Published in final edited form as:

Biochim Biophys Acta Mol Cell Res. 2018 September ; 1865(9): 1356–1367. doi:10.1016/j.bbamcr.2018.06.014.

Glutamine-dependent lysosome homeostatic changes induced by starvation and lysosome inhibition

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Abstract

Lysosomes are a major organelle for degrading macromolecules. When deprived of nutrients, cells activate the autophagy and lysosome biogenesis pathways to recycle cytoplasmic materials and to increase lysosomal degradation capacity for survival, respectively. We have identified a condition in which cells accumulated enlarged lysosomes upon starvation and lysosome inhibition. Selective autophagy and inhibition of the mechanistic target of rapamycin (mTOR) in combination with lysosome inhibition were not able to induce this phenomenon. Conversely, knocking out autophagy genes, ATG5 or ATG7, had no effects on the enlarged lysosome formation. This suggests that the enlarged lysosome formation is an autophagy independent process. Remarkably, adding glutamine to the treatment can prevent formation of the enlarged lysosomes and dissipate the pre-existing ones. Furthermore, the nucleus/cytoplasm translocation of the transcription factor EB (TFEB), but not mTOR activity, correlates with the formation/dissipation of enlarged lysosomes. Knockdown of TFEB, however, suggests that TFEB-mediated lysosome biogenesis is not directly involved in the process. These results indicate that there is a novel mechanism by which lysosome homeostasis can be regulated under certain stress conditions.

Keywords

glutamine; TFEB; mTOR; autophagy; lysosomes

Introduction

The lysosome is the major degradation and recycling center in the cell. It degrades macromolecules that are delivered from endocytic and autophagic pathways. Recent studies have shown a direct involvement of lysosomes in nutrient sensing and the regulation of important signaling pathways that control cellular homeostasis [1–3].

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Competing interests

The authors declare no competing financial interests.

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One of the conditions that trigger cells to activate the autophagy pathway is starvation. When nutrients are available, the mechanistic target of rapamycin complex 1 (mTORC1) situates on the lysosomal surface, where it activates various pathways that result in cell growth and proliferation [4]. On the other hand, when nutrients are scarce, the mTOR kinase becomes inactive. Dephosphorylation of one of the mTOR substrates, ULK1, initiates the autophagy pathway [5, 6]. During the autophagic process, cytoplasmic materials destined for degradation are sequestered into newly-formed autophagosomes, which then fuse with lysosomes to form autolysosomes where the recruited materials are degraded [7].

In addition to initiating autophagy, starvation can also activate lysosome biogenesis through the transcription factor EB (TFEB). TFEB is a master regulator of lysosome biogenesis and autophagy [8, 9]. The nuclear translocation and consequent transcription factor activity of TFEB are dependent on its phosphorylation status, which requires timely coordination between the kinases and phosphatases [9, 10]. Inactivation of mTOR leads to dephosphorylation of the transcription factor EB (TFEB) by the phosphatase calcineurin [11]. The dephosphorylated TFEB translocates to the nucleus and up-regulates genes involved in lysosome biogenesis and autophagy.

One of the common approaches for studying autophagy is to monitor autophagic flux by comparing the starvation response in the absence or presence of lysosome inhibitors [12, 13]. Several reagents have been used as lysosome inhibitors, most of them owing to their property of neutralizing lysosomal pH. For instance, lysosomotropic weak bases, such as chloroquine (CQ), can raise the pH of lysosomes; V-ATPase inhibitors, such as bafilomycin A (baf A), block the proton pump activity and consequently raise lysosomal pH. Large vacuoles have been observed in Chinese hamster ovary cells upon nutrient deprivation and lysosome inhibition by various reagents, such as CQ and baf A [14]. In this report, we describe a similar morphological change of lysosomes upon starvation and lysosome inhibition in several cell lines. Enlarged lysosomes were observed when cells were incubated with a stringent starvation condition (without amino acids or serum) in the presence of CQ. Further investigation revealed this phenomenon is autophagy-independent and glutamine-dependent.

Results

While investigating starvation-induced autophagy, we identified a condition that causes the formation of enlarged vacuoles. When cells were incubated with Hank's balanced salt solution (HBSS) and chloroquine (CQ), we observed that large vacuoles accumulated in the perinuclear region of the cytoplasm (Fig 1A, HBSS+CQ). Cells incubated with HBSS or CQ alone did not induce such cellular structures. This phenomenon was observed in different cell lines, including human skin fibroblasts, HeLa (Fig 1A) and COS7 cells (Fig S1). HBSS is one of the most commonly used solutions for stringent starvation treatment, whereas CQ is a weak base commonly used for neutralizing acid compartments and, as a consequence, inhibiting acid hydrolase activities in lysosomes. No previous reports have focused on this phenomenon, therefore we set out to further investigate this induced structure.

To understand the identity of these enlarged vacuoles, we immunostained the cells with various organelle markers. As shown in Fig 1B, the lysosomal membrane protein, Lamp1, decorated membranes of the enlarged vacuoles, indicating these vacuoles were derived from lysosomes (arrows). Starvation, such as HBSS treatment, induces autophagy and hence autophagosome formation. P62 acts as a cargo receptor for ubiquitinated autophagic substrates by recruiting the substrates to the autophagosomes [15] and is subsequently degraded in autolysosomes. We examined whether the Lamp1-positive enlarged lysosomes contained P62. Compared to the control, the P62 signals were increased in CQ, HBSS, and HBSS+CQ treatment conditions. While starvation induced autophagy and strong P62-positive puncta, in the presence of CQ, P62 was further accumulated, indicating block of the autophagy flux. Interestingly, P62 was not localized inside the enlarged lysosomes (Fig 1B). Most of the P62-puncta were localized to the darker structures in the phase contrast images (arrowheads). This indicates that fusion between autophagosomes and lysosomes was inhibited under the treatment condition. This is consistent with previous reports showing that CQ inhibits autophagosomes fusion with lysosomes [14]. Beside the enlarged lysosomes induced by the HBSS+CQ treatment, large lamp1-labeled lysosomes were also observed in cells treated with CQ alone (Fig 1B, CQ). This has been observed previously by others as well [16–18]. Measuring the diameter revealed CQ-induced structures (average diameter ~0.6 μm in HeLa, ~0.8 μm in human fibroblasts) were smaller in size than the ones induced by HBSS+CQ (average diameter ~0.9 μm in HeLa, ~1.2 μm in human fibroblasts) (Fig 1C). We also examined ultrastructures of the enlarged lysosomes by transmission electron microscopy (TEM) (Fig 1D). TEM data supported light/fluorescence microscopy findings. CQ and HBSS+CQ treated cells had larger membrane-limited vacuoles than observed in control cells. HBSS+CQ treated cells had the largest vacuoles. Within these vacuoles, lamellar structures and smaller membrane limited structures were observed. In addition, many HBSS+CQ vacuoles contained dense staining regions

