activity has enabled us to uncover several novel steps in this pathway.

Results

Effects of inhibitors of the autophagic-lysosomal pathway on LC3 lipidation and degradation

The functional dynamics of the autophagy-related protein LC3 during autophagy was investigated in freshly isolated rat hepatocytes, which display a high autophagic activity when incubated in amino acid-free buffer; pyruvate being included to ensure an adequate energy supply [5,16,23]. The protein synthesis inhibitor, cycloheximide, was used to prevent influx of newly synthesized LC3, thus simplifying the analysis. Cycloheximide did not detectably impair general hepatocytic functionality during the experimental incubations used in the present study [10].

As shown in Fig. 1A, LC3-I rapidly disappeared in both control and cycloheximide-treated hepatocytes, being converted, by lipidation, to LC3-II, which increased during the first hour of incubation. The 4x more efficient immunostaining of LC3-II relative to LC3-I [10] gives a false impression of an increase also in total LC3 levels, which are actually declining [10]; see also the quantifications in Fig. 4A. The LC3-II levels subsequently remained relatively stable in control cells, reflecting an equilibrium between degradation and new synthesis of LC3, whereas a gradual decline was observed in the presence of cycloheximide, indicating that the protein was rapidly degraded. Lactacystin, an inhibitor of proteasomal protein degradation used here mainly as a control, did not interfere with either lipidation (the rapid decrease in LC3-I) or LC3 turnover (the decline in total LC3 protein levels during
cycloheximide treatment) when compared with control cells (Fig. 1B). Proteasomes would thus not appear to play any role in hepatocellular LC3 processing or degradation.

3MA, a well-established inhibitor of autophagic sequestration activity [16], effectively reduced the rapid fall in LC3-I both with and without cycloheximide, indicating a suppression of LC3 lipidation (Fig. 1C). This is as expected: As a general inhibitor of PI 3-kinases [24,25], 3MA would inhibit the Vps34-dependent assembly of phagophores, in which LC3 attachment by lipidation is the final step [7]. However, in addition, 3MA strongly suppressed the decline in LC3-II seen in the presence of cycloheximide, suggesting that the post-lipidation autophagic-lysosomal processing of already completed, LC3-conjugated phagophores was also dependent on PI 3-kinase activity. More than one 3MA-sensitive step would thus seem to be required for phagophore assembly and autophagosome formation.

In contrast, TG, a Ca2+ pump inhibitor [26] that effectively suppresses early autophagic sequestration (probably by depleting some intracellular Ca2+ store) [17,18], did not prevent LC3-I lipidation, but inhibited the turnover of LC3-II (Fig. 1D). The Ca2+-dependent step in autophagy is thus clearly localized after the LC3 lipidation step. TG would, therefore, be expected to cause an accumulation of mature phagophores, as previously suggested [18].

Vinblastine, a general inhibitor of microtubule-dependent processes (including the translocation of intracellular organelles), had no effect on LC3-I lipidation, but strongly suppressed LC3-II turnover (Fig. 1E). This is consistent with its known ability to block the autophagic-lysosomal flux at intermediate transport and fusion steps, and to cause autophagosome accumulation [19,27].

The weak bases ammonia (NH4Cl) and propylamine, which suppress proteolysis in lysosomes (and amphisomes) by raising the intravacuolar pH to neutrality [21,28,29], blocked the turnover of LC3-II effectively, while having no detectable effects on LC3-I lipidation (Fig. 1F-G). This was also the case with leupeptin (Fig. 1H), an inhibitor of amphisomal and lysosomal cysteine proteinases, and of amphisome-lysosome fusion [14,20].

Since inhibitors of early autophagic sequestration steps (3MA, TG), of intermediate fusion steps (vinblastine) and of late degradation steps (ammonia, propylamine, leupeptin) all suppress LC3 turnover, it seems clear that this protein is primarily degraded by the autophagic-lysosomal pathway in rat hepatocytes during the first few hours of cycloheximide treatment.

