Mitochondrial Sodium/Calcium Exchanger (NCLX) Regulates Basal and Starvation-Induced Autophagy Through Calcium Signaling

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Abstract

In addition to their central role in energy metabolism, mitochondria can shape intracellular calcium (Ca^{2+}) signaling through the concerted activity of Ca^{2+} uptake to the matrix through the mitochondrial calcium uniporter, and efflux from the matrix mediated by the mitochondrial Na^{+}/Ca^{2+} exchanger, NCLX. Ca^{2+} levels in the cytosol are known to regulate both metabolism and autophagic activity. Here, we describe a novel and direct relationship between NCLX, cytosolic Ca^{2+}, and autophagic activity. We find that conditions that stimulate autophagy *in vivo* and *in vitro* such as caloric restriction and nutrient deprivation upregulate NCLX expression in hepatic tissue and cells. Conversely, siRNA-mediated knockdown of NCLX impairs basal and starvation-induced autophagy, by decreasing LC3 II flux. Similarly, acute inhibition of NCLX activity by CGP 37157 affects early steps of autophagy activation in hepatocyte-derived cells and mouse embryonic fibroblasts, resulting in reduced autophagic activity. Inhibition of NCLX caused decreased cytosolic Ca^{2+} levels, and pre-incubation with intracellular Ca^{2+} chelator BAPTA-AM promotes similar results to those of CGP 37157 on autophagy. BAPTA-AM also completely abolished the effects of NCLX inhibition on autophagy, demonstrating that mitochondrial Ca^{2+} efflux regulates autophagy through the modulation of cytosolic Ca^{2+} signaling. Collectively, our results demonstrate that the mitochondrial Ca^{2+} extrusion pathway NCLX is an important regulatory node linking nutrient restriction and autophagy regulation through the modulation of cytosolic Ca^{2+} levels.

Abbreviations

AMPK: AMP-activated protein kinase; ATG: autophagy-related; BafA1: Bafilomycin A1; CGP: CGP 37157; CM: complete media; ER: endoplasmic reticulum; HBSS: Hanks' balanced salt solution; KD: knockdown; LC3: membrane-bound microtubule-associated protein light chain 3; mTORC1: mammalian target of rapamycin complex 1; MCU: mitochondrial calcium uniporter; NCLX: mitochondrial $Na^{+}/Li^{+}/Ca^{2+}$ exchanger.

Introduction

Mitochondria are highly dynamic organelles that can adapt their functions to changes in the cellular environment [1,2]. In return, they provide signals that regulate cytoplasmic processes, leading to coordinated responses [3]. Among these, calcium (Ca^{2+}) signaling is known to regulate and be regulated by mitochondrial activity [4]. Indeed, the capacity to take up and release Ca^{2+} ions makes mitochondria capable of directly participating in a myriad of cellular Ca^{2+}-sensitive regulation processes [4,5].

Mitochondrial Ca^{2+} uptake is mediated by the mitochondrial Ca^{2+} uniporter (MCU) complex [6], while Ca^{2+} efflux can occur through two specific transporters: a mitochondrial H^+/Ca^{2+} exchanger, with a disputed molecular identity [5,7]; and the mitochondrial $Na^+/Li^+/Ca^{2+}$ exchanger (NCLX), which releases matrix Ca^{2+} in exchange for Na^+ ions. With the identification of the NCLX gene in 2010 [8], several subsequent studies uncovered important physiological functions of this transporter, including regulation of insulin secretion, cardiomyocyte automaticity, and lactate and glutamate secretion in astrocytes, all by shaping Ca^{2+} signals [9-12]. In pathology, decreased NCLX expression was observed in Alzheimer's disease, colorectal cancer, and heart failure patient tissues, while the rescue of its activity led to improved phenotypes [13-15]. These studies suggest that impaired NCLX activity is central in the regulation of pathways with extensive therapeutic potential.

Autophagy, a highly conserved intracellular pathway leading to lysosomal degradation of cellular components [16], is widely recognized as a pivotal process involved in maintaining tissue homeostasis. In macroautophagy (which we will refer to as autophagy from now on), these components are sequestered by autophagosomes, which in turn fuse with lysosomes to promote the digestion of cargo such as organelles or proteins [16]. Due to its ability to remove unwanted elements and promote specific or general nutrient availability as an output, autophagy is necessary for quality control, tissue renovation, and metabolic regulation [16,17].

Ca^{2+} ions are known second messengers in metabolic signaling and display an important role in autophagy regulation [18-20]. However, the genesis and consequences of Ca^{2+} signaling within

this regulatory mechanism are very context-dependent and not yet fully understood [21,22]. This is intrinsically related to the fact that Ca^{2+} signals are shaped at subcellular levels by ion transporters in different organelles. In fact, endeavors to study the role of specific Ca^{2+} transporters located in the endoplasmic reticulum (ER) or lysosome, rather than overall cellular Ca^{2+} levels, have strongly contributed toward untangling particular aspects of autophagic regulation [23,24]. Considering the presence of mitochondria in close contact with these organelles and Ca^{2+} transfer between them [25-27], it is likely that mitochondrial Ca^{2+} transport participates in Ca^{2+}-mediated autophagy regulation. In fact, the transfer of Ca^{2+} from inositol trisphosphate receptor (IP_3R) in the ER to mitochondria through the MCU was shown to suppress basal autophagy in an AMP-activated protein kinase (AMPK)-dependent manner [28]. On the other hand, the question of how mitochondrial Ca^{2+} efflux through NCLX can affect autophagy has not yet been addressed.

Thus, considering the emerging relevance of NCLX in a myriad of physiological and pathological processes in which autophagy is similarly important, and considering the importance of Ca^{2+} transporters in autophagic regulation, this work focused on investigating the interplay between NCLX activity and autophagy.

Results and Discussion

NCLX expression is increased by nutrient restriction

Autophagy is mainly activated in response to decreased nutrient availability [17]. Interestingly, works published by our group have shown that mitochondrial Ca^{2+} transport is strongly regulated by nutritional interventions such as caloric restriction, which is also well known to promote autophagy [29-31]. As a starting point to understand the interplay between mitochondrial Ca^{2+} transport and autophagy, we investigated whether NCLX levels are sensitive to calorie restriction. We consistently observed that mitochondria isolated from the livers of mice that underwent 4 months of caloric restriction contained higher levels of NCLX protein than animals on an *ad libitum* diet (**Figure 1A**). To test if this effect could be reproduced in an *in vitro* model of autophagy activation,

we submitted AML12 cells, a non-tumoral cell line derived from murine hepatocytes [32], to serum and amino acid starvation using Hanks' balanced salt solution (HBSS). Starvation resulted in a time-dependent increase in NCLX mRNA (**Figure 1B**). On the other hand, MCU mRNA levels were not changed under the same conditions (Fig S1), demonstrating that this effect is specific to NCLX, rather than a more global change in mitochondrial Ca^{2+} transport machinery. Overall, our results show that the specific expression of NCLX is highly sensitive to nutrient availability in hepatic cells, suggesting a physiological role in nutrient sensing.

Decreased NCLX expression causes impaired autophagic activity

Since we found NCLX expression to be increased during nutrient restriction, we tested whether reducing NCLX levels could affect starvation-induced autophagy by promoting NCLX genetic knockdown (KD) in hepatic AML12 cells transfected with NCLX-targeting siRNA (siNCLX). NCLX mRNA levels were significantly reduced 48 and 72 hours after transfection when compared to cells transfected with negative control siRNA (siNC) (**Figure 2A**). Consequently, NCLX protein levels were also decreased by siRNA-mediated KD (**Figure 2B**). As a readout of autophagic activity, we initially measured the levels of LC3 II and LC3 I proteins. As expected, siNC cells incubated with HBSS for 1 hour showed increased LC3 II and reduced LC3 I compared to cells incubated with complete media (CM), indicating autophagy activation (**Figure 2C**). In siNCLX cells, LC3 II levels were not upregulated by HBSS treatment (**Figure 2C**), which reflects an inhibition of autophagy. Curiously, LC3 I levels were lower in siNCLX cells even in the absence of starvation (**Figure 2C**), thereby suggesting a modulation of total LC3 by NCLX. Besides autophagic activity, LC3 levels may be affected by either proteasome degradation [33] or gene expression. We did not observe alterations in proteasome activity by NCLX KD (Fig S2A), while LC3A and LC3B mRNA were reduced compared to siNC (Fig S2B).