CQ is lysosomotropic and its accumulation can cause swelling of the lysosomes [19]. Therefore, we examined whether starvation with other means of lysosome inhibition can induce enlarged lysosomes. Similar to HBSS+CQ, HBSS+baf A treatment can induce enlarged lysosomes as well, though the structures were less prominent when observed by phase contrast microscopy (Fig S2). In contrast, starvation together with protease inhibitors leupeptin and pepstatin did not induce enlarged lysosomes (Fig S2). This suggests that the phenomenon is induced by a change in pH and not due to inhibition of proteases. Next, we examined whether the enlarged lysosomes originated from the endocytic pathway by immunostaining CD63, a marker protein of the intraluminal vesicles (ILVs) in the late endosome/multivesicular body (MVB). With Limp2 labeling lysosomal limiting membrane, CD63 can be observed inside the lysosomes (Fig 1E). This indicates that the enlarged lysosomes were of late endosome origin. We then examined whether the enlarged lysosomes colocalize with early or recycling endosomes. EGFP-tagged Rab5, Rab7, or Rab11 were expressed as marker proteins of early, late, and recycling endosomes (Fig 1F). Rab7 had the highest degree of colocalization with the enlarged lysosomes, further supporting the late endosome origin for the observed enlarged lysosomes.

Since starvation activates non-selective autophagy to degrade bulk cytoplasmic materials, we next examined if selective autophagy can also induce the “enlarged lysosomes” that we

observed with starvation and lysosome inhibition. To do this, we treated cells with carbonyl cyanide m-chlorophenyl hydrazine (CCCP), an oxidative phosphorylation uncoupler, to induce mitophagy. Treatment of CCCP led to mitochondrial fragmentation, indicating the drug treatment was effective (Fig 2A CCCP and CCCP+CQ, IMMT staining on top panels). Accumulation of the autophagy marker LC3-II in CCCP+CQ treatment was also observed (Fig 2B). Despite mitochondrial damages, no visible enlarged lysosomes were observed in any of the CCCP treatment conditions. This indicates that the enlarged lysosome phenotype is most likely a starvation-specific phenomenon.

At least two major signaling events occur when cells are starved, mTORC1 inactivation and autophagy activation. We examined if either of these two events was responsible for the enlarged lysosome formation. Instead of starvation treatment, Torin 1 was used to directly inactivate mTOR kinase. While treatments of Torin 1 and Torin 1+CQ inhibited mTORC1, as monitored by the status of the substrate S6K phosphorylation on Thr389 residue, there were no enlarged lysosomes formed after 8 hours of incubation (Fig 2C, D). These results suggest that alteration of mTORC1 activity during prolonged starvation is not responsible for the phenotype observed. Next, we examined involvement of the autophagy process in enlarged lysosome formation. ATG5 and ATG7 are essential genes for autophagosome formation [20, 21]. As shown in Fig 3A and B, ATG5^{-/-} as well as ATG7^{-/-} MEFs were capable of producing enlarged lysosomes upon HBSS+CQ treatment. We also performed transient siRNA knockdown on several autophagy proteins with similar results (not shown). Together, the results indicate that the enlarged lysosome phenomenon is independent of the mTOR and autophagy pathways.

To determine if nutrient-deprivation was responsible for the observed phenotype, we supplemented specific nutrients to the HBSS+CQ treatment. HBSS contains a low level of glucose (1 g/L). Supplementing with extra glucose (to the same level as high glucose DMEM medium, 4.9 g /L) had no effect on the enlarged lysosome formation (Fig 4A, HBSS +CQ+glucose). HBSS does not contain pyruvate or any amino acids. Therefore, we examined the effects of supplementing pyruvate to the treatment. Similar to glucose, supplementing pyruvate did not affect the enlarged lysosome morphology induced by HBSS +CQ (Fig 4A, HBSS+CQ+pyruvate). This indicates that the formation of enlarged lysosomes is not due to the absence of these energy sources. By contrast, when glutamine was supplemented to the HBSS+CQ treatment, no enlarged lysosomes were formed (Fig 4A, HBSS+CQ+Gln). Glutamine has been shown to activate mTORC1 via lysosomal V-ATPase [22]. Therefore, we examined the mTORC1 status. Surprisingly, adding glutamine did not prevent mTORC1 inactivation by starvation (Fig 4B). This indicates that glutamine does not convey the signal through mTORC1. This is consistent with Torin 1 treatment showing that mTORC1 activity is not correlated with enlarged lysosome formation. Next, we examined whether removal of glutamine from DMEM while still supplying other amino acids in DMEM was sufficient to induce enlarged lysosomes (Fig 4C). Remarkably, DMEM+CQ-Gln treatment was able to induce enlarged lysosome formation. In contrast to the HBSS+CQ and HBSS+CQ+Gln treatments, cells still had mTORC1 activity under DMEM+CQ-Gln treatment (Fig 4B). DMEM+CQ treatment, on the other hand, maintained mTORC1 activity and was not able to induce enlarged lysosomes. We tested several other amino acids,

including Arg, Asn, His, Ser, Gly, and Lys, but none of them were able to prevent or dissipate the enlarged lysosomes (Fig S3).

