universität wien

DIPLOMARBEIT / DIPLOMA THESIS

Titel der Diplomarbeit / Title of the Diploma Thesis

„Exploring the crosstalk between selective autophagy and the unfolded protein response during endoplasmic stress conditions“

verfasst von / submitted by

Alexander Gajic

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Magister der Pharmazie (Mag.pharm.)

Wien, 2021 / Vienna, 2021

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 449
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Diplomstudium Pharmazie
Betreut von / Supervisor:	Yasin Dagdas, PhD

Table of contents

Abstract

This work is a part of [1].

The endoplasmic reticulum is the cells largest organelle. Maintaining its homeostasis is key to proliferation and survival. As it is also host of protein folding and glycosylation it is crucial for the functioning of complex organisms. Various control mechanisms have been identified that promote ER homeostasis. In the last years selective ER autophagy (ER-phagy) has been identified as a key quality control mechanism, however it remains elusive on how ER-phagy interacts with other systems of ER quality control.

This study provides with C53 a cytosolic protein that is alleviating the cell of proteotoxic stresses in the endoplasmic reticulum and malfunctioning translation at stalled ribosomes via autohagy. C53 senses ER luminar perturbations via a tripartite protein complex that is further discussed in [1]. The key trigger to C53 activation is ribosome stalling. C53 is present in both plants and mammals and has the same role in both kingdoms of life and therefore is part of an ancient mechanism.

Diese Arbeit ist Teil von [1].

Das endoplasmische Retikulum ist das größte Organell der Zelle. Die Regulierung dessen Homöostase ist zum Wachstum und Überleben der Zelle unerlässlich. Durch die im ER stattfindende Proteinfaltung und Glycosylierung ist es zudem ein Schlüssel zur Funktion komplexer Organismen. Mehrere Kontrollmechanismen der ER Homöostase wurden bereits identifiziert. In den letzten Jahren hat sich selektive ER Autophagie (ER-phagie) als wichtiger Mechanismus der endoplasmischen Qualitätskontrolle hervorgetan, jedoch ist das Zwischenspiel zwischen ER-phagie und anderen Qualitästkontrollsystemen noch weitgehend unerforscht.

In dieser Arbeit wird mit C53 ein cytosolisches Protein identifiziert, dass in der Lage ist proteotoxische Stresszustände und stockende Translationen zu erkennen und über Autophagie abzubauen. C53 erkennt ER luminale Störungen über einen dreiteiligen Proteinkomplex der in [1] näher beschrieben wird. Der Schlüssel zur Aktivierung von C53 ist eine stockende Translation an ER-assoziierten Ribosomen. C53 ist sowohl in Pflanzen als auch im Tierreich präsent und übernimmt in beiden Domänen des Lebens diese Aufgaben und ist daher Teil eines antiken Mechanismus.

Acknowledgements

My gratitude goes to Yasin Dagdas, for taking the gamble and hiring me, a pharmacist, into his team of biologists even though it was clear that it would not be easy for any of us. Furthermore, Yasin needs to be thanked for his supervision and the way he did supervise me. This controlled freedom allowed me to train and develop skills that exceed all expectations I ever had. Also, I want to acknowledge his constant input and pushing at specific times so that the team could finish up the C53 paper just in time.

I also want to extend my gratitude to Madlen Stephani and to Lorenzo Picchianti for extended supervision and the techniques and skills they taught me, even though it could be a challenge at some times.

Furthermore, I want to thank the collaborators in the group and the group in general, even if the projects did not overlap. I have had the time of my life this past year and a half working with these people, both, scientifically and non-scientifically. Thank you for coffee, cake, and lunch breaks, snack times in the lab, social hours and all the lab activities we did. For all the good talks and the cultural exchange we had.

To Iris Bachtrog I owe special thanks. The basis of my current skill when working in tissue culture laboratories is all her achievement. Also, she was never tired of some delightful chats when working hood to hood or in front of the labs.

Gratitude needs to extend to the VBC, the GMI, IMBA and the in-house facilities they provided. Working there is a privilege, as all the maintenance work provided by the facilities allowed to focus my energy to my work instead of stacking tips or preparing and autoclaving media. Therefore thanks to the molecular biology service, the media kitchen, BioOptics, the GMI lab support as well as Lewis and Ingrid.