To confirm autophagy inhibition by NCLX KD, we repeated the experiment in the presence of the lysosomal inhibitor, Bafilomycin A1 (BafA1). Consistently, cells with reduced NCLX

expression showed significantly less LC3 II accumulation than siNC cells in the presence of BafA1 (**Figure 2D**). Accordingly, LC3 II net flux (BafA1-treated minus BafA1-untreated samples) was reduced in siNCLX cells (**Figure 2E**).

As an additional strategy, we used an AML12 cell line stably expressing mCherry-GFP-tagged LC3B which allows the visualization of autophagosome ($mCherry^+/GFP^+$ puncta) and autolysosome ($mCherry^+/GFP^-$ puncta) formation through fluorescence microscopy [34,35]. After 1 hour incubation in HBSS, siNCLX cells showed similar levels of autophagosomes to controls, but reduced autolysosome puncta (**Figure 2F**), hence supporting that autophagic activity is compromised in these cells. Interestingly, since LC3 II levels were lower in siNCLX cells (**Figure 2C**), we expected autophagosome puncta be similarly reduced, but did not see this in these cells, probably because the transduction of the LC3 system used as a probe affects the outcome. Since LC3 is essential for proper autophagosome formation [36], and we observed a reduction of its expression in siNCLX cells (Fig S2), ectopic expression of LC3 may compromise the expected effect. Nonetheless, our results with mCherry-GFP-tagged LC3B cells clearly demonstrate that reduced NCLX expression compromises autophagic activity during starvation.

Since reduced expression of NCLX led to changes in LC3 levels, we hypothesized that basal autophagy, in the presence of nutrients and serum (complete media), could be affected. Indeed, although steady-state (without BafA1) levels of LC3 II were unchanged, its accumulation in the presence of BafA1 was also lower in siNCLX cells (**Figure 2G**), which also resulted in decreased net flux (**Figure 2H**). Additionally, siNCLX cells presented reduced autolysosome puncta, either under control conditions or with BafA1; autophagosome puncta were reduced after BafA1 treatment (**Figure 2I**). Of note, the level of autophagy inhibition by NCLX KD was higher in starved than basal conditions, suggesting that the NCLX role is more prominent in the latter, while also still present without starvation.

Thus far, our results demonstrated that cells under autophagic stimulation upregulate NCLX expression, while cells with reduced expression of this mitochondrial transporter have decreased

autophagic activity and are less sensitive to starvation stimulus. Collectively, these findings indicate a direct relationship between NCLX levels and autophagic activity in hepatic cells.

Acute NCLX inhibition decreases autophagy activation by starvation

Our data shown up to now indicate that chronic inhibition of NCLX affects autophagic machinery. However, the time frame required for silencing studies means the effect of NCLX KD on autophagy could be indirect. To address the hypothesis that Ca^{2+} efflux from the mitochondria could directly participate in the regulation of autophagy activation, we used the strategy of acute pharmacological inhibition of NCLX activity by CGP 37157 (CGP). To validate that the drug was effective under our conditions, we measured Ca^{2+} levels within the mitochondrial matrix using the genetically-encoded probe mito-GEM-GECO1 [37]. Cells incubated with HBSS in the presence of DMSO or 10 µM CGP were imaged upon the addition of 100 µM extracellular ATP to stimulate ER Ca^{2+} release (**Figure 3A**). ATP elicited mitochondrial Ca^{2+} uptake, resulting in a rapid increase in intramitochondrial Ca^{2+} levels followed by a slower decay towards basal levels (**Figure 3A, light grey line/column**), corresponding to the mitochondrial Ca^{2+} efflux rate. Ionomycin, a Ca^{2+} ionophore, was added at the end of all traces as an internal control. Importantly, cells in the presence of CGP (**Figure 3A, blue line/column**) had equal mitochondrial Ca^{2+} increases upon ATP stimulation, but did not present a measurable efflux rate after the Ca^{2+} peak, thereby confirming specific NCLX inhibition.

When CGP was concomitantly added to AML12 cells during incubation with HBSS, we observed a reduction in autophagic activity, as shown by lower accumulation of LC3 II in the presence of BafA1 and lower net flux (**Figure 3B**). The same effect was observed using control versus CPG-treated mouse embryonic fibroblasts (MEFs, **Figure 3C**), showing that NCLX-dependent regulation of autophagy is not specific to hepatic cells. Interestingly, while HBSS led to a reduction in LC3 I levels due to LC3 lipidation, we noticed that the presence of CGP partially inhibited this effect, promoting the accumulation of LC3 I (Fig. S3). This suggests that NCLX is acting at early steps of

autophagy. To confirm this, we investigated the intracellular localization of FIP200 and ATG16L1, both upstream of LC3 I lipidation during autophagy activation. As expected, cells incubated with HBSS showed increased FIP200 and ATG16L1 puncta formation (**Figure 3D**). Conversely, the presence of CGP during incubation with HBSS significantly inhibited puncta formation (**Figure 3D**), showing that NCLX activity regulates the recruitment of autophagy initiation machinery during starvation.

Impaired autophagy promoted by NCLX inactivation is a result of reduced cytoplasmic Ca^{2+}

Among the direct downstream effects of NCLX inhibition are the accumulation of Ca^{2+} in the mitochondrial matrix and decreased Ca^{2+} release into the cytosol (**Figure 3A**, [8, 11]). Together with the ER and plasma membrane, mitochondrial Ca^{2+} uptake is responsible for Ca^{2+} clearance from the cytosol during signaling events [4]. In mitochondria, controlled increases in Ca^{2+} concentrations are known to promote oxidative phosphorylation, as long as they are within a specific "Goldilocks" concentration range [38]. This concentration-dependent stimulation of oxidative phosphorylation is due to the lack of activation of vital matrix metabolic enzymes when mitochondrial Ca^{2+} is too low, and opening of the mitochondrial permeability transition pore (mPTP) and inhibition of respiration when Ca^{2+} levels are too high [38]. In this sense, Ca^{2+} efflux pathways are important to prevent Ca^{2+} buildup within the mitochondrial matrix, avoiding toxicity [39].

Prior effects of NCLX in the literature have been mechanistically associated with changes in intra or extramitochondrial Ca^{2+} [8-15], both of which are plausible mechanisms capable of modulating autophagic activity. In order to identify mechanisms in which NCLX impacted on autophagy, we measured ATP synthesis through oxidative phosphorylation in intact AML12 cells using extracellular flux analysis. We found that neither treatment with CGP nor NCLX KD resulted in significant alterations in basal or ATP-linked mitochondrial oxygen consumption rates (ATP-linked OCR) (**Figure 4A**, Fig. S4A), a result consistent with the finding that NCLX inhibition maintains intramitochondrial calcium concentrations under control conditions within the

"Goldilocks" range, without impacting on oxidative phosphorylation [38]. Thus, it is unlikely that decreased autophagy activation in the absence of NCLX is related to alterations in intramitochondrial Ca^{2+} concentrations or subsequent changes in cellular ATP turnover.

We then questioned if NCLX could regulate cytosolic Ca^{2+} levels, which were assessed through Fura-2 fluorescence ratios upon stimulation with 100 µM ATP and in the presence of DMSO (control) or CGP (**Figure 4B**). Interestingly, although acute inhibition of NCLX did not affect the ATP-induced Ca^{2+} peak (**Figure 4B**), clearance of Ca^{2+} from the cytosol was faster, as shown by a substantial reduction in the half-life decay and the area under the curve (AUC) (**Figure 4B**). Indeed, decreased cytoplasmic Ca^{2+} levels due to NCLX inhibition have been observed in prior studies [11,12]. Consistently, siNCLX cells presented decreased Ca^{2+} release after ATP stimulus (Fig S4B [9,12,40,41]). In KD cells, the half-life decay was strikingly higher (Fig S4B), indicating that other Ca^{2+} clearance pathways are strongly affected by decreased NCLX expression, and further associating reduced NCLX activity to impaired cytoplasmic Ca^{2+} signals. Indeed, NCLX KD has been extensively associated with decreased cellular Ca^{2+} entry and, consequently, low Ca^{2+} levels in the ER [10,12,42-44].