To examine the role of glutamine in this process, we first treated cells with HBSS+CQ for 8 hours to induce enlarged lysosomes and then added glutamine to the cells containing pre-existing enlarged lysosomes. To our surprise, within 2 to 4 hours of glutamine addition, nearly all enlarged lysosomes dissipated (Fig 4D). Lysosome localization returned from perinuclear to normal peripheral cellular distribution. The percentage of cells with enlarged lysosomes upon the addition of Gln was quantified (Fig 4E). Furthermore, the phosphorylation state of S6 kinase was not affected by the addition of glutamine after starvation treatment (Fig 4B). This implies glutamine can provide a signal to counteract the starvation signal and block or reverse the enlarged lysosome formation independent of mTORC1.

Our experiments thus far implicated an mTOR-independent mechanism for enlarged lysosome formation and dissipation. We wondered whether and how TFEB localization correlates with the enlarged lysosome formation. While most of the TFEB was cytoplasmic under nutrient-rich conditions, in HBSS+CQ and DMEM+CQ-Gln treatments (two conditions that induced enlarged lysosomes), TFEB was localized to the nucleus (Fig 5A). This is not surprising, as treating cells with CQ alone triggers TFEB nuclear translocation [9, 10]. However, when supplementing glutamine to the HBSS+CQ treatment, TFEB was mostly cytosolic (Fig 5B, HBSS+CQ+Gln). Quantification of TFEB nuclear to cytoplasmic intensity ratio shows that the nuclear pool of TFEB was dramatically reduced in the HBSS+CQ+Gln condition (Fig 5C). This suggests that TFEB was either unable to move to the nucleus, or the nuclear pool was degraded. Immunoblotting of TFEB showed that in the HBSS+CQ and DMEM+CQ-Gln treatments, there was a down-shift of the TFEB pattern, which correlated with the dephosphorylated forms of TFEB (Fig 5D)[10]. However, in the HBSS+CQ+Gln condition, the TFEB pattern was similar to the nutrient-rich condition, suggesting the cytoplasmic TFEB we observed was unable to move to the nucleus due to its phosphorylation state. Note that under the HBSS+CQ+Gln condition, mTORC1 was inactivated (Fig 4B) and thus was unable to phosphorylate TFEB at Ser142 or Ser221 [9, 10, 23]. It is therefore possible that the dephosphorylation of TFEB was stalled. Another possibility is that it involves a different kinase, such as Akt, to phosphorylate and keep TFEB in the cytoplasm. An mTORC1-independent and Akt-dependent TFEB regulation has been reported [24]. Therefore, we examined the Akt activation status in our treatment conditions (Fig S4). The activation status of Akt among different treatments were similar to that of mTORC1, and thus Akt is unlikely to be the kinase that keeps TFEB in the cytoplasm. Next, we examined if there was a nuclear pool of TFEB under the HBSS+CQ+Gln treatment. To this end, we expressed TFEB-EGFP in HeLa cells and applied bortezomib (BZ) to our treatments to inhibit proteasome degradation. While most of the TFEB-EGFP signal was in the cytoplasm under the HBSS+CQ+Gln condition, in the presence of BZ, TFEB-EGFP can be seen in the nucleus in ~ 80% of the TFEB-EGFP expressing cells (Fig 5E). Quantification of TFEB nuclear to cytoplasmic intensity ratio shows that in the presence of BZ, the nuclear pool of TFEB increased. This suggests that in the presence of glutamine, the proteasomes actively degraded the nuclear pool of TFEB. It is plausible that glutamine provided a cue to the cells that there was no need to activate

lysosome biogenesis and therefore the nuclear pool of TFEB was degraded. This coincides with the lack of enlarged lysosome formation under HBSS+CQ+Gln treatment (Fig 4A), suggesting the lysosome biogenesis was a pre-requisite for enlarged lysosome formation.

We also followed TFEB distribution when glutamine was reintroduced to dissipated preexisting enlarged lysosomes. Within two hours of adding glutamine, TFEB started to appear in the cytoplasm (Fig 5B). Pronounced cytoplasmic signals can be observed after 4 hours of glutamine addition. As shown in Fig 4B, mTORC1 stayed inactivated under these conditions. This supports our earlier results that the effects of glutamine on the enlarged lysosome formation and dissipation were independent of mTORC1 status.

In light of our results, we decided to investigate whether a recently characterized lysosomal glutamine transporter SNAT7 (SLC38A7) is involved in the glutamine-dependent effects on enlarged lysosomes we observed [25]. Comparable to control siRNA, knocking down SNAT7 did not prevent the glutamine-dependent inhibition of the enlarged lysosome formation (Fig 6A and B, HBSS+CQ+Gln), nor did it block the glutamine-induced dissipation of the enlarged lysosomes (HBSS+CQ 8h → +Gln 4h).

As TFEB nucleus/cytoplasm translocation correlates with formation/dissipation of enlarged lysosomes, we examined whether depleting TFEB and TFE3, two members of the MIT/TFE family known to regulate lysosome biogenesis [26, 27], can block enlarged lysosome formation and/or disappearance. Knocking down TFEB and TFE3 simultaneously did not inhibit enlarged lysosome formation or dissipate the pre-existing ones (Fig 6C). This suggests that TFEB nucleus/cytoplasm translocation occurs in parallel to enlarged lysosome formation/dissipation, and is dispensable for the formation and dissipation.

Discussion

In this report, we demonstrated that cells form enlarged lysosomes in response to stringent starvation and lysosomal inhibition by CQ. Inhibition of lysosomal proteases by leupeptin and pepstatin treatment was not sufficient to induce the phenomenon, suggesting that change of lysosomal pH is required. This is supported by our observation that starvation in combination with baf A, a V-ATPase inhibitor, can also induce enlarged lysosomes. Cells treated with CQ alone have been shown to induce large lysosomes [16–18]. We were able to observe the differences in size between the enlarged lysosomes induced by CQ alone and by HBSS+CQ treatment in our system.

