Proteaphagy in mammalian cells can function independent of ATG5/ATG7

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Abbreviations:

ACN: Acetonitrile
ATG: autophagy-related
BOD-TMR-Epox: Bodipy-TMR-epoxomycin
BTZ: bortezomib
CMA: chaperone-mediated autophagy
DHFR: dihydrofolate reductase
HEK: human embryonic kidney
HMGCoA: 3-hydroxy-3-methylglutaryl-Coenzyme A
KEGG: Kyoto Encyclopedia of Genes and Genomes
LAMP: lysosome-associated membrane protein
LC3: microtubule-associated protein light chain 3
MEF: mouse embryonic fibroblast
NBR1: Neighbor of BRCA1 gene 1 protein
NCO4: nuclear receptor coactivator 4
NUFIP1: nuclear FMR1-interacting protein 1
PSMA: proteasome subunit alpha
PSMB: proteasome subunit beta
PSMC: regulatory proteasome subunit
PSMD: 26S proteasome regulatory subunit
SILAC: stable isotope labeling in cell culture
SNARES: soluble N-ethylmaleimide-sensitive-factor attachment receptors
SQSTM1: sequestosome-1/p62
TOLLIP: Toll-interacting protein
UPS: ubiquitin-proteasome system
Abstract.

The degradation of intra- and extracellular proteins is essential in all cell types and mediated by two systems, the ubiquitin-proteasome system (UPS) and the autophagy-lysosome pathway. This study investigates the changes in autophagosomal and lysosomal proteomes upon inhibition of proteasomes by bortezomib (BTZ) or MG132. We find an increased abundance of more than 50 proteins in lysosomes of cells in which the proteasome is inhibited. Among those are dihydrofolate reductase (DHFR), ß-Catenin and 3-hydroxy-3-methylglutaryl-coenzym-A (HMGCoA)-reductase. Since these proteins are known to be degraded by the proteasome they seem to be compensatorily delivered to the autophagosomal pathway when the proteasome is inactivated. Surprisingly, most of the proteins which show increased amounts in the lysosomes of BTZ or MG132 treated cells are proteasomal subunits. Thus an inactivated, non-functional proteasome is delivered to the autophagic pathway. Native gel electrophoresis shows that the proteasome reaches the lysosome intact and not disassembled. Adaptor proteins, which target proteasomes to autophagy, have been described in Arabidopsis, Saccharomyces and upon starvation in mammals. However, in cell lines deficient of these proteins or their mammalian orthologues, respectively, the transfer of proteasomes to the lysosome is not impaired. Obviously, these proteins do not play a role as autophagy adaptor proteins in mammalian cells. We can also show that chaperone-mediated autophagy (CMA) does not participate in the proteasome delivery to the lysosomes. In autophagy-related (ATG)-5 and ATG7 deficient cells the delivery of inactivated proteasomes to the autophagic pathway was only partially blocked, indicating the existence of at least two different pathways by which inactivated proteasomes can be delivered to the lysosome in mammalian cells.
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Introduction

Two distinct mechanisms, the autophagy-lysosome pathway and the UPS, mediate protein degradation within the cell. Lysosomes are membrane-bound organelles mediating the degradation of intracellular macromolecules like proteins, lipids and oligosaccharides. Endocytosed cargo and autophagocytosed cell components are degraded by a wide array of acid hydrolases. The delivery of substrates to the lysosomal lumen is mostly accomplished by vesicle transport, through endosomes and phagosomes for extracellular material or autophagosomes for the degradation of intracellular material. Alternatively, lysosomal substrates can be delivered by CMA or endosomal microautophagy. The generation of autophagosomes starts by the formation and growth of a double-layered isolation membrane enabled by specialized autophagy proteins like the microtubule-associated protein light chain 3 (LC3) in the cytoplasm, engulfing cargo that is to be degraded. This engulfment can be mediated by adaptor proteins for specific cargo in case of selective autophagy, as has been shown for ribosomes in case of the nuclear FMR1-interacting protein 1 (NUFIP1) or nuclear receptor coactivator 4 (NCOA4) for ferritin degradation, respectively. Often selective autophagy of a specific cargo is preceded by its ubiquitination, which in turn enables attachment to ubiquitin-binding domain-containing proteins like sequestosome-1/p62 (SQSTM1) or Neighbor of BRCA1 gene 1 protein (NBR1). After closing of the autophagosomal membrane, autophagosomes and lysosomes fuse by the action of several proteins like soluble N-ethylmaleimide-sensitive-factor attachment receptors (SNARES) and the inner autophagosomal membrane including its content is degraded by lysosomal hydrolases.

In contrast to the organelles lysosomes and autophagosomes, proteasomes reside as 2.5 mega Dalton protein complexes within the cytoplasm. They are composed of the 20S core complex containing 4 hetero-hepteromeric rings, each containing either seven α- or β-subunits. Six catalytic subunits in total, two of each β1, β2 and β5 subunits with caspase-like, trypsin-like and chymotrypsin-like proteolytic properties mediate the cleavage of the peptide bonds within the barrel of the 20S core complex. The 19S regulatory complex can be further...
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divided into lid and base parts, and its subunits mediate e.g. binding of ubiquitinated substrates and the removal of ubiquitin before entry of the substrate into the narrow barrel of the 20S proteosome. Proteasomes exclusively degrade proteins and due to their structure can only do so when proteins are unfolded and selectively labeled with polyubiquitin chains on their lysine residues. This ubiquitination is mediated by a sequential action of the E1-ubiquitin-activating enzymes, which activate the C-terminal Gly residue of ubiquitin and then transfers it to the E2-ubiquitin carrier family. From there it is attached to a lysine residue of the target protein by highly specific members of the E3-ubiquitin ligase family. While initially the autophagy/lysosome system and the proteasome were regarded as two independent systems within the cell, in recent years it has become increasingly obvious that those systems are interconnected as both can degrade ubiquitinated substrates. Many proteins have been shown to be degraded by both pathways, and an increased autophagic protein degradation in case of proteasome impairment indicates compensatory mechanisms.