**Thapsigargin and other inhibitors do not disrupt lysosomes**

We previously reported that TG strongly inhibited autophagic cargo sequestration in isolated hepatocytes, and in a fully reversible manner [17]. It has nevertheless been suggested [30] that this effect of TG could be a methodological artefact, reflecting a drug-induced disruption of lysosomes and a consequent loss of sequestered cargo from the sedimentable cell fraction (cell corpses) used in our autophagy assay [5]. However, as shown in Fig. 2, neither TG nor
any other autophagy perturbant tested (3MA, vinblastine, okadaic acid, leupeptin, cycloheximide, propylamine) caused any detectable leakage of the lysosomal enzyme, cathepsin B, from the sedimentable cell corpses (CC) into the non-sedimentable cell sap (CS) fraction. Hepatocytic lysosomes (including the fully active autolysosomes present, e.g., after 100 min with cycloheximide) thus remained fully intact under all conditions. As a control, freeze-thawing of the cell disruptate prior to fractionation caused a complete release of cathepsin B from the cell corpse lysosomes into the cell sap fraction (upper left-hand panels).

A two-step mechanism for inhibition of autophagic sequestration by 3-methyladenine

The fact that 3MA, unlike TG, interfered with both LC3-I lipidation and LC3-II turnover (Fig. 1C) would suggest that 3MA inhibited autophagy at two different steps. This possibility was further examined by comparing the effects of 3MA and TG at the level of autophagic cargo (LDH) sequestration.

As shown in Fig. 3A, both 3MA and TG (tested in the absence of cycloheximide) almost completely inhibited macroautophagic cargo sequestration as measured by the accumulation of LDH in the presence of leupeptin [5]. Reversal experiments (preincubation with 3MA or TG, followed by washout and reincubation with leupeptin only), showed that both inhibitor effects were entirely reversible (Fig. 3B); in the case of 3MA there was even an overshoot indicative of autophagic capacity accretion during the preincubation. The reversibility of the 3MA effect makes it suitable for block-release experiments, unlike more potent, but irreversible, inhibitors of autophagic sequestration such as wortmannin [30].

Re-addition of inhibitors during the second incubation period again blocked LDH sequestration effectively, and with complete reciprocity (Fig. 3B). The ability of the post-lipidation inhibitor TG to prevent reversal of the 3MA effect is not surprising, but the ability of 3MA to prevent reversal of the TG effect was unexpected. This observation supports the contention made above, i.e., that 3MA inhibits autophagy both at the LC3 lipidation step and at a post-lipidation step. This second effect of 3MA could be exerted after, or at the TG-sensitive step, perhaps reflecting a dual involvement of PI(3)P and Ca^{2+} at this step.

The reciprocity between 3MA and TG was also evident in cycloheximide-treated cells (Fig. 3C). Both inhibitors strongly suppressed LDH sequestration during a 100-min incubation with cycloheximide (left-hand panel) as well as after a 200-min preincubation with cycloheximide. The reversal of sequestration after preincubation with 3MA, again with an overshoot (shown to be statistically significant), was prevented equally well by 3MA and TG, as was the reversal after preincubation with TG.

The post-lipidation effects of 3MA and TG on autophagic cargo sequestration thus reflect robust mechanisms that persist over time, even facing the absence of protein synthesis and rapidly declining LC3 levels. The strength of these effects would be compatible with post-
lipidation events being rate-limiting for the overall autophagic-lysosomal flux under many conditions.

**Autophagic-lysosomal LC3 flux analysed by block-release experiments**

To enable quantitative studies of autophagic-lysosomal LC3 flux in isolated hepatocytes, we employed a novel immunoblotting procedure that accurately detects and quantifies both the cytosolic form, LC3-I, and the lipidated, membrane-conjugated form, LC3-II [10]. Using cycloheximide to block new LC3 synthesis, the subsequent decline in total LC3 levels (LC3-I plus LC3-II) could be used as a measure of overall LC3 degradation, which proceeded with a half-life of ~2 h (Fig. 4A and ref. 10). As in the study of cargo sequestration (Fig. 3B), two consecutive 100-min incubations were used for the block-release experiments. The two LC3 forms were initially present at approximately equal levels in control cells (Fig. 4A), but LC3-I fell more rapidly during the first 100-min period due to its lipidation to LC3-II. In the second 100-min period, the two forms were apparently in equilibrium, and declined at similar rates (Fig. 4A).