I also want to thank my friends and family for keeping my head up when work was demanding. Especially Isabel, who set me up on this endeavor in the first place. Nicolas for being my partner, even though it did not last he was a constant motivator. Veronika and Tilmann for listening to my constant talking about autophagy and pretending it was interesting, even though for them it probably was not. Sven and Christian for constantly keeping my head over water in these challenging time that this thesis was finished.

Introduction

Autophagy

Autophagy is a self-degradative process that is important for balancing sources of energy at critical times in development and in response to nutrient stress. Autophagy also plays a housekeeping role in removing misfolded or aggregated proteins, clearing damaged organelles, such as mitochondria, endoplasmic reticulum and peroxisomes, as well as eliminating intracellular pathogens. Thus, autophagy is generally thought of as a survival mechanism, although its deregulation has been linked to non-apoptotic cell death. Autophagy can be either non-selective or selective in the removal of specific organelles, ribosomes and protein aggregates, although the mechanisms regulating aspects of selective autophagy are not fully worked out.[2]
Specificity is ensured by so called autophagic receptors which interact on one hand with their respective cargo and on the other hand with an ATG8 (Autophagy related protein 8) molecule via an ATG8-Interacting-Motiv (AIM) (Figure 1). This work focuses on the description of a novel autophagy receptor, C53 (also known as CDK5RAP3, LZAP), which has stalled ribosomes as its primary cargo.

Mechanism of autophagy

Induction of autophagy results in recruitment of ATGs to a specific subcellular location termed the phagophore assembly site (PAS) and nucleation of an isolation membrane that forms a cup- shaped structure termed the phagophore. Gradual elongation of the curved isolation membrane results in expansion of the phagophore into a sphere around a portion of the cytosol. The isolation membrane eventually seals into a doublemebraned vesicle, termed the auto- phagosome, thereby trapping the engulfed cytosolic material as autophagic cargo. After clearance of most ATGs and delivery along microtubules to the lysosome, the outer membrane of the autophagosome fuses with the lysosomal membrane to form an autolysosome. This fusion results in the release of a single- membrane autophagic body into the lysosomal lumen, which is followed by the degradation of the autophagic body together with its cargo by the autolysosomal hydrolytic milieu. [3]

For this work the most important player in the autophagic cascade is the ATG8 protein family. This family includes microtubule-associated protein light chain 3 (LC3) proteins and γ-aminobutyric acid receptor-associated proteins (GABARAPs) in mammals. These proteins are key players in the phagophore expansion and sequestration of cargo by interacting with respective carco receptors. [3] This interaction happens via an ATG8-Interacting-Motiv (AIM) or LC3-Interacting-Region (LIR).