To test whether the changes in cytoplasmic Ca^{2+} homeostasis could lead to defective autophagy, we pre-incubated AML12 cells for 1 hour with the cell-permeant Ca^{2+} chelator BAPTA-AM before incubation with HBSS. Interestingly, pre-incubation with BAPTA-AM resulted in similar autophagy impairment to that observed in the absence of NCLX activity, as shown by the accumulation of LC3 I and a significant reduction of LC3 II in the presence of BafA1 (**Figure 4C**). To test if NCLX inhibition effects on autophagy require cytosolic Ca^{2+} signaling, we verified the concomitant effects of CGP and BAPTA-AM, and did not observe any additive effect of CGP in cells pre-incubated with the Ca^{2+} chelator (**Figure 4C**). Collectively, these results demonstrate that mitochondrial Ca^{2+} efflux through NCLX is involved in cytosolic Ca^{2+} signaling events required for the early steps of autophagy activation.

Intracellular Ca^{2+} signals are generated during amino acid and serum starvation, which is known to be required for autophagy activation [45]. However, the origin of this Ca^{2+} and its downstream effects are still debated. Mechanistically, the elevation in cytoplasmic Ca^{2+} has been proposed to activate autophagy through the CaMKKI-β-AMPK-mTORC1 pathway [45,46]. On the other hand, recent results show that starvation-induced transient increases in Ca^{2+} concentration generated on the ER surface trigger liquid-like FIP200 puncta formation in autophagosome formation sites, necessary for autophagy activation. Interestingly, Ca^{2+} efflux from lysosomes showed the same effect [47]. In agreement, we show here that Ca^{2+} efflux from mitochondria through NCLX also regulates FIP200 puncta formation during starvation. Accordingly, ER-mitochondria contact sites are proposed as a location for autophagosome formation [48], thus supporting the presence of NCLX in autophagosome formation sites. The schematic summary of our findings regarding the interplay between NCLX and autophagy is shown in **Figure 4D**.

Our findings add to the current knowledge by showing that NCLX is both regulated by autophagy-inducing nutrient deprivation and contributes to autophagy activation, under basal and induced conditions, as seen by both silencing and acute pharmacological inhibition experiments. These results indicate that NCLX is an important new regulatory node, integrating autophagy control by Ca^{2+} and cellular responses to nutrient availability.

Methods

Chemicals, reagents and antibodies

siRNA molecules, insulin-transferrin-selenium (ITS) solution, fetal bovine serum (FBS), DMEM/F-12, high glucose DMEM, HBSS, Ripa Buffer, Lipofectamine™ RNAiMAX, Lipofectamine™ 2000, Opti-MEM™, High-Capacity cDNA Reverse Transcription Kit, Platinum™ SYBR™ Green qPCR SuperMix-UDG, protease and phosphatase inhibitor cocktails, Pierce™ BCA Protein Assay Kit, Fura-2 AM, Ca^{2+} Green™-5N, and ProLong™ Diamond were purchased from

Thermo Fisher Scientific (Waltham, MA, USA). CGP 37157 (1114) was purchased from Tocris/Bio-Techne (Bristol, UK). BAPTA-AM (A1076), Bafilomycin A1 (SML1661), and Rapamycin (R0395) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ionomycin (11932) was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Primary antibodies against β-actin (8226; 1:5,000) were purchased from Abcam (Cambridge, UK). Primary antibodies against LC3 A/B (12741; 1:1,000), LC3B (2775; 1:1,000), and α-tubulin (3873; 1:10,000) were purchased from Cell Signaling Technology (Danvers, MA, USA). NCLX antibody (orb317904; 1:500) was purchased from Biorbyt (Cambridge, UK).

Caloric restriction diet and mitochondrial isolation

Eight-week-old male Swiss mice were divided randomly into *ad libitum* (AL) and caloric restriction (CR) groups, eating 40% less calories than AL animals, with micronutrient supplementation, for 4 weeks [30]. The diet was adjusted weekly according to the AL animals' food intake. Both chow diets were prepared by Rhoster® (Campinas, SP, Brazil). All animal experiments were approved by the animal ethical committee and followed National Institutes of Health guidelines for animal welfare.

After the diet period, mitochondria were isolated from the liver of mice as described previously [49]. Briefly, the livers were minced, washed repeatedly with ice-cold PBS and suspended in isolation buffer (250 mM Sucrose, 10 mM HEPES, 1 mM EGTA, 1 mM EDTA, 1 mg/ml BSA fatty acid-free). Tissues were homogenized using a Potter-Elvehjem tissue grinder, and mitochondria were isolated by differential centrifugation.

Cell cultures

AML12 cells were purchased from ATCC® and cultivated in complete media (CM) consisting of DMEM/F-12 media supplemented with 10% FBS (v/v), 1% antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin), and 1% ITS (Gibco™, 41400045). HEK 293FT cells (kindly donated by Prof.

Alexandre-Bruni Cardoso, Universidade de São Paulo) and wild-type MEFs [50,51] (a gift from Noboru Mizushima, University of Tokyo) were cultivated in High Glucose DMEM media supplemented with 10% FBS (v/v) and 1% antibiotics. Cells were kept in a humidified incubator with a 5% CO_2 atmosphere, at 37°C, and trypsinized every 48-72 hours. The passage number was controlled up to 35. HBSS (Gibco™, 14175095) supplemented with 120 mg/L $CaCl_2$, 30 mg/L $MgCl_2$, and 50 mg/L $MgSO_4$ was used for serum and amino acid starvation experiments.

Small interference RNA (siRNA) transfection

AML12 cells were transfected with siRNAs targeting NCLX (Ambion®, ID s100747) or with a Negative Control (NC) sequence (Ambion®, 4390843) at a final concentration of 20 nM. Transfections were performed using the Lipofectamine™ RNAiMAX (Invitrogen™, 13778030) reagent through a reverse transfection protocol. Briefly, Lipofectamine™ and siRNAs were mixed in Opti-MEM™ media and incubated for 5-10 minutes at room temperature. Cells were suspended in transfection media (DMEM/F-12, 10% FBS, 1% ITS, without antibiotics) and added to the plate well on top of the Lipofectamine™-siRNA complexes. The transfection media was replaced by CM 24 hours later, and experiments were performed 48-72 hours after transfection. Importantly, for experiments performed at 72 hours, the cells were trypsinized and re-seeded in new culture plates 24 hours earlier.

RT-qPCR

RNA was isolated from cells using Trizol® reagent following the manufacturer's instructions. The concentration of isolated RNA was quantified using a NanoDrop® spectrophotometer and 1-2 µg was used for cDNA synthesis with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, 4368814). qPCR reactions were conducted using a 7500 Real-Time PCR System (Applied Biosystems™) with Platinum™ SYBR™ Green qPCR SuperMix-UDG (Invitrogen™, 11733046), 10 ng of cDNA, and the specific primers described in Supplementary Table 1. The Ct

values were analyzed through the $2^{(-\Delta\Delta Ct)}$ method and the housekeeping gene or combination of genes was chosen by testing the expression of *Hmbs*, *B2m* and *Actb* in each experimental group. The compared stability value was calculated using NormFinder Software [52]. The mean Ct value between *Hmbs* and *B2m* expression was used as housekeeping for experiments containing siRNA-mediated knockdown. For experiments containing HBSS time curve treatments, a Ct value of *B2m* was used.