This HBSS+CQ induced phenomenon is not solely caused by mTORC1 inactivation, as Torin 1, an inhibitor for mTOR, was not able to produce the same phenomenon. Nor was it induced by autophagy, as blocking autophagy by knocking out ATG5 or ATG7 did not prevent the formation of enlarged lysosomes. This is consistent with our observation that the enlarged lysosomes induced by HBSS+CQ treatment did not contain the autophagosome protein P62. We also showed that the presence of glutamine is the sole determinant that can prevent the formation of enlarged lysosomes or dissipate the preexisting enlarged lysosomes. Consequently, removing glutamine from DMEM can have the same effects as the stringent starvation solution HBSS in its ability to induce enlarged lysosome formation when

combined with CQ treatment. Several amino acid sensors and transporters acting upstream of mTORC1 have been identified recently, including a lysosomal glutamine transporter SNAT7 [1–3, 25, 28, 29]. When we depleted SNAT7 by siRNA, we did not observe changes in the glutamine response, suggesting a different glutamine transporter is involved. However, another possibility could be that, as the transient siRNA knockdown of SNAT7 was not complete and the residual amount of SNAT7 was sufficient to transport glutamine across the lysosomal membrane. Further testing, e.g. using CRISPR/Cas9 to knock out the SNAT7 gene expression, will be required to draw a definitive conclusion. SNAT7 belongs to the system N transporters of SLC38 family [30]. Besides glutamine, the system N transporters can also transport asparagine and histidine. Some can transport serine as well. We included these amino acids in our analysis. Intriguingly, glutamine is the only amino acid that has the ability to block enlarged lysosome formation.

In this report, we also found that TFEB nuclear and cytoplasmic translocation correlates with enlarged lysosome formation and dissipation. To elucidate a possible mechanism, TFEB and TFE3 were depleted by siRNA transfection. Our results suggest that the presence of TFEB and TFE3 are not required for the enlarged lysosome formation. Similarly, the glutamine-induced enlarged lysosome dissipation does not depend on cytoplasmic translocation of TFEB and TFE3. It appears that the combined treatment of lysosome inhibitor CQ and stringent starvation triggers a TFEB response in parallel to the enlarged lysosome formation. Alternatively, it is possible that other MIT/TFE members, such as MITF, can have a redundant role in lysosome biogenesis [31]. As a result, depletion of TFEB and TFE3 may not be sufficient to block lysosome biogenesis or other transcriptional activities that are necessary for enlarged lysosome formation.

TRPML1/mucolipin 1 is a lysosomal calcium channel. The release of calcium by TRPML1 can regulate the activity of the phosphatase calcineurin and in turn the dephosphorylation of TFEB [11]. In addition, mice lacking TRPML1 exhibit larger lysosomes [32]. Similarly, enlarged lysosomes were observed in TRPML1 deficient human fibroblasts [33]. As aforementioned, it is possible that the dephosphorylation of TFEB is stalled when glutamine is added to the treatments. It will be interesting to test if glutamine sends the signal through calcium flux in lysosomes and whether TRPML1 connects the TFEB nucleus/cytoplasm translocation and enlarged lysosomes formation/dissipation we observed. Moreover, ARF1 GTPase has been shown to be involved in glutamine-induced mTORC1 activation at the lysosome [22]. It will be useful to investigate whether ARF1 is required for the glutamine effects identified in our system.

We observed that the addition of glutamine can dissipate pre-existing enlarged lysosomes. This brings up a possibility that glutamine may have a role in lysosome reformation. Besides continuous fusion with late endosomes or autophagosomes to form hybrid compartments for substrate degradation, lysosomes also maintain their identity by a process termed lysosome reformation. Autophagic lysosome reformation requires mTOR reactivation and lysosome activity [34]. Considering in our system that lysosome activity was inhibited and the reversal of enlarged lysosomes occurred without mTOR reactivation, it is likely the glutamine effect observed in our system involves a different mechanism other than ALR. Lysosome reformation from endolysosomes has also been described [35–37]. Further investigation will

be needed to understand whether the glutamine effect we observed is similar to the reformation process reported for endolysosomes.

Understanding the connection between glutamine, TFEB activity, and enlarged lysosome formation will be essential to comprehend lysosomal homeostatic changes upon nutrient deprivation and compromised lysosome activity. Starvation and lysosomal inhibition are common manipulations for studying cellular responses, such as autophagy. Understanding how lysosome homeostasis is maintained under these conditions will help in making proper interpretations of the data when applying these manipulations to cells. It can also provide insights in diseases with compromised lysosomes, such as lysosomal storage disorders.

Materials and Methods

Reagents and antibodies

Hank's balanced saline solution (HBSS), pyruvate, and L-glutamine were purchased from Corning. Chloroquine (CQ) was purchased from MP Biomedicals. Bafilomycin A (baf A), leupeptin, and Torin 1 were purchased from Cayman chemical. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) was purchased from Enzo Life Sciences. Bortezomib was purchased from LC Laboratories. Pepstatin, glycine, lysine and serine were purchased from Sigma. Glucose, asparagine, and histidine were purchased from Fisher. Arginine was purchased from Acros Organics. The siRNAs against TFEB, TFE3, and SNAT7 (siGenome SMARTpool) and the control scramble siRNA were purchased from Dharmacon. Rabbit antibodies used in this study were against LC3B (Abcam), P62, S6K, pT389-S6K, ATG5, ATG7, TFEB, TFE3 (Cell Signaling Technology), SNAT7 (Sigma), and Limp2 (Novus Biologicals). Mouse antibodies used in this study were against GAPDH (Developmental Studies Hybridoma Bank (DSHB)), Lamp1 [38] (DSHB), and CD63 [39] (DSHB). TFEB-EGFP was a gift from Shawn Ferguson (Addgene plasmid #38119) [10]. HRP-conjugated secondary antibodies for Western blotting were purchased from Jackson Laboratory. Secondary antibodies conjugated to Alexa Fluor were purchased from Molecular Probes.