In this study, we analyzed the impact of proteasomal inhibition on the composition of the proteome of lysosomes and autophagosomes. The activity of the proteasome was inhibited by either BTZ or MG132, and the composition of a lysosome-enriched fraction was analyzed by mass spectrometry. Among other proteins this revealed an increased presence of proteasome complexes within the lysosomal compartment where they are degraded. An in-depth investigation to specify the detailed mechanism revealed that inactivated proteasomes are most likely degraded by macroautophagy and not by chaperone-mediated autophagy, but do still reach lysosomes when either ATG5 or ATG7 are deficient. This process is neither dependent on Toll-interacting protein (TOLLIP); the adaptor protein for proteaphagy in yeast, nor SQSTM1, and is not accompanied by an increased association of any known macroautophagy adaptor protein to inactivated proteasomes.

Materials and Methods

Antibodies: The following antibodies were used: anti-lysosome-associated membrane protein (LAMP)-2 and anti-LAMP1 (Hybridoma Bank, University of Iowa, USA, Clones
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ABL93 and H4B4 and Clone H4A3, respectively), anti-Cathepsin D (Santa Cruz Biotechnology, Dallas, United States), anti-Proteasome 19S S4 (Abcam, Cambridge, United Kingdom), anti-Proteasome 26S S3 (Abcam, Cambridge, United Kingdom), anti-Proteasome subunit α (PSMA) 7 (Proteintech, Rosemont, United States), anti-Proteasome subunit β (PSMB) 5 (Cell Signaling Technologies), anti-PSMD4 (Proteintech, Rosemont, United States), anti-Tubulin (Sigma-Aldrich, St. Louis, United States), anti-GAPDH (Santa Cruz Biotechnology, Dallas, United States), anti-Actin (Sigma-Aldrich, St. Louis, United States), anti-TOLLIP (Abcam, Cambridge, United Kingdom), anti-LC3 (Novus Biologicals, Centennial, United States), anti-SQSTM1 (Abcam, Cambridge, United Kingdom), goat anti-rat IgG Alexa488 (Thermo Fisher Scientific, Waltham, United States).

Cell lines and treatments: Human embryonic kidney (HEK) 293 cells were obtained from the German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, MEF cells were generated in-house as described previously10. Both cell lines were cultured in Dulbecco’s modified Eagle’s medium (Thermo Fisher Scientific, Waltham, United States) supplemented with 10 % fetal calf serum (Thermo Fisher Scientific, Waltham, United States), 2 mM L-Glutamine (Thermo Fisher Scientific, Waltham, United States) and 100 U/ml Penicillin-Streptomycin in a humidified incubator at 37 °C and 5% CO2. Unless otherwise noted, for inhibition of the proteasomal activity cells were treated with either 25 µM MG132 (Merck KGaA, Darmstadt, Germany) or 1 µM BTZ (Merck KGaA, Darmstadt, Germany for 5 h, control samples were incubated with the respective amount of dimethyl sulfoxide (DMSO).

Plasmids and generation of CRISPR-CAS knockout cell lines: Knockout clones for ATG5, ATG7, TOLLIP and SQSTM1 were basically generated as described previously 11. In brief, HEK293 cells were transfected with TurboFect™ according to the manufacturer’s instructions with the respective target site sequence in lenti-gRNA-GFP-PPT and CAS9 cDNA on pRZ-Flag-mCherry2A-CAS9, which were a kind gift of Prof. Veit Hornung, University of Munich, Germany. Transfection efficiency was verified by fluorescence microscopy and cells were individualized to form single colonies. PCR amplification and data evaluation was performed exactly as described11. pEGFP-LC3 (human) was a gift from...
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Toren Finkel (Addgene plasmid # 24920; [n2t.net/addgene: 24920; RRID:Addgene_24920, Watertown, United States])

Stable isotope labeling by amino acids in cell culture (SILAC) and isolation of lysosomes with magnetic beads: All SILAC reagents were obtained from Thermo Fisher Scientific, Waltham, United States and Eurisotop, Saint-Aubin, France. For lysosomal proteome analysis, HEK293 cells were cultivated for six passages in SILAC-DMEM supplemented with 10% fetal bovine serum containing either 87.8 mg/ml l-arginine HCl, 181.2 mg/ml l-lysine for light labeling of cells or l-arginine$^{13}$C$_6$N$_4$ and l-lysine$^{13}$C$_6$N$_2$ for heavy labeling of cells. The isolation of lysosomal fractions with magnetic beads was performed as described recently.

Isolation of autophagosomes: SILAC labeled HEK cells were seeded on Poly-L-lysine (Sigma-Aldrich, St. Louis, United States) coated dishes one day before transfection with pEGFP-LC3. 48 hours post-transfection, cells were treated with either 1 µM BTZ or DMSO control for 5 h. Cells were washed with PBS twice, harvested in PBS and pelleted 10 min at 4°C and 500 x g. The supernatant was removed and cells were suspended in isolation buffer (20 mM HEPES, 1mM EDTA, 0.25 M sucrose, pH: 7.4). Cells were homogenized 25 times by a Dounce homogenizer and centrifuged down for 10 min at 4°C and 1000 x g. The protein amount of the supernatants was determined by the DC protein assay (BioRad, Hercules, United States) and equal protein amounts of BTZ-treated and -untreated samples were mixed with 50 µL of isolation buffer pre-washed GFP-Trap (ChromoTek, Planegg-Martinsried, Germany) magnetic agarose bead slurry. Mixed samples rotated for 1 h. Beads were again washed 3 times and GFP-LC3 was eluted by heating the beads for 5 min at 95°C in 1 x Laemmli-buffer.