Western blots

Cells were washed with 1X ice-cold PBS and lysed in RIPA buffer containing protease and phosphate inhibitor cocktail (Thermo Scientific™, 78443). Cell lysates were sonicated using a probe sonicator and protein concentration was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific™, 23227). Samples were prepared in 1X Laemmli buffer (2% SDS, 10% glycerol, 0.062 M Tris pH 6.8, 0.002% bromophenol blue, 5% 2-mercaptoethanol), loaded onto SDS-PAGE for gel electrophoresis separation, and transferred overnight to PVDF membranes using a wet transfer system. Membranes were blocked in TTBS (20 mM Tris pH 7.5, 150 mM NaCl and 0.1% Tween 20) containing 3-5% BSA (w/v) before overnight incubation with primary antibodies at 4°C. After washing with TTBS, membranes were incubated with fluorescent IRDye® secondary antibodies (1:15,000) for 1 hour at room temperature. Fluorescence was detected using the ChemiDoc™ Imaging System (Bio-Rad) and band densitometry was quantified using Fiji ImageJ software.

mCherry-EGFP-LC3B expression, imaging, and analysis

To evaluate autophagic activity, we generated an AML12 cell line stably expressing mCherry-EGFP-tagged LC3B protein. Retroviruses were produced by transfecting HEK 293FT cells with a pBABE-puro-mCherry-EGFP-LC3B plasmid (Addgene, 22418; gifted by Jayanta Debnath) [35] and a PCL-Eco packaging plasmid (Addgene, 12371; gifted by Inder Verma) [53]. 48-72 hours after transfection, the media from the HEK 293FT cells was used to transduce AML12 cells through

multiple rounds of infection. 24 hours after the last infection, cells were kept in CM containing 10 µg/mL puromycin for five days. After selection, cells were cultivated in CM supplemented with 1 µg/mL puromycin, which was removed from the media during experiments.

For experiments, AML12-LC3 cells were transfected with specific siRNAs using the same protocol described in the "*Small interference RNA (siRNA) transfection"* section. 48 hours after transfection, cells were trypsinized, seeded on poly-D-lysine-coated 12 mm coverslips (Thorlabs, CG15NH) and incubated for additional 24 hours before treatments. After treatments, media was removed, and cells were fixed in 4% paraformaldehyde for 15 minutes at room temperature. Coverslips were washed once with 1X PBS containing 0.1 M Glycine, washed two times with 1X PBS and incubated with DAPI (0.1 µg/mL) for 10 minutes at room temperature. Finally, coverslips were washed three times with 1X PBS and mounted onto glass slides using ProLong™ Diamond Antifade Mountant (Invitrogen™, P36965). Images were acquired using a Leica DMi8 microscope with an HC PL APO CS2 63x/1.40 OIL UV objective. At least 100 cells per group were imaged and each channel was acquired through 2 µm on the Z-axis dimension (total of 6 steps). Images were processed using the Leica 3D BlindDeblur deconvolution tool and $mCherry^+/GFP^+$ (autophagosome), $mCherry^+/GFP^-$ (autolysosome), and cell number (DAPI) were quantified using Fiji ImageJ software.

Proteasome activity

72 hours after siRNA transfection, cells were trypsinized, washed once with 1X cold PBS and resuspended in experimental buffer (5 mM $MgCl_2$, 50 mM Tris pH 7.5). Next, cells were lysed using an insulin syringe and centrifuged for 10 min at 10000 × g, at 4°C for debris separation. Protein concentration was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific™, 23227) and 30 µg of protein (in 100 µL of experimental buffer) was added to each well for the assay. To measure proteasome (chymotrypsin-like) activity, cell extracts were incubated with 250 µM fluorogenic peptide Suc-LLVY-AMC and the increase in AMC fluorescence was monitored for 10 minutes at 37°C, using a Flex Station 3 (Molecular Devices) microplate fluorescence

spectrophotometer (λex = 341 nm, λem = 441 nm). The slope of fluorescence increase over time was quantified using GraphPad Prism 7 software.

Mitochondrial Ca^{2+} efflux in intact cells

To estimate mitochondrial Ca^{2+} efflux rates in live cells, we measured intramitochondrial free Ca^{2+} levels by transfecting AML12 cells with the CMV-mito-GEM-GECO1 plasmid (Addgene, 32461; gifted by Robert Campbell) [37]. Briefly, cells were seeded in a glass bottom culture dish (Greiner Bio-One, 627870) and, 24 hours later, the media was replaced with transfection media (DMEM/F-12, 10% FBS, 1% ITS, without antibiotics). The plasmid was transfected using Lipofectamine™ 2000 Transfection Reagent (Invitrogen™, 11668019) at a 1:1 µg DNA to µL Lipofectamine™ ratio, following the manufacturer's protocol. The media was replaced for CM 24 hours after transfection and the cells were imaged after an additional 24 hours. For imaging, the media was replaced for HBSS (5.5 mM D-glucose, 120 mg/L $CaCl_2$, 30 mg/L $MgCl_2$, 50 mg/L $MgSO_4$, 60 mg/L KH_2PO_4, 48 mg/L Na_2HPO_4, 400 mg/L KCl, 8 g/L NaCl, 350 mg/L $NaHCO_3$, 5 mM HEPES) containing either DMSO or CGP 37157 and fluorescence was acquired in a Zeiss LSM 780-NLO confocal microscope using a 405 nm diode laser and a C-Apochromat" 63x/1.20 DIC objective, in an environment kept at 37°C, with 5% CO_2 atmosphere. During acquisition, cells were imaged every 4 seconds for at least 15 minutes. 100 µM ATP was added to stimulate intramitochondrial Ca^{2+} increases and 10 µM ionomycin was used to compare the maximum fluorescence ratio between groups. Analysis was performed using the Fiji ImageJ software and results are shown as the ratio between emissions at 455 nm and 511 nm relative to the basal ratio (R_1/R_0). Mitochondrial efflux rates were calculated as the slope after the ATP-stimulated Ca^{2+} peak, using the GraphPad Prism 7 software.

Confocal immunofluorescence

For the visualization of FIP200 and ATG16L1 puncta, MEF cells were immunostained as follows. Briefly, cells were seeded onto 13 mm glass coverslips and treated as indicated. After treatments, the media was removed, and cells were fixed with 4% formaldehyde for 10 minutes at room temperature. Next, coverslips were washed with PBS and cells were permeabilized with a PBS solution containing 0.5% Triton X-100 for 10 minutes incubation. Cells were then washed 3 times with PBS and incubated with blocking solution (PBS, 5% Normal Goat Serum, 0.05% Tween) for 1 hour at room temperature. Primary antibodies for FIP200 (Cell Signaling Technology, 12436S;1:100) and ATG16L1 (Cell Signaling Technology, 8089S; 1:100) were incubated overnight at 4°C. Cells were washed 3 times with PBS containing 0,05% Tween (PBS-T), incubated for 1 hour at room temperature with secondary antibodies (Alexa Fluor® 488, 1:1000) and washed again 3 times with PBS-T. Coverslips were mounted using ProLong™ Gold Antifade Mountant with DAPI (Invitrogen™, P36935). Images were acquired using a Zeiss LSM700 confocal microscope with a Plan-Apochromat 63x/1.40 Oil DIC M27 objective. Quantification of puncta number per cell was performed using Fiji ImageJ software.

ATP-linked respiration

Mitochondrial oxygen consumption linked to ATP production was measured using a Seahorse Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA, EUA) as described previously [38]. Briefly, 3×10^4 cells per well were seeded in CM 24 hours before analysis. On the next day, cells were washed three times with DMEM/F-12 media supplemented with 1% antibiotics and 5 mM HEPES (without bicarbonate and FBS) and were incubated in the same media for 1 hour in the absence or presence of 10 µM CGP 37157, at 37°C in a normal atmosphere. Oxygen consumption rates were measured under basal conditions and after the injection of 1 µM oligomycin to calculate ATP-linked respiration. After the experiment, cells were lysed in Ripa Buffer and total protein was

quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific™, 23227) for data normalization.