Cell culture and treatments

HeLa cells (ATCC CCL-2), human skin fibroblasts (Coriell institute for medical research), the mouse embryonic fibroblast (MEF) cell line ATG5 +/+, ATG5-/- [21], ATG7+/+, and ATG7-/- [20] (RIKEN BioResource Research Center) were grown and maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, HEPES, and gentamicin at 37°C with 5% CO₂. For various starvation and drug treatments, cells were seeded in 6-well culture plates with or without coverslips 24 hours before treatments. For drug treatments (CQ, CCCP, Torin 1, or combination), drugs were added to the complete media for 8 hours. For treatment with siRNA knockdown experiments, cells were transfected with siRNA (10 nM) using Lipofectamine RNAiMAX (Thermo Fisher) 48 hours before treatment. Cells were rinsed twice with phosphate saline buffer (PBS) prior to starvation treatments. All the starvation treatments were in the absence of FBS. The chemical concentrations used in various treatments were as follows: CQ, 50 µM; baf A, 20 nM; CCCP, 10 µM; Torin 1, 250 nM; bortezomib, 100 nM; glucose 4.9 g/L; pyruvate, 1 mM; L-glutamine, 4 mM; leupeptin 100 µM; pepstatin A, 5 µM; L-arginine, 0.4

mM; L-asparagine, 0.1 mM; glycine, 0.4 mM; L-histidine, 0.2 mM; L-lysine, 1 mM; L-serine, 0.4 mM. All sets of experiments were repeated at least three times to ensure reproducibility.

Immunoblotting

After treatments, cells were scraped and the pellets were kept at -80°C until use. Cell pellets were lysed using RIPA lysis buffer (50mM Tris, pH8.0, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (G Biosciences) and phosphatase inhibitors (50 mM NaF, 2 mM Na_3VO_4 , 10 mM $\text{Na}_4\text{P}_2\text{O}_7$). After incubation for 30 minutes on ice, extracts were centrifuged at 20,000g for 10 minutes at 4°C . The supernatant was collected as cell lysates. Protein concentrations were determined with Bradford assay when necessary. Aliquots of total extracts were incubated with sample buffer containing 100 mM DTT at 37°C for 10 min. Samples were separated by SDS-PAGE. Proteins were transferred to PVDF membranes (Millipore) followed by immunoblotting. ECL detection was performed according to the manufacturer's instructions (Millipore) and blots were imaged with G-Box (Syngene).

Immunofluorescence microscopy and statistical analysis

Cells were fixed with 4% formaldehyde for 10 minutes at room temperature, or 100% methanol for 10 minutes at -20°C . Blocking, permeabilization, antibody incubations, and washes were done using blocking solution (10% fetal calf serum, 0.1% saponin, and 0.02% sodium azide in PBS). The cells were imaged using either a Zeiss LSM-5 PASCAL (objective lens 100x/1.4 oil) or LSM-700 laser scanning confocal microscope (objective lens 40x/1.4 oil; ZEN 2012 microscope software). LSM 5 service pack and Zen 2012 were used for imaging acquisition. Photoshop software was used to adjust the imaging brightness and contrast. For TFEB localization quantification, the intensity of a 20 pixel $\times$ 20 pixel area (Fig 5C) or a 50 $\times$ 50 pixel area (Fig 5E) in the nucleus and cytoplasm was quantified in each cell using a photoshop histogram tool. Data were analyzed using a Student's t test with two-samples assuming equal variances, or by one way ANOVA followed by the Tukey's test.

Transmission electron microscopy

Cells were grown on 13 mm Nunc Thermanox coverslips. After treatments, cells were fixed with Karnovsky's fixative containing 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight at 4°C . Cells were then washed three times with 0.1 M cacodylate buffer, post-fixed in 2% osmium tetroxide for 1 hour at room temperature, washed, enblock stained in 2% aqueous uranyl acetate at room temperature, washed, dehydrated in a 50–100% ascending series of acetone, infiltrated and embedded in UltraBed resin (Electron Microscopy Sciences). Ultrathin sections (<95 nm silver to gold) were cut using ultramicrotome, examined with a CM 100 TEM (FEI Company) at 100 kV, and images captured with a Hamamatsu C8484 digital camera using a AMT image capture system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank the Microscopy Facility in the Division of Biology and the Confocal Core facility supported by the College of Veterinary Medicine at Kansas State University for their assistance and usage of these facilities.

Funding

This work was supported in part by grants from the National Institute of General Medical Sciences (P20GM103418) of the National Institute of Health and Terry Johnson Center for Basic Cancer Research at Kansas State University.