3MA pretreatment for 100 min suppressed both LC3-I lipidation and overall LC3 degradation (Fig. 4B). However, these effects of 3MA were not as complete as the inhibition of cargo sequestration (Fig. 3C), allowing LC3 turnover to proceed at approximately 50% of the control rate (in Fig. 4A) during 200 min in the presence of 3MA (Fig. 4B). A highly significant autophagic-lysosomal flux of LC3 could thus be maintained in the absence of a bulk cargo flux.

The rapid resurgence of overall LC3 degradation upon 3MA removal (Fig. 4B, CTR 100-200 min) was completely prevented by TG (100-200 min), which would thus seem to be more effective than 3MA in blocking progression of the LC3 flux into the degradative part of the autophagic-lysosomal pathway. NH₄Cl, particularly in combination with 3MA, also suppressed the resurgence of LC3 degradation effectively (NH₄Cl + 3MA, 100-200 min). Remarkably, NH₄Cl eliminated the LC3-I-preserving effect of 3MA during the second incubation period, perhaps by suppressing LC3 recycling as discussed below.

After release from a TG block, NH₄Cl prevented the resurgence of LC3-II degradation as effectively as did re-added TG (without significantly affecting LC3-I levels), indicating that the observed degradation was accomplished by an autophagic-lysosomal mechanism. (Fig. 4C). In contrast, 3MA preserved cellular LC3-I, but allowed LC3-II degradation to proceed at essentially the same rate as in control cells (Fig. 4C). Since cargo sequestration was effectively inhibited by 3MA under these conditions (Fig. 3B) the degradative autophagic-lysosomal LC3 flux could clearly proceed independently of autophagic-lysosomal cargo flux. Presumably, this means that not only cargo-filled autophagosomes, but also phagophores that do not carry any detectable cytosolic cargo can fuse with amphisomes/lysosomes to have their associated LC3-II degraded.
While both 3MA and TG prevented autophagosome formation (Fig. 3), the subsequent fusion step was apparently inhibited by TG only (Fig. 4C). TG is probably capable of inhibiting the fusion of both autophagosomes and phagophores with amphisomes/lysosomes, but it has not yet been possible to test this possibility independently of the drug’s effective prevention of phagophore closure.

During preincubation with NH₄Cl, LC3 was lipidated as efficiently as in control cells, but little LC3 degradation took place (Fig. 4D). Upon reincubation, LC3-II (presumably residing in lysosomes) was rapidly degraded under NH₄Cl-free conditions, but protected by re-added NH₄Cl, whereas the LC3-I level remained essentially unaltered. The LC3-II degradation that took place after release from NH₄Cl inhibition was also reduced by TG (Fig. 4D), which was unexpected considering the prelysosomal point(s) of action of this drug. One possible explanation might be that a fraction of the LC3 recycles from amphisomes/lysosomes, and that TG prevents its re-entry during a second autophagic-lysosomal round. This possibility is supported by the effect of 3MA, which caused a significant net increase in LC3-I following release from the NH₄Cl block (Fig. 4D). Since there cannot be any de novo formation of LC3 in the presence of cycloheximide, new LC3-I can only come from delipidated, recycled LC3-II. In the continued presence of NH₄Cl, 3MA did not cause any LC3-I accumulation, which might reflect an ability of NH₄Cl to suppress LC3 recycling. Presumably, some recycling (delipidation–relipidation) of LC3 also takes place during normal (inhibitor-free) operation of the autophagic-lysosomal pathway in rat hepatocytes.

**Some open phagophores are physically associated with amphisomes or lysosomes**

Since TG would be expected to cause an accumulation of mature phagophores, a density gradient analysis might provide some information about the properties of these elusive organelles, using the autophagic membrane marker, LC3-II, for their detection. A subcellular fractionation of homogenized cell corpses on isotonic iodixanol gradients showed that the initial density distribution of LC3-II was biphasic, most of the marker distributing broadly in the dense region (1.08-1.11 g/ml), but with a minor fraction at 1.05 g/ml (Fig. 5A). The lysosomal marker, cathepsin B exhibited a distribution overlapping with that of LC3-II, but extending further into the denser region of the gradient (Fig. 5A).