Cytosolic Ca^{2+} measurements

Initially, cells were incubated with a mix of 5 µM Fura-2 AM (Invitrogen™, F1221) and 0.025% Pluronic® F-127 (diluted in CM) for 10 minutes at 37°C for Fura-2 loading. Next, cells were trypsinized, centrifuged, washed once with 1X PBS, and resuspended in HBSS (5.5 mM D-Glucose, 120 mg/L $CaCl_2$, 30 mg/L $MgCl_2$, 50 mg/L $MgSO_4$, 60 mg/L KH_2PO_4, 48 mg/L Na_2HPO_4, 400 mg/L KCl, 8 g/L NaCl, 350 mg/L $NaHCO_3$, 5 mM HEPES). The cell number was determined and 1×10^6 cells were added to a cuvette to a final volume of 2 mL. Importantly, for experiments using pharmacological inhibition, DMSO (equal volume, as a control) or CGP 37157 were added to the cuvette before the fluorescence measurement started. The cuvette was placed in a F4500 Hitachi Fluorimeter at 37ºC with continuous stirring and Fura-2 fluorescence was detected at λex = 340/380 nm and λem = 505 nm. The addition of 100 µM ATP was used to stimulate an increase in cytosolic Ca^{2+} levels, which were analyzed as the ratio between fluorescence emission at 340 nm and 380 nm excitation (340/380) relative to the basal ratio (R_1/R_0). Peak height, area under the curve (AUC) and half-life after the peak were calculated using GraphPad Prism 7 software.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 software. At least three independent biological replicates were conducted for each experiment, and these are represented by individual dots in the graphs. Dots of the same color/pattern in different groups represent paired biological replicates. Data comparing two groups were analyzed with Student's t-test. Data comparing three different groups were analyzed with one-way randomized blocks ANOVA followed by Bonferroni's multiple comparison test. Data comparing two factors (e.g., siRNA and Bafilomycin A1) were analyzed with two-way randomized blocks ANOVA followed by either Bonferroni's (when

comparing only column effects) or Tukey's (when comparing rows and column effects) multiple comparisons test. Results are shown as means ± SD and * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, ns = not significant.

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Disclosure statement

V.I.K is a Scientific Advisor for Longaevus Technologies. Other authors declare that they have no conflicts of interest with the contents of this article.

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Figures legends

Figure 1. NCLX expression is increased by nutrient restriction. (**A**) Western blot analysis of NCLX protein levels in mitochondria isolated from the livers of Swiss mice after 4 months of *ad libitum* (AL) or caloric restriction (CR) diets (n = 7 per group). Data were analyzed by two-tailed unpaired t test. (**B**) RT-qPCR for NCLX mRNA in AML12 cells incubated with either complete media (CM) or HBSS for 3, 6, and 24 hours (n = 4). Data were analyzed by two-way RM ANOVA followed by Bonferroni's multiple comparisons test. Bar graphs show means ± SD. Dots in each group represent biological replicates. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = not significant.

Figure 2. Decreased NCLX expression causes impaired autophagic activity. AML12 cells transfected with NCLX (siNCLX) or negative control (siNC) siRNAs were analyzed by: (**A**) RT-qPCR for NCLX mRNA, 48 (n=6) and 72 (n=5) hours after transfection. Data were analyzed by two-tailed paired t test. (**B**) Western blot of NCLX protein levels 72 hours after transfection. (**C**) Western blots for LC3 II and LC3 I after 1 hour incubation with either complete media (CM) or HBSS (n = 6). (**D**) Western blot of LC3 II after 1 hour incubation with HBSS in the absence or presence of 100 nM Bafilomycin A1 (BafA1) during the last 30 min of incubation (n = 6). Data were analyzed by two-way RM ANOVA followed by Tukey's multiple comparisons test. (**E**) LC3 II net flux in siNC and siNCLX cells incubated with HBSS. Data were analyzed by two-tailed unpaired t test. (**F**) AML12 stably expressing the mCherry-EGFP-LC3B probe, showing representative images and

quantification of autophagosomes ($mCherry^+/GFP^+$; yellow puncta) and autolysosomes ($mCherry^+/GFP^-$; red puncta) per cell after 1 hour incubation with HBSS (n = 4). Data were analyzed by two-tailed unpaired t test. (**G**) Western blot for LC3 II in complete media (CM) conditions and in the absence or presence of 100 nM Bafilomycin A1 (+BafA1) for 1 hour (n = 6). Data were analyzed by two-way RM ANOVA followed by Tukey's multiple comparisons test. (**H**) LC3 II net flux in siNC and siNCLX cells incubated with complete media. (**I**) AML12 stably expressing the mCherry-EGFP-LC3B probe, showing representative images and quantification of autophagosomes ($mCherry^+/GFP^+$; yellow puncta) and autolysosomes ($mCherry^+/GFP^-$; red puncta) per cell (n = 4). Data were analyzed by two-way RM ANOVA followed by Tukey's multiple comparisons test. Data are presented in columns with means ± SD. Dots in each group represent biological replicates and paired biological replicates are represented by equally colored symbols. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = not significant.

Figure 3. Acute NCLX inhibition decreases autophagy activation by starvation. (**A**) Measurements of intramitochondrial Ca^{2+} levels to evaluate efflux rates after 100 µM ATP stimulation in the presence of vehicle (DMSO) or 10 µM CGP 37157 (CGP) (n = 3). Data were analyzed by two-tailed paired t test. (**B**) Western blot analysis of LC3 II in AML12 cells after 1 hour incubation with HBSS in the absence or presence of Bafilomycin A1 (BafA1) during the last 30 min of treatment (n = 6). Data were analyzed by two-way RM ANOVA followed by Tukey's multiple comparisons test. LC3 II net flux of DMSO- and CGP-treated cells were analyzed by two-tailed unpaired t test. (**C**) MEF cells; experiments performed as in B (n = 4). (**D**) Immunofluorescence analysis of FIP200 and ATG16L1 puncta formation in MEF cells (n = 3). Data were analyzed by one-way RM ANOVA followed by Bonferroni's multiple comparisons test. Data are presented in columns with means ± SD. Dots in each group represent biological replicates and paired biological replicates are represented by equally colored symbols. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = not significant.

Figure 4. Impaired autophagy is a result of reduced cytoplasmic Ca^{2+}. Oxygen consumption rates (OCR) linked to ATP synthesis (oligomycin-sensitive respiration) of AML12 cells treated with either DMSO or CGP (n = 4). (**B**) Cytosolic Ca^{2+} measurements using Fura-2 AM after stimulation with 100 µM ATP; bar graphs showing quantifications of Ca^{2+} peaks, the half-life of one phase decay after the peak and area under the curve (AUC) (n = 4). Data were analyzed by two-tailed paired t test. (**C**) Western blots for LC3 I and LC3 II in AML12 cells pre-incubated with 50 µM BAPTA-AM for 1 hour prior to incubation with HBSS for 1 hour in the presence either DMSO or CGP (n = 6). Data were analyzed by two-way RM ANOVA followed by Tukey's multiple comparisons test. Data are presented in columns with means or means ± SD. Dots in each group represent biological replicates and paired biological replicates are represented by equally colored symbols. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$, ns = not significant. (**D**) Schematic representation of our hypothesis over NCLX regulation of autophagy.

Extended Figure 1. RT-qPCR for MCU mRNA in AML12 cells incubated with either complete media (CM) or HBSS for 3, 6, and 24 hours (n = 4).

Extended Figure 2. AML12 cells transfected with NCLX (siNCLX) or negative control (siNC) siRNAs were analyzed by RT-qPCR for LC3A and LC3B mRNA (n =6). Data were analyzed by two-tailed paired t test. Data are presented in columns with means ± SD. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = not significant.

Extender Figure 3. Western blots for LC3 II and LC3 I of AML12 cells after 1 hour incubation with either complete media (CM) or HBSS in the presence of either DMSO or CGP (n = 6). Data were analyzed by two-way RM ANOVA followed by Tukey's multiple comparisons test. Data are presented in columns with means ± SD. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = not significant.

Extended Figure 4. (**A**) Oxygen consumption rates (OCR) linked to ATP synthesis (oligomycin-sensitive respiration) of AML12 cells transfected with NCLX (siNCLX) or negative control (siNC)

siRNAs (n = 4). (**B**) Cytosolic Ca^{2+} measurements using Fura-2 AM after stimulation with 100 µM ATP; bar graphs showing quantifications of Ca^{2+} peaks, the half-life of one phase decay after the peak and area under the curve (AUC) (n = 5). Data were analyzed by two-tailed paired t test. Data are presented in columns with means ± SD. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = not significant.

Tables

Supplementary Table 1. RT-qPCR primers.