Isolation of proteasomes: The immunoprecipitation of proteasome complexes was performed with the proteasome purification kit (Enzo Life Sciences, Farmingdale, United States) according to the manufacturer’s instructions. In brief, HEK cells were treated with 25 µm MG132 for 5h, harvested by scraping in binding buffer (25 mM HEPES, pH 7.4, 10% glycerol, 5 mM MgCl$_2$, 1 mM ATP, 1mM DTT) and homogenized in a Dounce homogenizer.
25 times. 100 µg of protein were used for incubation with proteasome binding affinity matrix containing immobilized anti-20S β5 antibody. Elution was performed by boiling the beads for 5 min at 95°C in 1 x Laemmli-buffer\textsuperscript{14}.  

**Sample preparation for mass spectrometry:** For proteome analysis of lysosomal, autophagosomal and proteasomal fractions, samples were processed for mass spectrometry analysis using in gel digestion as described previously\textsuperscript{13}. In brief, isolated fractions were denatured and alkylated with acrylamide\textsuperscript{15} to a final concentration of 1% for 30 min at RT before separation into different subfractions by a SDS-gel. The gel was then washed twice with water and stained with Coomassie Blue for one hour. After destaining the gel with distilled water, the eluate band profile was divided into 10 fragments. Slices were excised and subjected to tryptic in gel digestion. In brief, slices were washed consecutively with water, 50% acetonitrile (ACN), and 100% ACN. The slices were washed again and dehydrated with ACN. Dried slices were incubated with 400 ng sequencing grade trypsin at 37°C overnight. The peptide extract was separated and remaining peptides extracted with increasing concentrations of ACN. Peptides were dried in a vacuum concentrator and stored at -20°C.  

**LC-MS/MS measurement:** For mass spectrometric measurement, the samples were loaded directly onto the analytical column (ESI spray tip produced in house with a Sutter P2000 laser puller device from 360 µm OD, 100 µm ID fused silica capillary packed with 5 µm particles [Dr. Maisch, ReproSil C-18 AQ]) in 100 % A (water with 0.1 % FA) using a Thermo EASY-nLC 1000 at a flow rate of 1 µl/min. The column was washed for 10 min with 100 % A at a flow rate of 1 µl/min. Peptides were eluted with a linear gradient from 100 % A to 65 % A/35 % B (ACN with 0.1 % FA) in 60 min. Peptides eluting from the column were ionized in the positive ion mode using a capillary voltage of 1600 V and analyzed using a Thermo Orbitrap Velos mass spectrometer. One survey scan at a mass range of m/z 400 to m/z 1200 and a resolution of 30,000 was acquired in the Orbitrap mass analyzer followed by fragmentation of the 10 most abundant ions in the ion trap part of the instrument. The repeat count was set to one and the dynamic exclusion window to 60 s.
Experimental design and statistical rationale: In total, the isolation of lysosomes, autophagosomes and proteasomes and analysis of their proteome was performed for 4 independent biological replicates of proteasome inhibition treatment with SILAC label switching and five replicates of MG132 treatment to reach statistical significance of results. For In-gel digestion, each of the samples was divided in ten (lysosomes), eight (proteasomes), or six (autophagosomes) fractions. Measurement was performed as one technical replicate. Western Blots and immunofluorescence assays were performed in at least three biological replicates. The proteasome activity assay was performed in three biological replicates with technical triplicates each.

Data analysis of lysosomal fractions, isolated autophagosomes and proteasomes: The raw files were processed with Proteome Discoverer (Thermo Scientific, Version 2.3.0.523) and searched with the Mascot search engine (Version 2.6.1) against Swissprot (swissprot_2018_07 containing 23910 sequences, taxonomy: Homo sapiens), and cRAP database Version (2015_01, https://www.thegpm.org/crap/). Only Swissprot entries were used for further evaluation. Propionamide was set as fixed modification, as variable modifications protein N-acetylation, methionine oxidation, arginine (13 C (6) 15 N (4)) and lysine (13 C (6) 15 N (2)) isotopic labeling were considered. Up to two missed cleavages were accepted. The search was performed with a mass tolerance of 8 p.p.m mass accuracy for the precursor ion and 0.6 Da for the fragment ions. Search results were processed with Proteome Discoverer and filtered with a false discovery rate of 0.01. Low scoring spectrum matches were searched again with semitryptic specificity with up to one missed cleavage and a mass tolerance of 10 p.p.m mass accuracy for the precursor ion and 0.6 Da for the fragment ions. The consensus workflow in Proteome Discoverer was performed with normalization on total peptide amount (lysosomal fraction), on the specific protein amount of LC3 (isolated autophagosomes) or the specific protein amount of PSMB5 (isolated proteasomes). Quantification was based on pairwise ratio based ratio calculation as recommended by Thermo Fisher Scientific Proteome Discoverer Software for this type of mass spectrometric data, for which modified peptides with oxidation (M) and propionamide
(C), and N-terminal acetylation were included. Statistical significance was determined using background-based student’s t-test with Benjamini-Hochberg correction as performed by Proteome Discoverer. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD015243.