After 100 min of incubation with cycloheximide under autophagy-promoting (i.e., amino acid-free) conditions, the cathepsin B marker had moved conspicuously from the dense to the light region of the gradient (Fig. 5B), indicating that small, dense lysosomes now had turned into large, light autolysosomes as they became engaged in autophagic-lysosomal degradation of cytoplasm [32]. Although the reduced levels of LC3-II at this time made its detection quite difficult, it would seem that no significant change in the LC3-II distribution took place. The relative paucity of LC3 in the major cathepsin B-containing fractions might indicate that little
or no LC3-II was associated with the active autolysosomes, probably a reflection of its rapid intralysosomal degradation.

TG largely prevented the lysosomal density shift, in accordance with its ability to suppress autophagy (Fig. 5C). LC3-II, now preserved and easily detectable, retained its initial distribution, which would, presumably, reflect the actual density distribution of mature phagophores. Upon release from the TG block (Fig. 5D), the lysosomes (cathepsin B) shifted to the low-density (active) position, whereas LC3-II largely disappeared, reflecting its autophagic-lysosomal degradation. These changes were completely prevented by the re-addition of TG (Fig. 5E).

Having thus established the dynamic properties of the phagophore population preserved by TG, the acidotropic drug propylamine [21] was added to shift the lysosomal density independently of autophagic-lysosomal pathway activity. As shown in Fig. 5F, propylamine (in the presence of TG) effectively shifted the whole lysosome population (cathepsin B) to lower densities. Surprisingly, a significant fraction of the LC3-II was light-shifted as well. This propylamine-induced LC3-II density shift would suggest that some of the phagophores accumulating under TG treatment were physically associated with acidic vacuoles (lysosomes or amphisomes).

Discussion

Binding of LC3 to the phagophore, a final step in phagophore assembly, is accomplished by the conjugation of LC3 to phosphatidylethanolamine in the phagophore membrane (“LC3 lipidation”), catalysed by Atg12-Atg5-Atg16L complexes [7,34]. Proper functioning of the phagophore assembly line is dependent on PI(3)P anchors, which serve to recruit Atgs, WIPIs and other involved proteins to the phagophore assembly site (PAS) and to the developing phagophore [34,35]. PI 3-kinase inhibitors like 3MA [24] accordingly disrupt phagophore assembly and hence the final LC3 lipidation. However, as shown in the present study, 3MA also inhibits autophagic sequestration (phagophore closure) at a second, later step (after LC3 lipidation and after or at the first TG-sensitive step), which may contribute to its strong suppression of overall macroautophagy [16]. Interestingly, studies of 293A cells [36] and of HeLa cells [37] have indicated that GABARAP family members rather than LC3 family members are required for phagophore closure, at a step downstream of LC3 recruitment/lipidation. In contrast, no such requirement was found in mouse embryo fibroblasts [38]. In rat hepatocytes and LNCaP human prostate cancer cells, we have recently shown that macroautophagic cargo sequestration can proceed without any involvement of LC3, but rather is dependent on Atg8 orthologues belonging to the GABARAP family [10]. The latter may well be required at the 3MA-sensitive closure step. However, although LC3 does not serve any essential function in macroautophagy, it can be a useful marker of autophagic organelle flux and localization as shown in the present study.
TG, a SERCA Ca\(^{2+}\) pump inhibitor [26], suppresses autophagy very effectively [17,18] by blocking an early post-lipidation step prior to phagophore closure. A fraction of the TG-arrested phagophores could be density-shifted by propylamine (Fig. 5F), and were thus apparently physically associated with acidic vacuoles. This may reflect an ability of some phagophores to dock to their future fusion partner already before closure. It is also conceivable that phagophores may actually be assembled *de novo* at the surface of an acidic vacule that functions as a PAS, and that at least some of them may complete the cargo sequestration process without ever leaving their site of origin [6]. Involvement of the lysosome in the control of early steps in autophagy is not without precedent, since the proton pump in the lysosomal membrane has been implicated as a mediator of amino acid signalling to the autophagy-suppressive mTORC1 complex [39].