Gene	Forward (5' - 3')	Reverse (5' - 3')
Map1lc3a	ATGAGCGAGTTGGTCAAGATC	CGTCTTCATCCTTCTCCTGTTC
Map1lc3b	TTATAGAGCGATACAAGGGGGAG	CGCCGTCTGATTATCTTGATGAG
Mcu	ACTCACCAGATGGCGTTCG	CATGGCTTAGGAGGTCTCTCTT
Slc8b1	TGTCACCTTCCTGGCCTTTG	CACCCCTGCACCAAACAGA
Actb	TCACTATTGGCAACGAGCG	AGGTCTTTACGGATGTCAACG
Hmbs	CAGCTACAGAGAAAGTTCCCC	AGGACGATGGCACTGAATTC
B2m	CTGGTCTTTCTATATCCTGGCTC	TGCTTGATCACATGTCTCGATC

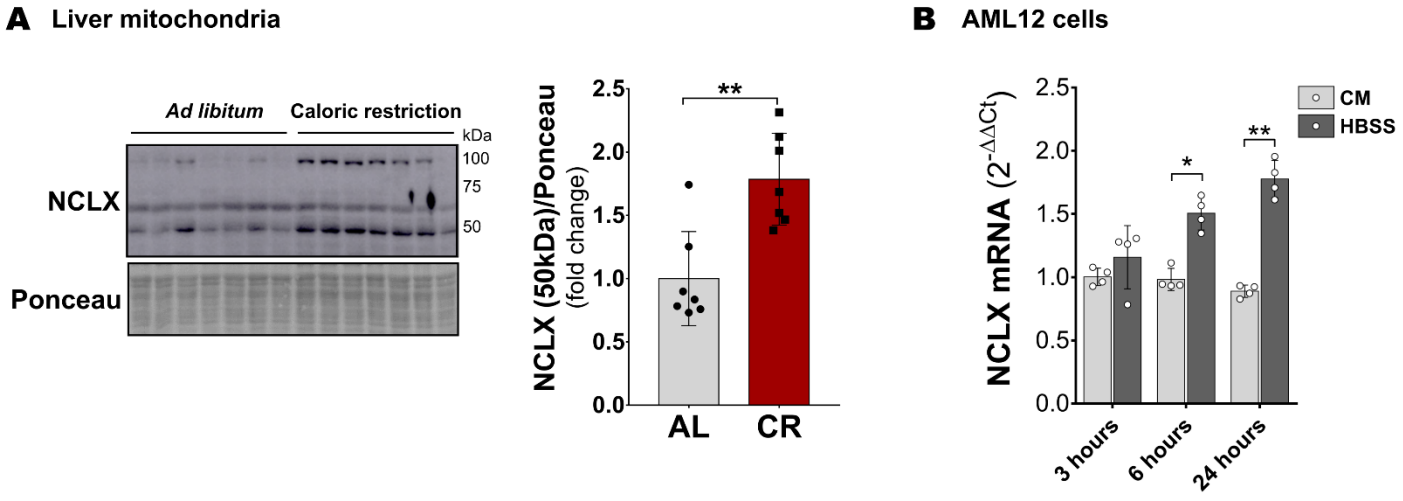

A Liver mitochondria
Ad libitum
Caloric restriction
kDa
100
75
50
NCLX
Ponceau
NCLX (50kDa)/Ponceau
(fold change)
2.5
2.0
1.5
1.0
0.5
0.0
**
AL
CR
B AML12 cells
NCLX mRNA (2^-ΔΔCt)
CM
HBSS
*
**
3 hours
6 hours
24 hours

Figure 1

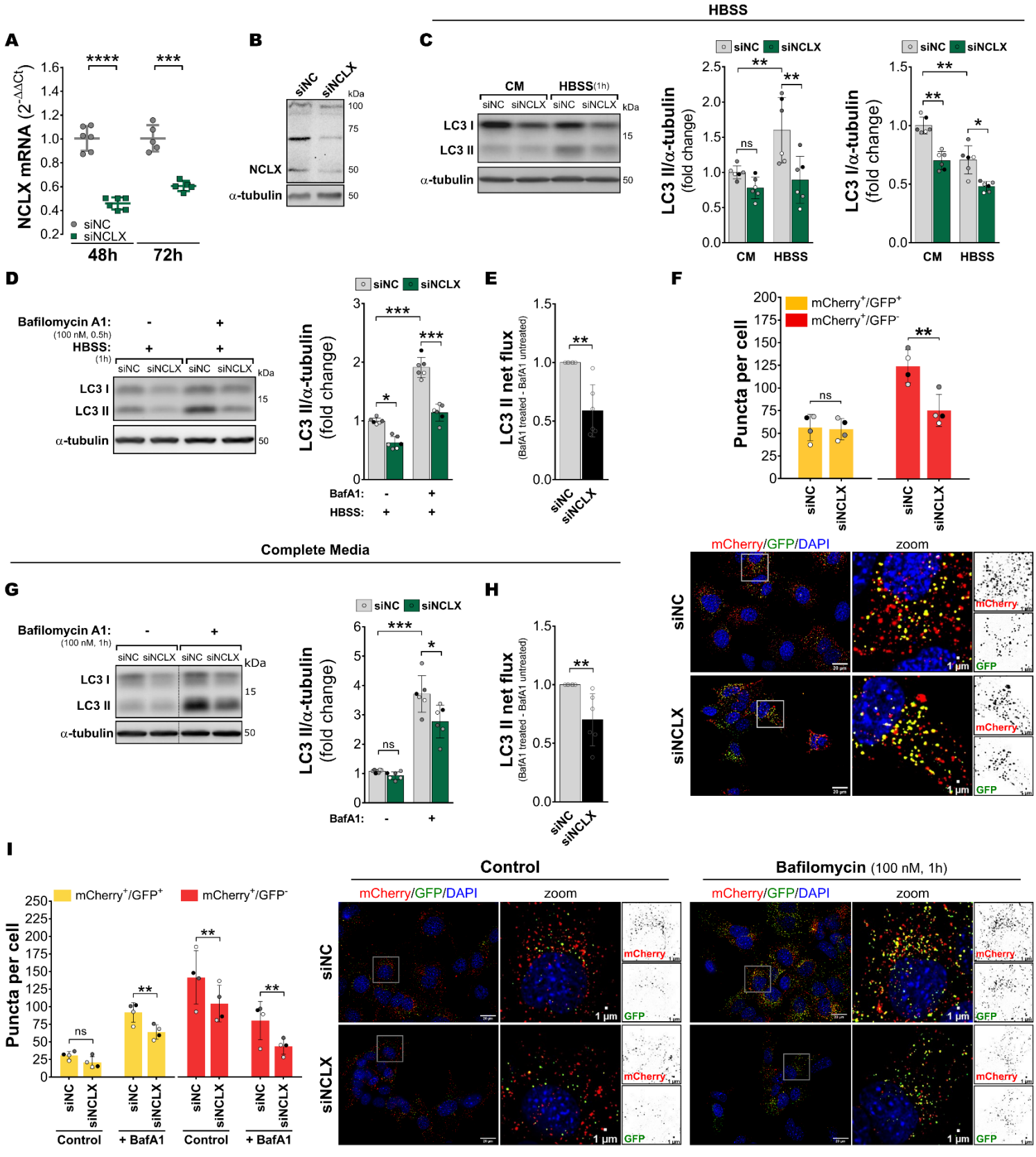

HBSS
A
NCLX mRNA ($2^{-\Delta\Delta Ct}$)
siNC
siNCLX
48h
72h
B
siNC
siNCLX
kDa
NCLX
α-tubulin
C
CM
HBSS(1h)
LC3 I
LC3 II
α-tubulin
LC3 II/α-tubulin (fold change)
LC3 I/α-tubulin (fold change)
D
Bafilomycin A1:
(100 nM, 0.5h)
HBSS:
(1h)
E
LC3 II net flux
(BafA1 treated - BafA1 untreated)
F
Puncta per cell
mCherry+/GFP+
mCherry+/GFP-
Complete Media
G
(100 nM, 1h)
BafA1:
H
mCherry/GFP/DAPI
zoom
mCherry
GFP
I
Control
Bafilomycin (100 nM, 1h)
+ BafA1