**Western Blotting:** The protein concentration of input and eluate fractions of lysosome isolation was determined using the DC protein assay. Equal protein amounts of different samples were diluted in 4x sample buffer (500mM Tris-HCl, pH 6.8, 4% SDS, 40% Glycerin, 40 mM DTT, Coomassie Blue G), heated to 95 °C for 5 min and loaded onto 12.5 % polyacrylamide gels. After electrophoretic separation proteins were transferred to nitrocellulose transfer membranes and afterwards free binding sites were blocked in 5 % milk powder in TBST. For immunostaining membranes were incubated over night with primary antibody diluted in 5 % BSA in TBST. Membranes were washed thrice in TBST for 5 min, incubated with HRP-conjugated secondary antibody in 5% BSA in TBST and specific binding was detected by enhanced chemiluminescence detection. For quantification densitometric analysis was performed by the Vilber Lourmat gel documentation and Fusion system.

**Proteasome activity assay:** The activity of the proteasome was determined with the Proteasome activity kit (abcam), according to the manufacturer’s instructions.

**Immunofluorescence:** Cells were grown on Poly-L-lysine-coated glass-bottomed coverslip dishes (Paul Marienfeld GmbH, Lauda-Königshofen, Germany). To follow the localization of inactive proteasomes cells were incubated with 2 µM activity-based probe Bod-TMR-epoxomicin (MVB003 or MV151) that was kindly provided by Overkleeft laboratory (Leiden University) for different time periods \(^{16,17}\). Cells were washed in PBS, fixed and permeabilized in ice-cold methanol at -20°C for 10 min and after two times washing in PBS blocked in 10 % fetal calf serum in phosphate buffered saline (Thermo Fisher Scientific, Waltham, United States for 1h. For immunostaining cells were exposed to appropriate primary antibodies in 1% FCS in PBS overnight at 4°C. Cells were washed thrice in PBS and
incubated with appropriate fluorescence dye-coupled secondary antibodies for 1h at room temperature. After two times washing in PBS and once in water cells were mounted in DAPI-containing mounting solution. Cells were imaged with either Zeiss LSM700 confocal microscope or Zeiss LSM800 with airyscan processing. Wild type and knockout cell samples were exposed to laser for identical time spans. Dynamic ranges were adjusted for optimal color representation.

Lysosome counting: We quantified lysosome structures from separate microscopic images, each containing one single cell. By using CellProfiler software (3.1.8) we detected individual lysosome structures by applying gaussian smoothing (sigma=1), automatic thresholding (minimum cross entropy method) and an intensity-based watershed for separating touching objects (sigma=12 for gaussian smoothing). For each detected lysosome object, the area (nr of pixels) and the average marker intensity was calculated. Objects with a diameter below 10 pixels were rejected.

Results
Analysis of proteomic changes in lysosome-enriched fractions after proteasome inhibition
To determine changes in the lysosomal protein composition caused by an impairment of the ubiquitin-proteasome system, a SILAC-based mass spectrometric analysis of lysosomes from cells treated with either MG132 or BTZ was performed. HEK293 cells were grown in complete DMEM containing either light or heavy amino acids for six passages before incubating them with superparamagnetic particles for 24 h followed by a 36 h chase to allow complete transport of magnetic particles to the lysosome. After removal of nuclei by centrifugation, postnuclear supernatants of light and heavy labeled cells were mixed before magnetic lysosome enrichment and mass spectrometry sample preparation (Fig. 1A). The efficiency of lysosomal enrichment was examined by lysosomal β-hexosaminidase activity, an exemplary result of which is displayed in figure 1 B, showing 70% of intact lysosomes were concentrated in the lysosome-enriched fraction. As the effect of proteasome inhibitors is dose-dependent, we tested different concentrations for a treatment time of 5 hours and chose 25 µm MG132 and 1 µm BTZ to reach a proteasome inhibition of around 60% (Fig.
The influence of proteasome inhibitor treatment on lysosome size and number was investigated and showed a slight increase in lysosomal size after MG132 treatment and a higher number of lysosomes per cell after BTZ treatment (Supplementary Figure 1 A, B). These changes may be related to changes in lysosomal homeostasis or autophagic flux followed by lysosome reformation induced by proteasome inhibition, as we also detected an increase in the protein amount of LC3-II (Supplementary Figure 1 C, D) after MG132 but not BTZ treatment. Among 2854 and 4419 proteins identified and quantified in the datasets of BTZ- and MG132-treated cells vs. controls (Supplementary Tables S1-S4), respectively, we found 259 and 360 proteins that are classified as lysosomal or lysosome-associated by GO-term (http://www.pantherdb.org), most of whom have unchanged protein levels. In both datasets we found a variety of proteins that showed a statistically significant change in their proteins amounts, as visualized in Figure 1 D and E. To exclude possible side effects caused by either proteasome inhibitor we filtered for those proteins regulated in both datasets with a corrected p-value < 0.05. This yielded 58 proteins upregulated in inhibitor-treated cells of both datasets, whereas only 6 proteins had reduced protein amounts in the lysosome-enriched fraction after proteasome inhibition (Figure 2 A-C, for lists of proteins see Supplementary Table S5). Among these 58 proteins we identified dihydrofolate reductase, the key enzyme of folate metabolism and thymidin biosynthesis, β-catenin, an important regulator of Wnt-signaling, and HMGCoA reductase, the main regulator of cholesterol biosynthesis. These proteins are known to be degraded by the proteasome pathway. The hxyphoxanthine-guanine phosphoribosyltransferase is an important enzyme of purine metabolism and the phosphoglycerate kinase 1 phosphorylates Beclin 1 and thereby induces macroautophagocytosis19. This suggests that the cell diverts at least some proteins to the autophagy pathway in case the proteasomal degradation is blocked.