Although 3MA effectively prevents bulk cytosolic cargo sequestration, the inhibitor nevertheless allows a significant degradative autophagic-lysosomal flux of LC3 (Fig. 4B). Furthermore, phagophores released from a TG block in the presence of 3MA remain completely unable to perform bulk sequestration (Fig. 3C), but are nevertheless capable of shipping LC3-II into the autophagic-lysosomal pathway for degradation (Fig. 4C). These results strongly suggest that phagophores can traverse the autophagic-lysosomal pathway without carrying cytosolic cargo.

Notably, TG could effectively prevent the resumption of LC3 flux after release from a 3MA block (Fig. 4B). These observations indicate the existence of a second TG-sensitive step, beyond 3MA-sensitivity, apparently required for the fusion of phagophores with amphisomes or lysosomes. It was recently reported that TG could inhibit the fusion of autophagosomes with lysosomes (or conceivably with amphisomes) in mouse embryonic fibroblasts [41], but without a bulk cargo assay it is difficult to distinguish between autophagosomes and phagophores that have not yet closed. Given the complete suppression of bulk cargo sequestration at the first TG-sensitive step in a number of different cell types [17,18], TG-arrested autophagic organelles are most likely to be phagophores. However, beyond this point it is quite plausible that the second TG-sensitive step may be required for autophagosomes as well as for phagophores to fuse with amphisomes/lysosomes.

How phagophores enter amphisomes/lysosomes is a matter of conjecture. One possibility is that they fuse as open phagophores, being internalized by a subsequent microautophagy-like invagination and vesiculation [41]. Alternatively, the phagophores may close before fusion in such a manner as to exclude surrounding cytosol. This has been suggested to be the case in so-called “selective-exclusive” autophagy, as *in vitro* reconstitution studies have indicated that cargo receptors may mediate a very tight apposition between the phagophore membrane and the sequestered cargo [42]. It is well established that in addition to its proposed role as a phagophore expander [37,43], LC3 is involved rather ubiquitously as a cargo recruiting protein in selective autophagy, binding various types of cargo (organelles, infectious agents, protein aggregates) through receptors like p62, NBR1, Nix etc. [8, 44-46].
During normal macroautophagy, phagophoric LC3 may conceivably add some selectivity to an otherwise nonspecific bulk cargo sequestration. The propensity of many variant forms of cytosolic proteins to bind selectively to phagophoric/autophagosomal membranes [11] would be consistent with such a possibility. Inhibition of bulk cargo sequestration may still allow the phagophores to scavenge specific cargo and carry it into lysosomes for degradation (along with LC3), thus possibly acting as a switch between a nonselective and a form of selective-exclusive autophagy.

Materials and Methods

Reagents
The polyclonal antibody used for immunoblotting of LC3 was made to order by BioGenes [10], whereas the cathepsin B antibody was a kind gift from David Buttle (Sheffield, UK). Supersignal® for amplification of LC3 staining was purchased from Thermo Scientific Pierce (#34094); Iodixanol (Optiprep, #1114542) and Nycodenz (#1002424) from Axis-Shield. Other chemicals were obtained as previously described [20].

Animals and cells
Isolated rat hepatocytes were prepared by two-step collagenase perfusion [47] of livers from 18-h starved male Wistar rats (250-300 g; Harlan UK Ltd.). The livers were excised under 4% isoflurane anesthesia, and the animals euthanized by bleeding while anesthetized. The use of animals was approved by, and in accordance with the ethical guidelines of The Norwegian National Animal Research Authority and FELASA (Federation of European Laboratory Animal Science Associations). The cells were suspended in buffered saline (suspension buffer) [47] containing extra Mg²⁺ (2 mM final conc.) and 15 mM pyruvate, and incubated at 37°C as 2-ml aliquots (~20 mg wet mass) in 6-cm suspension dishes (Sarstedt #83.1801.002) gently shaken on a rocking platform. All incubations were done in amino acid- and serum-free buffer (amino acid starvation conditions) in order to induce maximal autophagic activity.