Figure 2

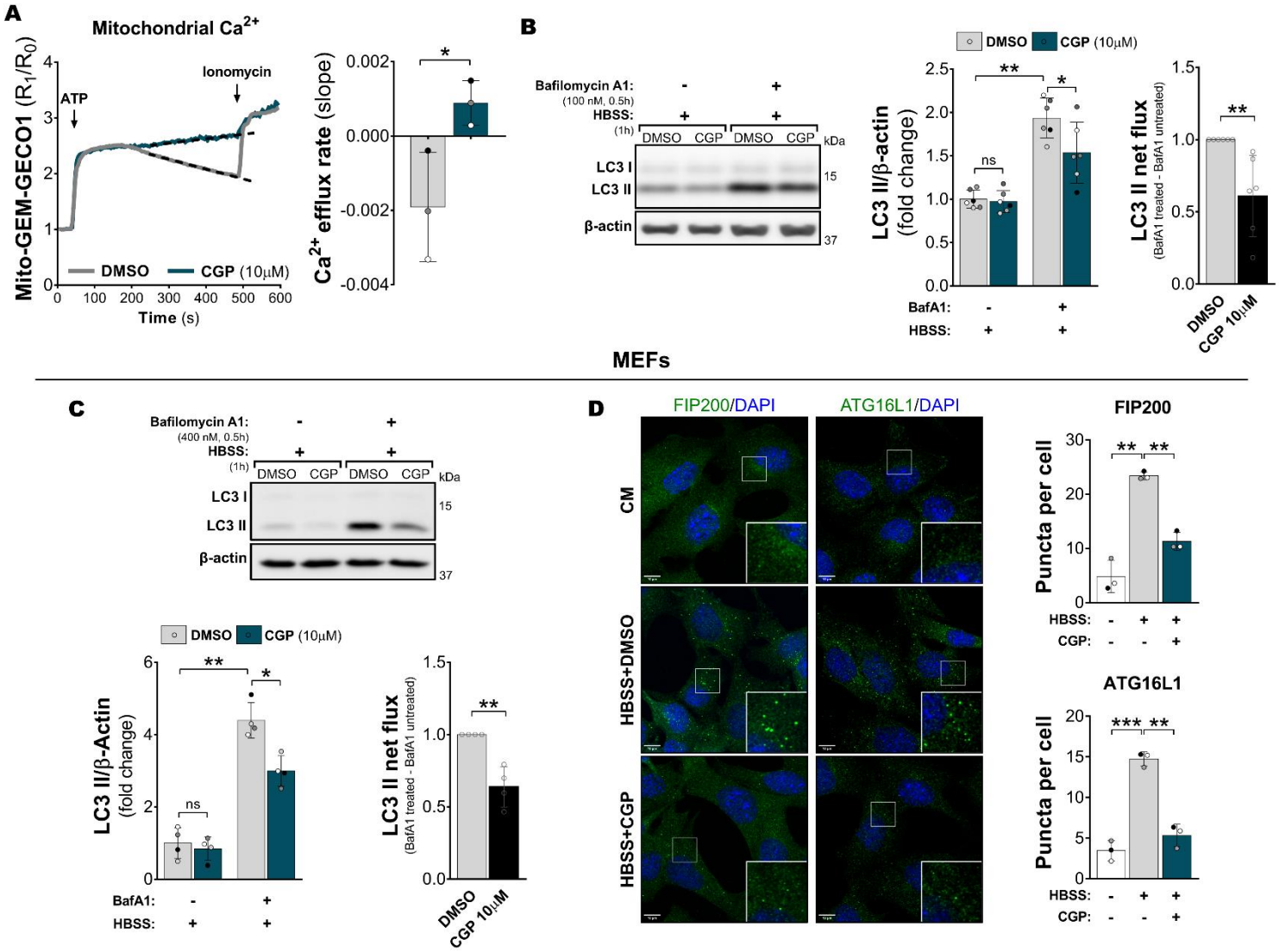

A
Mitochondrial Ca2+
Mito-GEM-GECO1 (R1/R0)
ATP
Ionomycin
DMSO
CGP (10μM)
Time (s)
Ca2+ efflux rate (slope)
B
Bafilomycin A1:
(100 nM, 0.5h)
HBSS:
(1h)
DMSO
CGP
kDa
LC3 I
LC3 II
β-actin
LC3 II/β-actin (fold change)
LC3 II net flux (BafA1 treated - BafA1 untreated)
BafA1:
HBSS:
MEFs
C
(400 nM, 0.5h)
D
FIP200/DAPI
ATG16L1/DAPI
CM
HBSS+DMSO
HBSS+CGP
FIP200
ATG16L1
Puncta per cell
CGP:

Figure 3

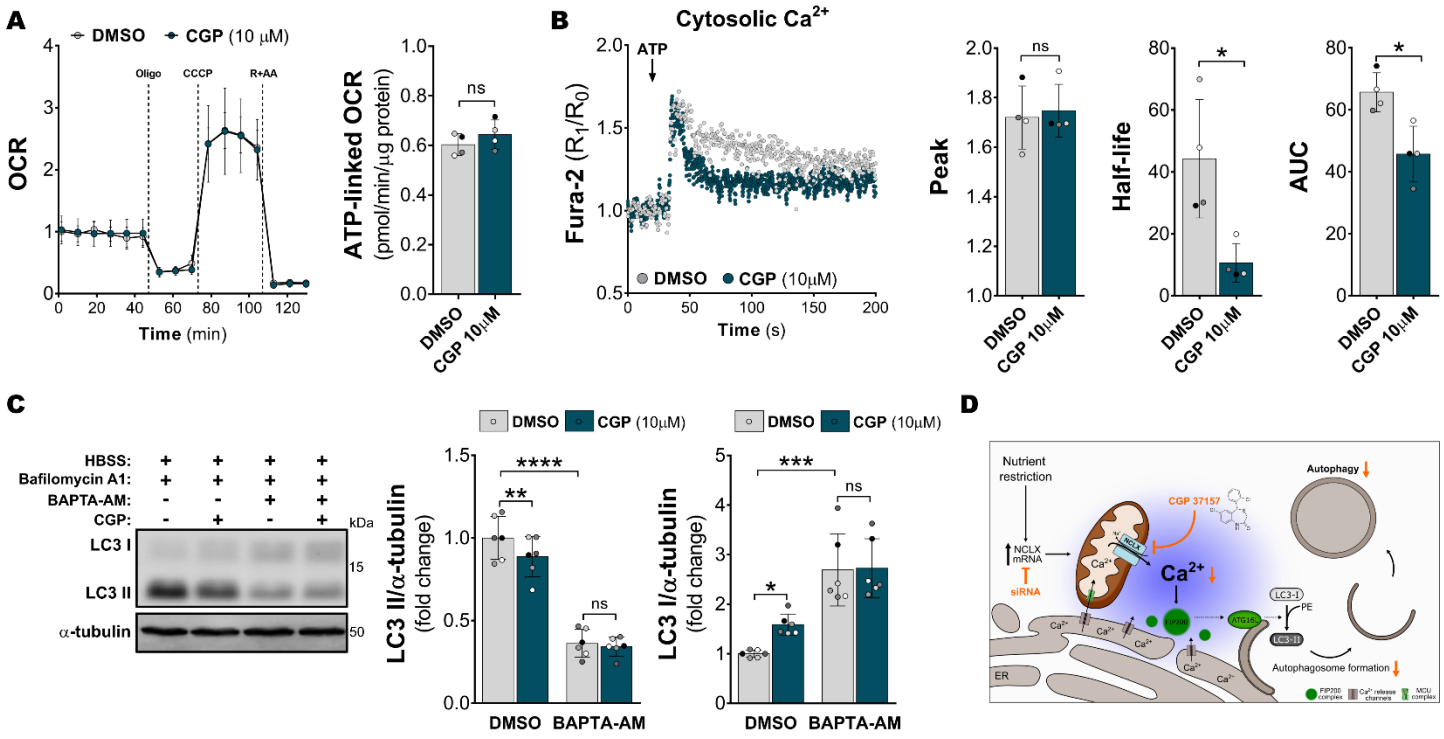
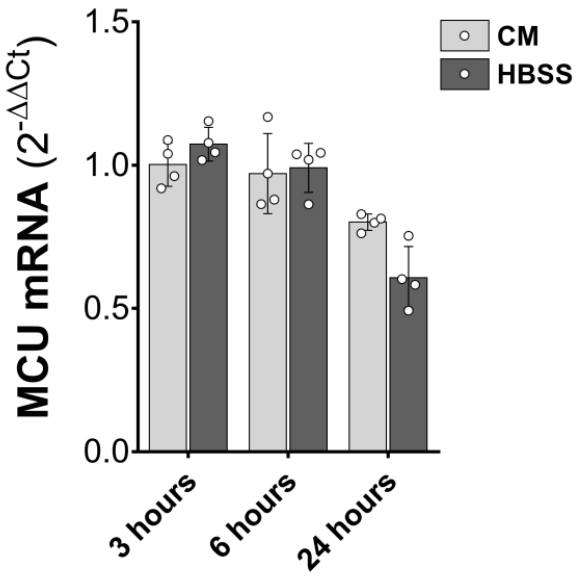
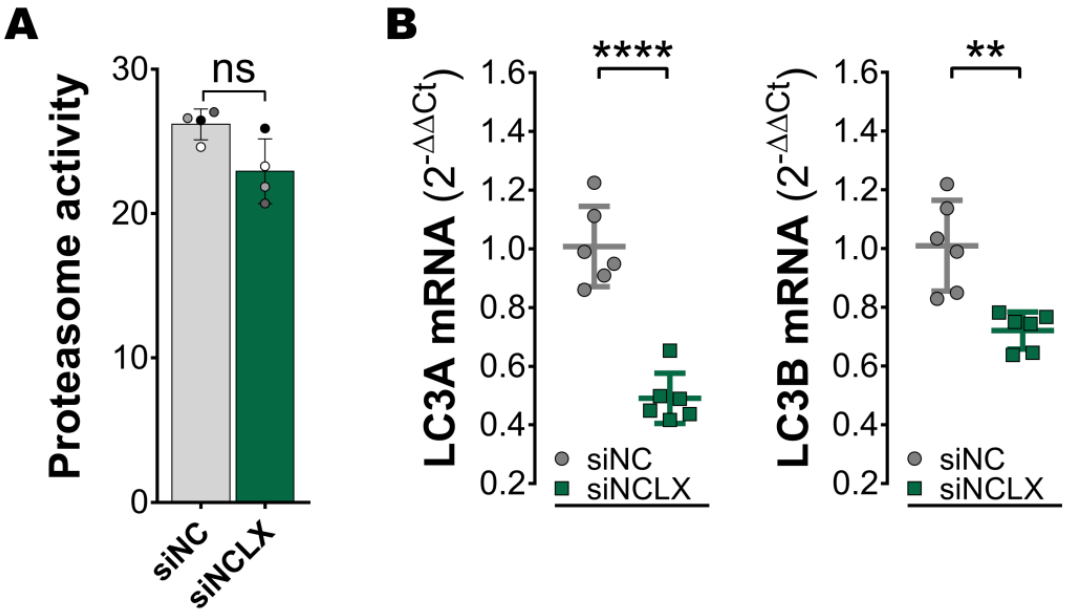