**Accumulation of inactivated proteasomes in lysosome-enriched fractions**

Surprisingly, 21 of the commonly upregulated proteins were proteasome subunits, and when analyzing enriched *Kyoto Encyclopedia of Genes and Genomes* (KEGG)-pathways among the upregulated proteins, the proteasomal ubiquitin-dependent catabolism was the most...
enriched biological process (Figure 2 B). Upon closer analysis, even more components of the proteasome complex were identified and quantified in both datasets, namely 42 proteins in the MG132 dataset and 32 proteins in the BTZ dataset. They showed an upregulation of around 50% (BTZ-treated cells) to 100% (MG132-treated cells), albeit not all subunits reached statistical significance. In MG132-treated cells, the extent of regulation of 20S core subunits was generally stronger than of 19S regulatory subunits, this could not be observed after BTZ treatment.

To verify the enrichment of proteasome subunits, postnuclear supernatants as well as lysosome-enriched fractions from MG132 and BTZ-treated cells were subjected to western blotting against the proteasome subunits PSMA7, PSMB5, PSMD4, PSMC1 and PSMD3 (Fig. 2 D-G). In postnuclear supernatant there was no significant difference detectable for any of the tested subunits between treated and untreated cells, while in lysosome-enriched fractions the accumulation of proteasomal subunits after proteasome inhibition could be verified. All subunits examined showed a statistically significant increase of protein amount between 150 and 580% in western blotting, with the exception of PSMD4 in BTZ-treated cells, where no difference could be detected.

Proteins within enriched lysosomal fractions can be either localized within the lysosome or at the lysosomal surface. Furthermore, due to the connection of the lysosome to other organelles and the cytoskeleton, as well as cytoplasmic carry-over, a considerable number of proteins detected in this fraction is probably not lysosomal. To verify the localization of the proteasome within the lysosome, we incubated cells with the fluorescently labeled proteasome inhibitor BOD-TMR-Epox to follow the fate of inactivated proteasomes. Upon microscopic analysis and colocalization with the lysosomal membrane protein LAMP2, an increasing accumulation of labeled proteasomes over time was clearly visible (Figure 3). While after 1.5 hours of incubation with BOD-TMR-Epox almost no co-localization of proteasomes and lysosomes was visible, after 2.5 and 5 hours labeled proteasomes were clearly localized within LAMP2-positive lysosomes. A localization within the lysosome indicates that the proteasomal subunits have been subject to autophagocytosis rather than
presenting a contamination in the mass spectrometric data set. Furthermore, the degradation of proteasomes is corroborated by co-incubation of BTZ with the lysosomal inhibitor leupeptin for different time points between 1 and 5 hours, showing an increasing accumulation of all tested subunits with prolonged leupeptin co-incubation (Supplementary Figure 2).

Proteasomes reach the lysosome by autophagosomes

The presence of proteasomes inside rat lysosomes has been already reported as early as the 90’s20, and recently a form of macroautophagy termed proteaphagy has been described in Arabidopsis thaliana and Saccharomyces cerevisiae21,22. The transport of autophagic cargo to the lysosome occurs mostly via macroautophagy, and to determine if this applies for proteasomes as well, LC3-positive autophagosomes were isolated via immunoprecipitation from GFP-LC3 overexpressing cells. A mass spectrometric analysis of isolated autophagosomes showed an enrichment of several proteins in autophagosomes from BTZ-treated cells vs. control (Supplementary Table S6-S7, Figure 4 A), including proteasome subunits. Western blot analysis revealed increased levels of PSMA7, PSMB5, PSMC1 and PSMD3 in autophagosomes isolated from MG132-treated HEK cells, equal protein loading was verified by ponceau staining after blotting (Figure 4B). As the proteasomes inside the cells are present as a 2.5 mega Dalton protein complex consisting of more than 30 subunits assembled into 19S and 20S proteasome particles7, they may either be degraded completely or after disassembly into different subunits. Native SDS Gel analysis showed increased levels of proteasomes at a size of around 750 kDa corresponding to the proteasome 20S core complex, indicating engulfment of the inactivated proteasome occurs before complete disassembly (Figure 4C).

In yeast, the inhibition of macroautophagy by knockout of ATG7 completely abolishes proteaphagy22. Mammalian ATG5 and ATG7 KO cells were generated from HEK cells by the CRISPR-Cas system11 and for both knockout cell lines conversion of LC3-I to its lipidated form LC3-II could no longer be observed (Supplementary Figure 3). These ATG5 and ATG7 knock-out cells were used to examine the extent of delivery of proteasomes to the lysosome
after BTZ treatment. Except for BTZ, leupeptin was also added to the cells to inhibit lysosomal proteolysis which should stabilize intralysosomal proteasomes and thus make their detection more sensitive. Results show that the lysosomal accumulation of inactive proteasomes is significantly diminished but not completely abolished. In ATG5 as well as in ATG7 KO cells, there is still an increase of proteasome subunits in the lysosome enriched fraction after proteasome inhibition, which reaches in between 40-60% of the level of wt cells. (Figure 4 D-F). This can also be seen in immunofluorescence, where a colocalization between proteasomes labeled with BOD-TMR-L3VS and LAMP2-stained lysosomes in ATG5 and ATG 7 deficient cells is still evident (Figure 5).