References

- [1]. Zoncu R, Bar-Peled L, Efeyan A, Wang S, Sancak Y, Sabatini DM, mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase, *Science*, 334 (2011) 678–683. [PubMed: 22053050]
- [2]. Rebsamen M, Pochini L, Stasyk T, de Araujo ME, Galluccio M, Kandasamy RK, Snijder B, Fauster A, Rudashevskaya EL, Bruckner M, Scorzoni S, Filipek PA, Huber KV, Bigenzahn JW, Heinz LX, et al., SLC38A9 is a component of the lysosomal amino acid sensing machinery that controls mTORC1, *Nature*, 519 (2015) 477–481. [PubMed: 25561175]
- [3]. Wang S, Tsun ZY, Wolfson RL, Shen K, Wyant GA, Plovanich ME, Yuan ED, Jones TD, Chantranupong L, Comb W, Wang T, Bar-Peled L, Zoncu R, Straub C, Kim C, et al., Metabolism. Lysosomal amino acid transporter SLC38A9 signals arginine sufficiency to mTORC1, *Science*, 347 (2015) 188–194. [PubMed: 25567906]
- [4]. Laplante M, Sabatini DM, mTOR signaling in growth control and disease, *Cell*, 149 (2012) 274–293. [PubMed: 22500797]
- [5]. Shang L, Chen S, Du F, Li S, Zhao L, Wang X, Nutrient starvation elicits an acute autophagic response mediated by Ulk1 dephosphorylation and its subsequent dissociation from AMPK, *Proc Natl Acad Sci U S A*, 108 (2011) 4788–4793. [PubMed: 21383122]
- [6]. Kim J, Kundu M, Viollet B, Guan KL, AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1, *Nat Cell Biol*, 13 (2011) 132–141. [PubMed: 21258367]
- [7]. Reggiori F, Ungermann C, Autophagosome Maturation and Fusion, *J Mol Biol*, 429 (2017) 486–496. [PubMed: 28077293]
- [8]. Settembre C, Di Malta C, Polito VA, Garcia Arencibia M, Vetrini F, Erdin S, Erdin SU, Huynh T, Medina D, Colella P, Sardiello M, Rubinsztein DC, Ballabio A, TFEB links autophagy to lysosomal biogenesis, *Science*, 332 (2011) 1429–1433. [PubMed: 21617040]
- [9]. Settembre C, Zoncu R, Medina DL, Vetrini F, Erdin S, Erdin S, Huynh T, Ferron M, Karsenty G, Vellard MC, Facchinetti V, Sabatini DM, Ballabio A, A lysosometo-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB, *EMBO J*, 31 (2012) 1095–1108. [PubMed: 22343943]
- [10]. Rocznik-Ferguson A, Petit CS, Froehlich F, Qian S, Ky J, Angarola B, Walther TC, Ferguson SM, The transcription factor TFEB links mTORC1 signaling to transcriptional control of lysosome homeostasis, *Sci Signal*, 5 (2012) ra42. [PubMed: 22692423]
- [11]. Medina DL, Di Paola S, Peluso I, Armani A, De Stefani D, Venditti R, Montefusco S, Scotto-Rosato A, Prezioso C, Forrester A, Settembre C, Wang W, Gao Q, Xu H, Sandri M, et al., Lysosomal calcium signalling regulates autophagy through calcineurin and TFEB, *Nat Cell Biol*, 17 (2015) 288–299. [PubMed: 25720963]
- [12]. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozana A, Adachi H, Adams CM, Adams PD, Adeli K, Adhihetty PJ, Adler SG, Agam G, Agarwal R, Aghi MK, et al., Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition), *Autophagy*, 12 (2016) 1–222. [PubMed: 26799652]
- [13]. Tanida I, Minematsu-Ikeguchi N, Ueno T, Kominami E, Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy, *Autophagy*, 1 (2005) 84–91. [PubMed: 16874052]

- [14]. Kawai A, Uchiyama H, Takano S, Nakamura N, Ohkuma S, Autophagosomelysosome fusion depends on the pH in acidic compartments in CHO cells, *Autophagy*, 3 (2007) 154–157. [PubMed: 17204842]
- [15]. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, Overvatn A, Bjorkoy G, Johansen T, p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy, *J Biol Chem*, 282 (2007) 24131–24145. [PubMed: 17580304]
- [16]. Yoon YH, Cho KS, Hwang JJ, Lee SJ, Choi JA, Koh JY, Induction of lysosomal dilatation, arrested autophagy, and cell death by chloroquine in cultured ARPE19 cells, *Invest Ophthalmol Vis Sci*, 51 (2010) 6030–6037. [PubMed: 20574031]
- [17]. Florey O, Gammoh N, Kim SE, Jiang X, Overholtzer M, V-ATPase and osmotic imbalances activate endolysosomal LC3 lipidation, *Autophagy*, 11 (2015) 88–99. [PubMed: 25484071]
- [18]. Chen PM, Gombart ZJ, Chen JW, Chloroquine treatment of ARPE-19 cells leads to lysosome dilation and intracellular lipid accumulation: possible implications of lysosomal dysfunction in macular degeneration, *Cell Biosci*, 1 (2011) 10. [PubMed: 21711726]
- [19]. Marceau F, Bawolak MT, Lodge R, Bouthillier J, Gagne-Henley A, Gaudreault RC, Morissette G, Cation trapping by cellular acidic compartments: beyond the concept of lysosomotropic drugs, *Toxicol Appl Pharmacol*, 259 (2012) 1–12. [PubMed: 22198553]
- [20]. Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, Ezaki J, Mizushima N, Ohsumi Y, Uchiyama Y, Kominami E, Tanaka K, Chiba T, Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice, *J Cell Biol*, 169 (2005) 425–434. [PubMed: 15866887]
- [21]. Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, Ohsumi Y, Tokuhisa T, Mizushima N, The role of autophagy during the early neonatal starvation period, *Nature*, 432 (2004) 1032–1036. [PubMed: 15525940]
- [22]. Jewell JL, Kim YC, Russell RC, Yu FX, Park HW, Plouffe SW, Tagliabracci VS, Guan KL, Metabolism. Differential regulation of mTORC1 by leucine and glutamine, *Science*, 347 (2015) 194–198. [PubMed: 25567907]
- [23]. Martina JA, Chen Y, Gucek M, Puertollano R, MTORC1 functions as a transcriptional regulator of autophagy by preventing nuclear transport of TFEB, *Autophagy*, 8 (2012) 903–914. [PubMed: 22576015]
- [24]. Palmieri M, Pal R, Nelvagal HR, Lotfi P, Stinnett GR, Seymour ML, Chaudhury A, Bajaj L, Bondar VV, Bremner L, Saleem U, Tse DY, Sanagasetti D, Wu SM, Neilson JR, et al., mTORC1-independent TFEB activation via Akt inhibition promotes cellular clearance in neurodegenerative storage diseases, *Nat Commun*, 8 (2017) 14338. [PubMed: 28165011]
- [25]. Verdon Q, Boonen M, Ribes C, Jadot M, Gasnier B, Sagne C, SNAT7 is the primary lysosomal glutamine exporter required for extracellular protein-dependent growth of cancer cells, *Proc Natl Acad Sci U S A*, 114 (2017) E3602–E3611. [PubMed: 28416685]
- [26]. Sardiello M, Palmieri M, di Ronza A, Medina DL, Valenza M, Gennarino VA, Di Malta C, Donaudo F, Embrione V, Polishchuk RS, Banfi S, Parenti G, Cattaneo E, Ballabio A, A gene network regulating lysosomal biogenesis and function, *Science*, 325 (2009) 473–477. [PubMed: 19556463]
- [27]. Martina JA, Diab HI, Lishu L, Jeong AL, Patange S, Raben N, Puertollano R, The nutrient-responsive transcription factor TFE3 promotes autophagy, lysosomal biogenesis, and clearance of cellular debris, *Sci Signal*, 7 (2014) ra9. [PubMed: 24448649]
- [28]. Wolfson RL, Chantranupong L, Saxton RA, Shen K, Scaria SM, Cantor JR, Sabatini DM, Sestrin2 is a leucine sensor for the mTORC1 pathway, *Science*, 351 (2016) 43–48. [PubMed: 26449471]
- [29]. Chantranupong L, Scaria SM, Saxton RA, Gygi MP, Shen K, Wyant GA, Wang T, Harper JW, Gygi SP, Sabatini DM, The CASTOR Proteins Are Arginine Sensors for the mTORC1 Pathway, *Cell*, 165 (2016) 153–164. [PubMed: 26972053]
- [30]. Broer S, The SLC38 family of sodium-amino acid co-transporters, *Pflugers Arch-Eur J Physiol*, 466 (2014) 155–172. [PubMed: 24193407]
- [31]. Ploper D, De Robertis EM, The MITF family of transcription factors: Role in endolysosomal biogenesis, Wnt signaling, and oncogenesis, *Pharmacol Res*, 99 (2015) 36–43. [PubMed: 26003288]