Cell disruption and subcellular fractionation
After incubation, the suspended hepatocytes were cooled to 0°C and washed twice (sedimented at 1,400 rpm for 5 min, resuspended in 4 ml) with electrolyte-free, isotonic (10%) sucrose for subcellular fractionation, or with phosphate-buffered saline (PBS) for direct lysis and immunoblotting.

For subcellular fractionation, six pooled parallel samples in 2.4 ml sucrose were electrodisrupted [48] by a single high-voltage pulse (2 kV/cm; 1.2 μF). The total cell disruptate (TOT) was either used directly, or separated (by low-speed centrifugation at 4,000 g for 30 min/4°C above an 8% buffered, isotonic Nycodenz cushion) into a "cell corpse" (CC) fraction that contained all sedimentable cellular components, and a nonsedimentable "cell sap" (CS) fraction [5]. If no further subcellular fractionation was to be undertaken, the
fractions were adjusted to equivalent volumes and buffer composition (10% sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.3), and, if required, frozen and stored at -70°C.

For density gradient fractionation, freshly prepared cell corpse pellets were homogenized with 1.5 ml ice-cold homogenization buffer (HB; 0.25 M sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.3). The homogenate was layered on top of an isotonic 11-ml gradient of 0-30% iodixanol (Optiprep) in HB. The gradient was centrifuged in a Sorvall SW40 rotor at 4°C for 20 min at 5,000 rpm and for 3 h at 40,000 rpm, then left overnight at 4°C. Twenty fractions (~0.63 ml each) were collected by upwards displacement by a dense fluid (Purdenz) and the fraction densities determined by refractive index measurements. A 200-µl aliquot from each fraction was mixed with 50 µl of 5x concentrated lysis buffer and lysed as outlined above, the lysate was concentrated to 100 on a spin column, and 8 µl of this concentrate was used for SDS-PAGE/immunoblotting.

**Cargo sequestration assay**

Autophagic cargo sequestration activity was measured as the ability of hepatocytes to transfer the cytosolic cargo marker LDH into sedimentable cell corpses, which contain all autophagic organelles in an intact state [4,5]. Maximal autophagic activity was ensured by incubating cells at 37°C in amino acid-free, pyruvate/Mg²⁺–fortified suspension buffer with leupeptin (0.3 mM) to prevent degradation of the sequestered LDH [5]. LDH was quantified by an enzymatic assay as previously described [4,5], using a multi-analyzer (MaxMat PL-II) from Erba Diagnostics. The net amount of LDH sequestered into cell corpses during the incubation period with leupeptin was expressed as per cent (or %/h) of the total cellular LDH measured in the disruptate. It should be noted that modified versions of the LDH sequestration assay can apparently be applied to any cell type [10,18], as recently described in detail [49].

**Immunoblotting**

For lysis and immunoblotting, each cell pellet (~20 mg) was dissolved in 700 µl of lysis buffer (final concentrations, 1% SDS, 5 mM EDTA, 5 mM EGTA, 10 mM sodium pyrophosphate, 0.3 mM leupeptin, 10 µM pepstatin A, 15 µM E-64, 50 µM bestatin, 0.8 µM aprotinin, 3 mM 4-(2-aminoethyl)benzenesulfonyl fluoride and 20 mM Tris, pH 7.2), was added to each cell pellet, and after lysis for 1 h at 0°C the samples were frozen and stored at -70°C. Before SDS electrophoresis, each lysate was sonicated for 10 s and a 100-µl aliquot was mixed with 20 µl of 6x concentrated sample buffer (final concentrations, 2% SDS, 100 mM DTT, 0.002% bromophenol blue, 5% glycerol and 50 mM Tris-HCl, pH 6.8). Eight µl of this mixture was used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Note that the sample heating step (5 min at 95°C; routinely used in immunoblotting to reduce nonspecific background staining) was omitted from our procedure in order to eliminate cross-staining with mitochondrial nucleoside diphosphate kinase, which co-banded in the position of LC3-I at 17 kDa [10].
SDS gel electrophoresis was performed using Criterion™ precast gels (567-1125, Bio-Rad Laboratories) with an associated midi-tank system (Criterion™ Cell). Gels were routinely run at 200 V for 45 min with 8-μl samples (~20 μg protein) in sample buffer. The running buffer contained 0.1% SDS, 192 mM glycine and 25 mM Tris-HCl. Molecular mass standards were included in all gels (Novex® Sharp, LC5800, Life Technologies). The separated proteins were transferred onto PVDF blotting membranes (ISEQ00010, Millipore) using a wet-blotting cell (Criterion™) at 100 V for 60 min and a CAPS transfer buffer (10 mM CAPS buffer, pH 11, with 2% ethanol). Following transfer, membranes for detection of LC3 were fixed in 100% methanol for 3 min and then washed in mqH2O and TBS-T (0.1% Tween 20, 0.8% NaCl and 20 mM Tris-HCl, pH 7.6) before blocking. The membranes were blocked by treatment with 5% (w/v) dry milk in TBS-T for 1 h, then incubated overnight at 4ºC with the respective primary antibodies, usually diluted with TBS-T containing 5% (w/v) dry milk. After washing three times with TBS-T, the membranes were incubated for 1 h at room temperature with the respective secondary antibodies conjugated to horseradish peroxidase in TBS-T/dry milk, and finally washed three more times with TBS-T.