A
DMSO
CGP (10 μM)
Oligo
CCCP
R+AA
OCR
Time (min)
ATP-linked OCR
(pmol/min/μg protein)
ns
DMSO
CGP 10μM
B
Cytosolic Ca2+
ATP
Fura-2 (R1/R0)
DMSO
CGP (10μM)
Time (s)
Peak
Half-life
AUC
C
HBSS:
Bafilomycin A1:
BAPTA-AM:
CGP:
kDa
LC3 I
LC3 II
α-tubulin
15
50
LC3 II/α-tubulin
(fold change)
LC3 I/α-tubulin
(fold change)
DMSO
BAPTA-AM
D
Nutrient restriction
NCLX mRNA
siRNA
CGP 37157
Ca2+
Autophagy
LC3-I
PE
LC3-II
ATG16L
Autophagosome formation
ER

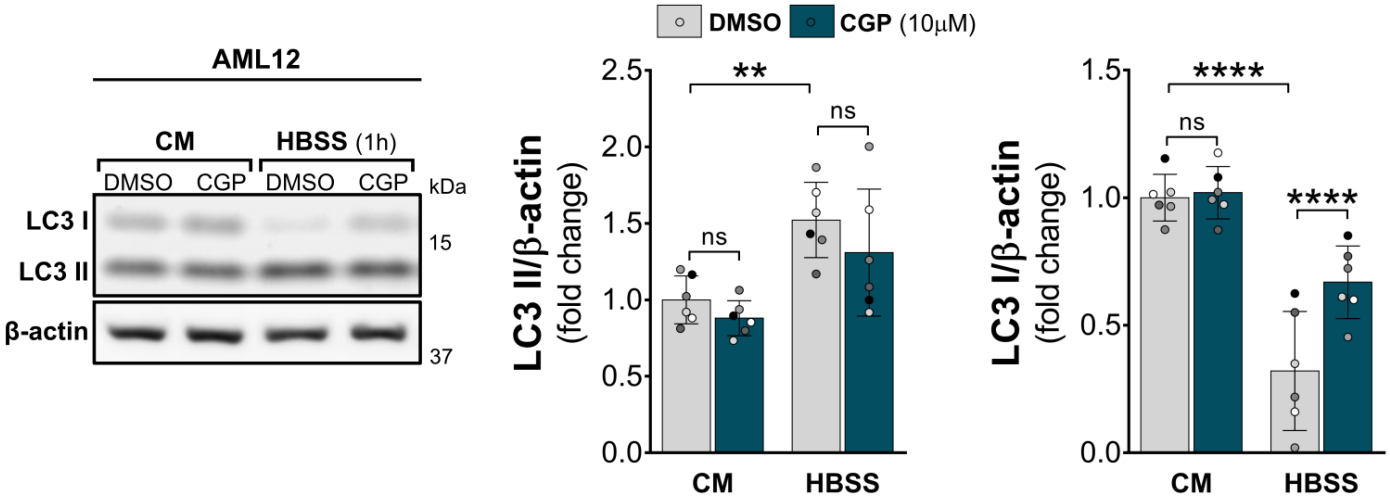
Figure 4



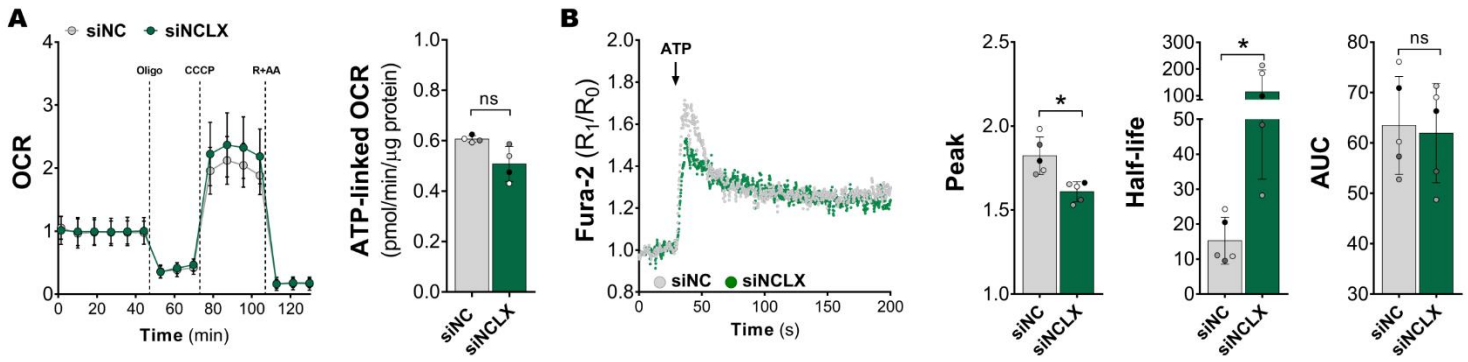
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4