- [32]. Venugopal B, Browning MF, Curcio-Morelli C, Varro A, Michaud N, Nanthakumar N, Walkley SU, Pickel J, Slaughter SA, Neurologic, gastric, and ophthalmologic pathologies in a murine model of mucopolipidosis type IV, *Am J Hum Genet*, 81 (2007) 1070–1083. [PubMed: 17924347]
- [33]. Cao Q, Yang Y, Zhong XZ, Dong XP, The lysosomal Ca(2+) release channel TRPML1 regulates lysosome size by activating calmodulin, *J Biol Chem*, 292 (2017) 8424–8435. [PubMed: 28360104]
- [34]. Yu L, McPhee CK, Zheng L, Mardones GA, Rong Y, Peng J, Mi N, Zhao Y, Liu Z, Wan F, Hailey DW, Oorschot V, Klumperman J, Baehrecke EH, Lenardo MJ, Termination of autophagy and reformation of lysosomes regulated by mTOR, *Nature*, 465 (2010) 942–946. [PubMed: 20526321]
- [35]. Bissig C, Hurbain I, Raposo G, van Niel G, PIKfyve activity regulates reformation of terminal storage lysosomes from endolysosomes, *Traffic*, 18 (2017) 747–757. [PubMed: 28857423]
- [36]. Miller A, Schafer J, Upchurch C, Spooner E, Huynh J, Hernandez S, McLaughlin B, Oden L, Fares H, Mucopolipidosis type IV protein TRPML1-dependent lysosome formation, *Traffic*, 16 (2015) 284–297. [PubMed: 25491304]
- [37]. Pryor PR, Mullock BM, Bright NA, Gray SR, Luzio JP, The role of intraorganellar Ca(2+) in late endosome-lysosome heterotypic fusion and in the reformation of lysosomes from hybrid organelles, *J Cell Biol*, 149 (2000) 1053–1062. [PubMed: 10831609]
- [38]. Schweizer A, Fransen JA, Bachi T, Ginsel L, Hauri HP, Identification, by a monoclonal antibody, of a 53-kD protein associated with a tubulo-vesicular compartment at the cis-side of the Golgi apparatus, *J Cell Biol*, 107 (1988) 1643–1653. [PubMed: 3182932]
- [39]. Hildreth JE, Derr D, Azorsa DO, Characterization of a novel self-associating Mr 40,000 platelet glycoprotein, *Blood*, 77 (1991) 121–132. [PubMed: 1984792]

Highlights

- Starvation with lysosome inhibition induces enlarged lysosome formation
- The formation of enlarged lysosomes is autophagy and mTOR independent
- The addition of glutamine prevents the enlarged lysosome formation
- The addition of glutamine dissipates the pre-existing enlarged lysosomes
- TFEB translocation correlates with formation/dissipation of enlarged lysosomes

Figure 1. Treatment of HBSS and CQ induces enlarged lysosome formation.

(A) Cells were incubated with CQ, HBSS, or HBSS+CQ for 4 hours (human skin fibroblasts, top) or 8 hours (HeLa, bottom) prior to fixation. The control (–) cells were left untreated. Phase contrast microscopy shows that cells with HBSS+CQ treatment accumulated enlarged vacuolar structures. (B) The lysosomal marker Lamp1 is localized to the enlarged vacuolar structures. Human fibroblasts were incubated with CQ, HBSS, or HBSS+CQ for 4 hours prior to fixation and immunostained for Lamp1 and P62. Arrows indicate the membrane localization of Lamp1. Arrowheads indicate that the P62 puncta

localized to the dark regions in the phase contrast images. An enlarged view of HBSS+CQ treated cells is shown in the last row. (C) The diameter of enlarged lysosomes in CQ and HBSS+CQ treated cells were quantified using ImageJ measure tool. Each value represents mean + SEM. Left panel: HeLa cells, about 165 lysosomes per condition were measured. Right panel, human fibroblasts (HF), 97 lysosomes in CQ and 403 lysosomes I HBSS+CQ treated samples were measured. (D) TEM images show the vacuoles (V) in control (–), CQ and HBSS+CQ treated HeLa cells. The diameter of vacuoles was analyzed (right panel). Endoplasmic reticulum (ER) are labeled for size reference. Scale bar, 200 nm. (E) The enlarged lysosomes contained intraluminal vesicles, labeled by antibodies against CD63 (top). Bottom graph shows the signal intensity profiles along the line drawn on the top panel. The arrowheads indicate CD63 signal peaks between lysosomal membranes marked by Limp2. (F) EGFP-tagged Rab5, Rab7, or Rab11 were expressed in HeLa cells. Cells were incubated with HBSS+CQ for 8 hours to induced enlarged lysosomes. Colocalization of Lamp1-labeled enlarged lysosomes with Rab5, Rab7, and Rab11 were analyzed using the ImageJ JACoP plugin Pearson's coefficient R. Each value represents mean + SEM. Scale bars in A, B, E, and F, 10 μ m.