**Known macroautophagy adaptor proteins and CMA are not involved in proteaphagy**

As specific autophagy usually relies on binding of the cargo by specific adaptor proteins, we aimed to identify potential adaptor proteins. Known adaptor proteins that have been identified previously have shown enhanced association with cargo under degradation-inducing conditions, as reported for the proteaphagy receptors Rpn10 in *Arabidopsis thaliana*\(^2\), coupling of ubiquitin conjugation to ER degradation protein 5 (Cue5) in yeast\(^2\), and SQSTM1 in human cells\(^2\). It was reported earlier that SQSTM1 may be involved in degradation of mammalian proteasomes under nutrient stress conditions\(^2\), and this may also hold true for inactivated proteasomes. We generated HEK CRISPR-Cas knock-out cell lines for SQSTM1 (Supplementary Figure 3) and isolated lysosome-enriched fractions from BTZ-treated and untreated cells. Western Blot analysis of proteasome subunit enrichment did not show an effect of SQSTM1 deficiency on the presence of proteasomes in the lysosome (Figure 6 A). In isolated autophagosomes, SQSTM1 was present but showed a BTZ/control ratio of 0.9 and was therefore not enriched together with proteasomes.

In yeast, the aggrephagy receptor protein Cue5 was shown to bind ubiquitinated proteasomes accumulated in cytoplasmic aggregates upon MG132 treatment, and deletion of Cue5 abolished transport of proteasomes to the vacuole\(^2\). The mammalian homologue of Cue5 is the toll-interacting protein (TOLLIP), a cytoplasmic soluble protein interacting with
proteins of the Atg8 family. We generated TOLLIP CRISPR-Cas knockout cells (Supplementary Figure 3), but in western blots of isolated lysosome-enriched fractions we could not detect differences in the protein expression levels of PSMA7 or PSMB5 (Figure 6 B) in comparison to wildtype cells. As PSMD4, the human orthologue of Arabidopsis Rpn10, does not possess the domain necessary for proteaphagy in Arabidopsis and its role in mammalian proteaphagy was already excluded by Marshall and colleagues, we did not include it in our analysis.

Another pathway to deliver proteins to the lysosome is chaperone-mediated autophagy (CMA), and it has been suggested that proteasomes may be delivered to lysosomes via this mechanism. Since CMA depends on the lysosomal membrane protein LAMP2, we investigated a possible involvement of CMA in LAMP2-deficient mouse embryonic fibroblast (MEF) cells. We incubated the cells with BOD-TMR-Epox and analyzed the accumulation of the inhibited proteasomes by microscopy. No differences were observed between wildtype and LAMP2-deficient cells (Figure 6 C). Thus, CMA is not involved in delivery of proteasomes to lysosomes.

No new proteaphagy adaptor protein could be identified

To identify yet unknown possible adaptor proteins we isolated proteasomes by anti-PSMB5 affinity purification and compared those proteins associated to the isolated proteasomes with and without proteasome inhibition by MG132 (Supplementary Table S8-S9). In four independent biological replicates, a total of 2549 proteins were quantified, 147 of which were associated to the GO terms proteasome complex and/or proteasomal ubiquitin-dependent catabolism (Supplementary Table S8). There were no differences in the protein amounts of any of the proteasomal subunits between the immunoprecipitates of MG132 treated- and untreated cells. A number of proteins showed reduced or increased abundances with the proteasome under proteasome inhibition. Most of those proteins have several ubiquitination sites and are thereby known proteasome substrates or associated proteins. None of the identified, statistically significant upregulated proteins has been reported to be a macroautophagy adaptor protein or interact with LC3. We additionally compared our dataset
with a dataset generated by Besche and colleagues\textsuperscript{27}, who isolated proteasomes from BTZ-treated and non-treated HEK293 cells expressing tagged proteasome subunits. Nine proteins were commonly upregulated in both datasets (Supplementary Table S8), among them the RING-type E3 ubiquitin transferase RNF181 and the proteasome activator complex subunit 3.

Discussion

The connections of the UPS and the lysosome-autophagy pathways are numerous and diverse. Both systems can partially compensate each other, as it has been extensively shown that inhibition of the proteasome can lead to upregulation of ATG genes. Pharmacological enhancement of 20S proteasome activity impairs autophagic flux by increasing degradation of Synaptosomal-associated protein 29 and Syntaxin-17\textsuperscript{28}. The present study revealed even more interconnections between both pathways.

Proteotoxic stress conditions can lead to a shift from proteasomal to autophagosomal degradation mediated by BAG family molecular chaperone regulator \textsuperscript{329}. Proteins that would normally be degraded by the UPS can be deflected to autophagic degradation upon inhibition of the proteasome by either MG132 or BTZ. Dihydrofolate reductase is a well-known substrate of the proteasome\textsuperscript{30-32}. Its degradation is obviously re-routed to the lysosome as the proteasome is blocked, but it could not be identified in our mass spectrometric analysis of autophagic vesicles. As it has been described to be able to enter the lysosome by CMA\textsuperscript{33}, this is likely its route of transport into the lysosomal lumen for degradation. B-catenin degradation has been extensively described and is usually performed by the proteasome\textsuperscript{34,35}. In our analysis it is not only enriched in lysosomes from MG132- and BTZ-treated cells, but also in isolated autophagosomes. This is in line with an earlier study by Petherick and colleagues\textsuperscript{36}, who have described a direct interaction of β-catenin with LC3 followed by autolysosomal degradation.

The autophagic degradation of proteasomes by lysosomes has initially been described in \textit{Arabidopsis thaliana}\textsuperscript{27}, where Rpn10, a subunit of the proteasome itself and whose human homologue is PSMD4, has been elegantly identified as the responsible adaptor protein.
which binds to ubiquitinated proteasomes and Atg8 with ubiquitin-interacting motifs. Additionally to being part of the proteasome complex, it exists as a cytosolic pool binding directly to Atg8 and proteasomes upon their inhibition. As the region identified as being responsible for this process is not present in the yeast/mammalian counterparts of Rpn10, and it has been shown to not or only weakly bind to yeast and human versions of Atg8, its function as a proteaphagy adaptor is probably limited to plants\textsuperscript{21,37}.