Visualization of bound secondary antibodies was accomplished by use of the Immobilon Western Chemiluminescent HRP Substrate (WBKLS0500, Millipore) or, for increased sensitivity, the Supersignal® west Femto chemiluminescent HRP substrate (34096, Thermo Fisher Scientific). Chemiluminescent signals from immunoblots were detected and quantified using the ChemiGenius 3 imaging system (Syngene, Cambridge, UK) and the associated software, GeneSnap and GeneTools. Proprietary image files were converted to TIF-file format for image presentation using the Adobe Photoshop software. In the case of LC3, the values obtained for the (unlipidated) LC3-I form were routinely multiplied by four to allow a valid quantitative comparison with the more immunoreactive (lipidated) form LC3-II, as previously described [10].

**Protein measurement**

Total protein was measured by the biuret method on a MaxMat PLII multi-analyzer (Erba Diagnostics). Absorbance was read at 550 nm wavelength using the accompanying Total Protein kit (RMPROT0125V).

**Acknowledgement**

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References


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**Figure legends**

**Fig. 1.** Effects of autophagic-lysosomal pathway inhibitors on LC3 lipidation and turnover in cycloheximide-treated hepatocytes. Hepatocytes were incubated at 37°C for up to 4 h with or without cycloheximide (CHX; 100 µM) and (A), no other inhibitors, or with the additional presence of (B), lactacystin (100 µM); (C), 3-methyladenine (10 mM); (D), thapsigargin (5 µM); (E), vinblastine (50 µM); (F), NH₄Cl (20 mM); (G), propylamine (10 mM) or (H), leupeptin (0.3 mM). After incubation, the cells were electrodisrupted, lysed in SDS buffer, and the whole-cell extracts were immunoblotted with an anti-LC3 antibody.

**Fig. 2.** Effects of thapsigargin and other inhibitors on lysosomal integrity. Hepatocytes were incubated for up to 2 h at 37°C with thapsigargin (TG; 5 µM), 3-methyladenine (3MA; 10 mM), vinblastine (VBL; 50 µM), okadaic acid (OA; 100 nM), leupeptin (0.3 mM), cycloheximide (CHX; 100 µM) or propylamine (PAM; 10 mM) as indicated. After incubation, the cells were electrodisrupted, and the protein levels of the lysosomal enzyme, cathepsin B, were measured by immunoblotting in extracts of the total disruptate (TOT), sedimentable cell corpses (CC) or nonsedimentable cell sap (CS). None of the treatments caused any detectable leakage of cathepsin B into the cell sap fraction. As a control, deliberate disruption of lysosomal membranes by freeze-thawing of control (CTR) cell disruptates did indeed cause the appearance of cathepsin B in the CS fraction as well as its corresponding disappearance from the CC fraction (top panel, second from the left).