Figure 2. Mitophagy and mTORC1 inhibition do not induce enlarged lysosome formation. (A) and (B) HeLa cells were incubated with CCCP, CCCP+CQ, or HBSS+CQ for 8 hours. In (A) mitochondria were immunostained with antibodies against a mitochondrial membrane protein, IMMT. Phase contrast microscopy shows CCCP and CCCP+CQ treatments did not induce enlarged lysosomes. In (B) the immunoblot shows LC3 protein levels of the treatments as indicated. In (C) CQ, Torin 1, or Torin 1+CQ was added to the complete media for 8 hours. Samples were processed for immunoblotting analysis. The protein levels of phosphorylated (Thr389) S6K, total S6K, and LC3 were immunoblotted. (D) HeLa cells

were treated with Torin 1, Torin 1+CQ, or HBSS+CQ for 8 hours prior to fixation. Phase contrast microscopy and immunofluorescence of Lamp1 show that Torin 1 and Torin 1+CQ treatments did not induce enlarged lysosomes. The control (–) cells were incubated with DMSO under nutrient-rich conditions. GAPDH was blotted as a loading control. Scale bar, 10 μ m.

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Figure 3. Autophagy activation is not required for enlarged lysosome formation.

Knockout of the essential genes for autophagy, Atg5 or Atg7, did not block enlarged lysosome formation induced by HBSS+CQ treatment. The immunoblots show Atg5^{-/-} MEFs (A) and Atg7^{-/-} MEFs (B) did not express Atg5 (A) and Atg7 (B), respectively. Phase contrast microscopy and immunofluorescence of Lamp1 show enlarged lysosome formation upon HBSS+CQ treatment in the absence of Atg5 or Atg7. The control (-) cells were left untreated. GAPDH was blotted as a loading control. Scale bar, 10 μ m.

Figure 4. Glutamine blocks the formation of enlarged lysosomes and dissipates the pre-existing enlarged lysosomes.

(A) HeLa cells were incubated with HBSS+CQ for 8 hours in the absence or presence of the supplements as indicated. Glucose (4.5g/L), pyruvate (1 mM), and glutamine (4 mM) were added to reach the concentrations in the growing media. Phase contrast microscopy shows that the addition of glutamine prevented the formation of enlarged lysosomes. (B) HeLa cells were incubated with the conditions as indicated. Samples were processed for immunoblotting analysis. The addition of glutamine during starvation treatments did not change the mTORC1 inactivation state. The protein levels of phosphorylated (Thr389) S6K

and total S6K were immunoblotted. (C) HeLa cells were incubated with DMEM+CQ with glutamine (DMEM+CQ), or DMEM+CQ without glutamine (DMEM+CQ-Gln) for 8 hours. Phase contrast microscopy and immunofluorescence of Lamp1 show the formation of enlarged lysosomes in the absence of glutamine. (D) HeLa cells were incubated with HBSS +CQ for 8 hours, and glutamine was added to the cells for another 2 or 4 hours. Phase contrast microscopy shows that the addition of glutamine dissipated the pre-existing enlarged lysosomes. Lamp1 immunofluorescence shows the distribution of lysosomes. GAPDH was blotted as a loading control. (E) The percentage of cells with enlarged lysosomes upon the addition of Gln was quantified and analyzed by one-way ANOVA followed by the Tukey's test. n=3 experiments, average 40 cells per condition per experiment. * P< 0.0001. Each value represents mean + SEM. The control (–) cells were left untreated. Scale bar, 10 μ m.

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Figure 5. TFEB nuclear and cytoplasmic translocation correlates with the formation and dissipation of enlarged lysosomes.

(A) HeLa cells were incubated with HBSS+CQ, or DMEM+CQ-Gln for 8 hours. (B) HeLa cells were incubated with HBSS+CQ+Gln for 8 hours, then added glutamine for another 2 or 4 hours as indicated. Immunofluorescence of endogenous Lamp1 and TFEB are shown. (C) Quantification of nuclear/cytoplasmic intensity ratio of TFEB. 100 cells of each condition from three independent experiments were quantified and analyzed by ANOVA followed by the Tukey's test. * $P < 0.0001$ (D) HeLa cells were incubated with the conditions as indicated. Samples were processed for immunoblotting analysis. The endogenous TFEB

was immunoblotted. GAPDH was blotted as a loading control. (E) HeLa cells were transfected with TFEB-EGFP plasmid for 24 hours. Cells were then incubated with HBSS +CQ+Gln in the absence or presence of bortezomib (BZ). Immunofluorescent microscopy shows Lamp1 and TFEB-EGFP localization. 10 cells of each condition were quantified and analyzed by a Student's t test. *P < 0.001. Each value represents mean + SEM. The control (–) cells were left untreated. Scale bar, 10 µm.

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Figure 6. Knockdown SNAT7 or TFEB/TFE3 has no effect on enlarged lysosome formation or dissipation.

HeLa cells were transfected with control siRNA (A), siRNA against SNAT7 (B), or siRNA against TFEB and TFE3 (C) 48 hours prior to treatments as indicated. In control (–), cells were left untreated. Immunoblots show the decreased protein levels of SNAT7 (B), TFEB and TFE3 (C). GAPDH was blotted as a loading control. Scale bar, 10 μ m.