In yeast, two general pathways of proteasome clearance can be distinguished: either by nutrient stress conditions like nitrogen starvation, or induced by proteasome inhibitor treatment. The latter seems to be a stepwise process starting with deposition of inactivated proteasomes in cytoplasmic aggregates followed by autophagic degradation\textsuperscript{23,38}.

In mammalian cells, the degradation of proteasomes also seems to follow different pathways. While the degradation of misassembled proteasome subunits is performed preferentially by the proteasome itself\textsuperscript{38}, the degradation of whole proteasome by autophagy during steady-state and starvation conditions was documented in several studies\textsuperscript{39,40}. Our results clearly show that also proteasomes inactivated by the inhibitors MG132 or BTZ are accumulated in autophagosomes and subsequently in lysosomes. Proteasome subunits were identified in a proteomic analysis of autophagosome-associated proteins\textsuperscript{39}, and 19S and 20S subunits showed reduced degradation rates in autophagy deficient cells lacking ATG5 or ATG7\textsuperscript{40}. The degradation of proteasomes by autophagy induced by prolonged starvation periods in HeLa cells was shown to be at least partially dependent on SQSTM1, whose siRNA knockdown led to a 25% reduction in colocalization between the proteasome and LC3B\textsuperscript{24}. This could not be verified by our study regarding inhibitor-induced proteaphagy, as we did not find any differences in the degree of proteasome accumulation in lysosomes after inhibitor treatment in SQSTM1 deficient cells. Therefore, the shuttling of proteasome subunits to the autophagosome by SQSTM1 may be restricted to nutrient-stress induced degradation of proteasomes.

The same study also reported an ubiquitination preceding proteaphagy, a mechanism that is probably common to both pathways as Besche and colleagues\textsuperscript{27} showed enhanced
ubiquitination of proteasome subunits after BTZ treatment and association of several ubiquitin ligases to inhibited proteasomes, one of which, RNF181, was also detected in our mass spectrometric analysis of MG132-treated isolated proteasomes. Therefore also inhibitor-induced proteaphagy in mammalian cells probably relies on ubiquitination of certain proteasome subunits.

While autophagy mutants in Arabidopsis and Yeast show a complete blockage of proteaphagy \(^{21,23}\), we could show a reduced accumulation of around 40\% after either ATG5 or ATG7 CRISPR-Cas knockout in HEK293 cells. It has been reported that Atg5-deficient MEF cells are able to form autophagosomes, and also autolysosomes without conversion of LC3-I to LC3-II. These alternative macroautophagy pathways can also degrade organelles in erythrocyte maturation\(^{41}\). The selective autophagy of mitochondria, termed mitophagy, has been shown to work by alternative macroautophagy pathways\(^{42}\). After deletion of either ATG5 or ATG7, proteaphagy is thus probably performed by those alternative mechanisms of autophagosome formation seemingly present in mammalian cells. Besides macroautophagy, an alternative route to the lysosomes via endosomes is possible, since it was just reported that active proteasomes can be internalized to endosomes/phagosomes to mediate antigen cross-presentation but do not stay there permanently\(^{43}\).

Proteasomes are obviously present as 20S complexes in lysosomal fractions. This is in line with the fact that they are probably disassembled into 19S and 20S complexes before being exported from the nucleus, their main localization besides the cytoplasm\(^{7}\). As by CMA only single proteins are transported into the lysosomal lumen by the lysosomal membrane protein LAMP2 and the chaperone HSP70 and LAMP2-deficient MEF cells do not show differences to wildtype cells regarding the presence of inactivated proteasomes within LAMP1-positive compartments (Figure 6), delivery of proteasome subunits to the lysosomal lumen by CMA seems unlikely.

The identity of a possible selective macroautophagy adaptor protein for proteasomes in cells remains enigmatic. While Cue5 was clearly identified in yeast as this adaptor protein and showed a massive enrichment on inactive proteasomes\(^{23}\), its human homologue TOLLIP
that has been implicated in aggrephagy and lipophagy\textsuperscript{25,44} does, according to our results, not seem to be responsible for delivery of inactivated proteasomes to the autophagosome. Nevertheless, a role of TOLLIP or SQSTM1 in proteaphagy can still not completely excluded, as other macroautophagy adaptors could substitute for them in the respective knockout cells. Furthermore, it should be noted that also nuclear proteins could be involved in proteasome delivery to the lysosomes, which could not be detected by our study as we used postnuclear supernatants for the isolation of organelles.

Dengjel and colleagues investigated the possibility of proteasome binding directly to LC3, but no direct interaction between proteasome subunits and LC3\textsuperscript{39} could be detected. Direct interactions between those proteins upregulated after proteasome inhibition and LC3 were also not reported so far, but could be investigated by immunoprecipitation or immunomagnetic separation.

Interestingly, in the model organism \textit{Dictyostelium discoideum} it could recently be shown that the 19S subunits PSMD1 and PSMD2 directly interact with ATG16, a component of the core autophagy machinery, and are degraded by autophagy dependent on ATG16\textsuperscript{45}. This interaction and the following autophagic degradation is dependent on the N-terminal portion of Atg16 containing an Atg5-interacting motif.