**Fig. 3.** A novel post-lipidation effect of 3-methyladenine blocks the resumption of autophagic cargo sequestration after release from a thapsigargin block. (A), Hepatocytes were incubated with leupeptin (0.3 mM) for up to 100 min at 37°C without further additions (○), with 5 µM thapsigargin (TG; ●) or with 10 mM 3-methyladenine (3MA; ■). (B), After 100 min of incubation, control cells (○), 3MA-pretreated cells (□) or TG-pretreated cells (△) were washed and reincubated for another 100 min with leupeptin only. Alternatively, 3MA-pretreated cells were also given 3MA (■) or TG (▼) during the reincubation, or TG-pretreated cells were also given 3MA (▼) or TG (▲). After incubation, the cells were electrodisrupted and the net amount of LDH sequestered into sedimentable cell corpses during the preceding 100 min was measured and expressed as % of the total cellular amount of LDH. Values represent single experiments or the means of 2-3 experiments. (C), left-hand panel: Hepatocytes were incubated for 100 min at 37°C with cycloheximide (CHX; 100 µM) and leupeptin (LPT; 0.3 mM) without or with the additional presence of 3MA or TG as indicated (open bars). Right-hand panel: Hepatocytes were preincubated for 200 min with CHX only (dotted bars), or with CHX plus 3MA (cross-hatched bars) or TG (hatched bars); then reincubated for another 100 min with CHX and LPT with no further additions (CTR), or with the additional inclusion of 3MA or TG as indicated. After incubation, the net sequestration of LDH during the last 100 min was measured and expressed as % of the total cellular amount of LDH per hour. Each value is the mean of two experiments, or the mean ± S.E. of 8-9 experiments as indicated in parentheses.
Fig. 4. Turnover, lipidation and reutilization of LC3 upon release from autophagic-lysosomal block points. Hepatocytes were incubated for two consecutive 100-min periods at 37°C in buffered saline with pyruvate (15 mM) and cycloheximide (100 µM) only (CTR), or with the additional inclusion of the reversible autophagic-lysosomal inhibitors 3-methyladenine (3MA, 10 mM), thapsigargin (TG, 5 µM) and NH₄Cl (20 mM) as indicated. The cells were washed between the two incubation periods. (A), no block imposed during the first 100 min; (B), 3MA present during the first 100 min; (C), TG present during the first 100 min; (D), NH₄Cl present during the first 100 min. After incubation, whole-cell extracts were immunoblotted with an anti-LC3 antibody, and comparable quantifications of LC3-I (open bars) and LC3-II (dotted bars) were performed. The values in each treatment group are expressed as % of their respective 0-min totals ± S.E. (error bars extending upwards for total LC3 and for LC3-I; downwards for LC3-II) of the number of independent experiments given in parentheses at each bar. *P<0.05; **P<0.01; ***P<0.001 vs. the 200-min control in the same treatment group according to the t-test. ¤ P<0.05 vs. 100 min TG (C) or 100 min NH₄Cl (D); ¤¤ P<0.01 vs. 200 min 3MA (C); ¤¤¤ P<0.001 vs. 0 min (B).

Fig. 5. Effects of thapsigargin and propylamine on the density distributions of autophagic organelles. Hepatocytes were incubated at 37°C with cycloheximide (CHX, 100 µM) and thapsigargin (TG, 5 µM) or propylamine (PAM, 10 mM) as indicated. After incubation and electrodisruption, cell corpse homogenates were fractionated on isotonic 0-30 % iodixanol density gradients and the fractions were immunoblotted for the cathepsin B or LC3-II. (A), Non-incubated hepatocytes; (B), cells incubated for 100 min with CHX; (C), cells incubated for 100 min with TG + CHX; (D), cells preincubated 100 min with TG + CHX, then reincubated for another 100 min with CHX only; (E), cells preincubated and reincubated with TG + CHX; (F), as in (E), but with PAM included during the reincubation (which causes density-shifting of all cathepsin B and part of the LC3-II).
Fig. 1
**LYSOSOMAL INTEGRITY (CATEPSIN B SEDIMENTABILITY)**

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**Fig. 2**
WITH CYCLOHEXIMIDE

A) NOT PREINCUBATED

B) PREINCUBATED - LPT

REINCUBATED + LPT

C) WITH CYCLOHEXIMIDE

Fig. 3
Fig. 4
Fig. 5