In conclusion, proteaphagy of inhibitor-inactivated proteasomes in human cells seems to follow an at least partially different pathway from that utilized by proteasomes in nutrient stress conditions. The conventional autophagy pathway probably mediates most of this degradation, but even if LC3-conjugation is blocked by ATG5 or ATG7 knockout, inactivated proteasomes still reach the lysosome by alternative pathways. The utilization of adaptor proteins for selective autophagy of the proteasome is species-specific, and there may very well be a direct binding of inactivated, probably ubiquitinated proteasomes to components of the forming autophagosome.

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Data Availability

The mass spectrometry datasets have been deposited to the Proteome Xchange Consortium (27) via the PRIDE partner repository with the dataset identifier PXD015243. All peptide and protein identifications and quantification are available in the Supplementary Tables S1-S9.

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**Figure 1: Analysis of the lysosomal proteome after proteasome inhibition.**

A) Experimental set-up of the study B) β-hexosaminidase enzyme assay. All fractions of the lysosome isolation procedure were subjected to N-acetyl-β-D-glucosaminide and the total enzyme activity was determined using Lambert-Beer’s Law. A representative experiment is shown. FT = flow through of magnetic column. C) Chymotrypsin-like protease activity was determined after treatment of HEK293 cells with MG132 or Bortezomib, respectively. The fluorogenic substrate LLVY-R110 was used, n=3, values are depicted ±SD, *=p<0.05 as determined by student’s t-test. D) and E) Volcano Plots of identified proteins in dataset of
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BTZ- or MG132 treated vs control cells, respectively. Values were normalized to total protein amount in the respective channel. P-values were determined using t-test with Benjamini-Hochberg correction using Proteome Discoverer. N=4 (D) or 5 (E). Proteins mentioned in the text and Top10 regulated proteasome subunits are marked in blue, proteins with p-Values <0.05 in red, others in black.

Figure 2: Enrichment of proteasomal subunits in lysosome-enriched fractions. Overlap of statistically significant (p<0.05) upregulated (A) or downregulated (C) proteins in BTZ-treated (left circle) and MG132-treated (right circle) datasets of lysosome-enriched fractions. B) Enriched KEGG pathways were analyzed for the intersection between upregulated proteins from BTZ- and MG132-treated datasets. D-G) Western Blots and their
densitometric quantification of proteasomal subunits in the lysosomal fraction and postnuclear supernatants after proteasome inhibition. **D;F** MG132-treated HEK cells. **E;G** BTZ-treated cells. Equal protein amounts of each fraction were separated, blotted and probed with antibodies against different proteasomal subunits and GAPDH (inputs) or cathepsin D/LAMP1 (lysosomes) as loading controls. One representative experiment out of at least 3 is shown. Densitometric quantification was performed for n=3-8 and significance was determined by paired student's t-test, *=p<0.05. Untreated control samples were set to 1.

**Figure 3: Immunofluorescence of MEF cells incubated with BOD-TMR- Epoxomycin.**

MEF cells were seeded on glass cover slips and incubated for different time periods with the fluorescent activity-based probe BOD- TMR-Epoxomycin. Afterwards cells were fixed with MeOH and stained with an antibody against LAMP2 and DAPI staining. Scale bar = 10 µm.
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Figure 4: Analysis of macroautophagy (A) Volcano Plot of identified proteins in dataset isolated autophagosomes of BTZ-treated vs control cells. Values were normalized to LC3 protein amount in the respective channel. P-values were determined using t-test with Benjamini-Hochberg correction using Proteome Discoverer. Proteins mentioned in the text and Proteasome subunits are labelled. (B) HEK293 cells were transiently transfected with GFP-LC3 an either treated with 25 µM MG132 or DMSO as control. Autophagosomes were isolated by GFP-Trap beads, eluates were separated, blotted and probed with antibodies against different proteasomal subunits. Ponceau staining is shown as loading control. (C)
Lysosome-enriched fractions were separated under native conditions, blotted and probed with an antibody against PSMA7. Three independent replicates are shown. D-E) Lysosomal and input (PNS) fractions were isolated from wt, ATG5 and ATG7 ko cells after 5h of treatment with 1 µM BTZ and 100 µM Leupeptin, equal protein amounts were loaded, separated, blotted and probed with antibodies against different proteasomal subunits and GAPDH (inputs) or cathepsin D (lysosomes) as loading controls. One representative experiment out of at least 3 is shown. F) Densitometric quantification was performed for n=3-8 and significance was determined by paired student’s t-test, *=p<0.05. Untreated wt control samples were set to 1.

Figure 5: Immunofluorescence of ATG5 and ATG7 KO HEK cells incubated with BOD-TMR-L3VS. HEK cells were seeded on glass cover slips and incubated for 2h time periods with the fluorescent activity-based probe BOD- TMR-L3VS. Afterwards cells were fixed with MeOH and stained with an antibody against LAMP2 and DAPI staining. Scale bar = 10 µm.
Figure 6: Potential proteaphagy adaptor proteins. Western Blot of proteasomal subunits in the lysosomal fraction and postnuclear supernatants after proteasome inhibition of p62 (A) and TOLLIP-deficient cells (B) after BTZ treatment. Equal protein amounts of each fraction were separated, blotted and probed with antibodies against different proteasomal subunits and Actin (inputs) or cathepsin D/LAMP1 (lysosomes) as loading controls. One representative experiment out of at least 3 is shown. Immunofluorescence of wt and LAMP2-deficient MEF cells incubated with BOD-TMR- Epoxomycin. MEF cells were seeded on glass cover slips and incubated for 5h with the fluorescent activity-based probe BOD- TMR- Epoxomycin. Afterwards cells were fixed with MeOH and stained with an antibody against...
LAMP1 and DAPI staining. Scale bar = 10 µm.