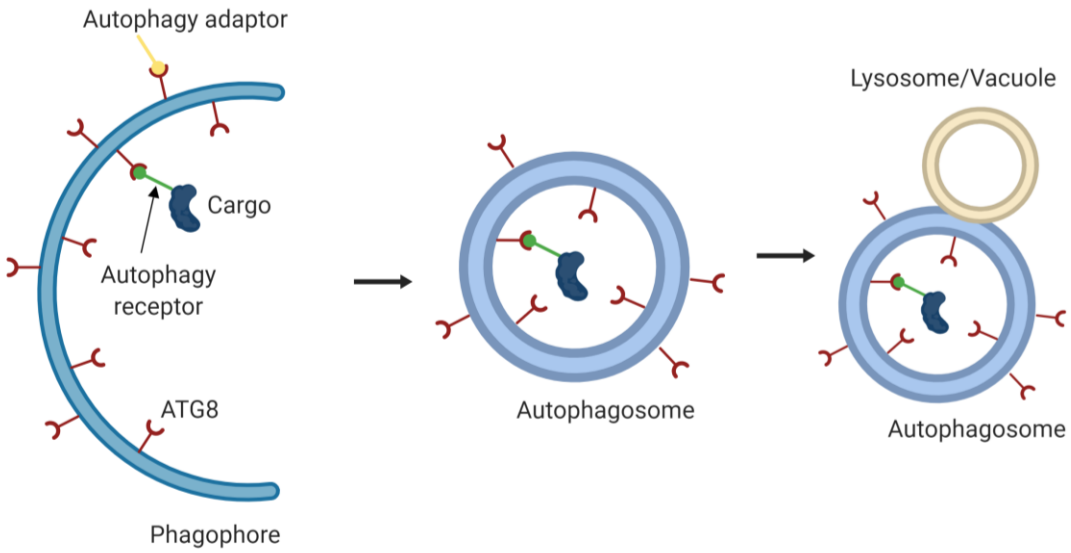

Figure 1:General (simplified) mechanism of autophagy: The phagophore engulfs eventual cargo which is recruited by autophagy receptors and forms the autophagosome. Proteins that interact with ATG8s on the outside of the autophagosome act as so-called autophagy adaptors and might handle trafficking. The destination of the autophagosome is the fusion with a lysosome (or vacuole in Plants/Yeast). The contents of the autophagosome are broken down and can be reused by the cell.

Autophagy and disease:

Autophagy, or a defect of the same, is also discussed to be a player in various diseases and metabolic processes, such as aging, adipositas and diabetes, inflammation and immunity, as well as neurodegenerative diseases like Alzheimer's or Parkinson's. Autophagic processes also play a role in control of the cell cycle and may therefore be a factor in cancerogenicity. [2,4]

Endoplasmic reticulum (ER) and ER stress

The ER is the largest organelle in the cell and is a major site of protein synthesis and transport, protein folding, lipid and steroid synthesis, carbohydrate metabolism and calcium storage. The multi-functional nature of this organelle requires a myriad of proteins, unique physical structures and coordination with and response to changes in the intracellular environment. Work from a variety of systems has revealed that the ER is composed of multiple different structural domains, each of which is associated with a specific function or functions. [5]

One of the major functions of the ER is to serve as a site for protein synthesis for secreted and integral membrane proteins, as well as a subpopulation of cytosolic proteins. Protein synthesis requires localization of ribosomes to the cytosolic face of the ER, and the canonical pathway that regulates protein synthesis involves co-translational docking of the mRNA:ribosome complex on the ER membrane. Translation of secretory or integral membrane proteins initiates in the cytosol, then ribosomes containing these mRNAs are recruited to the ER membrane via a signal sequence within the amino terminus of the nascent polypeptide that is recognized and bound by the signal recognition particle (SRP). The complex of mRNA:ribosome:nascent polypeptide:SRP is targeted to

the ER where it docks on the SRP receptor. Translation continues on the ER and the emerging polypeptide can co-translationally enter the ER through the translocon, which is a channel that contains several Sec proteins and spans the lipid bilayer. Also during this time, or in some cases once translation is complete, a signal peptidase cleaves the short signal peptide allowing the free protein to enter the ER lumen If the protein is destined to be an integral membrane protein, determined by the presence of a stretch of hydrophobic residues or stop-transfer membrane anchor sequence, translocation will pause. At this point the protein will be shifted laterally and become anchored within the phospholipid bilayer where it remains. Transmembrane proteins can either contain one hydrophobic stretch of amino acids, and are classified as single pass transmembrane proteins, or contain multiple regions that cross the membrane and are classified as multi- pass transmembrane proteins. If the protein is not destined to be integrated into the membrane, but instead enter the secretory pathway or the lumen of membrane-bound organelles, the protein begins the process of transport. Once translation is complete and the signal peptide has been cleaved the ribosomes are released back into the cytosol. For mRNAs translated by stably bound ER ribosomes, mRNAs are released and ribosomes may remain bound to the ER and participate in multiple rounds of translation. For cytosolic proteins translated on ER-bound ribosomes it is not clear how these mRNAs are recruited to the ER or what populations of ribosomes are utilized to initiate translation, although a recent study indicates that the ER-resident protein p180 may play a role in the translation-independent recruitment of mRNAs to the ER. Following protein synthesis and translocation into the ER lumen, a protein destined for secretion must undergo proper folding and modifications, with the aid of chaperones and folding enzymes. These modifications include N-linked glycosylation, disulfide bond formation and oligomerization. At this point the fate of the secretory proteins is determined. If the protein functions in the ER, for example as a chaperone, then proper folding will commence. If the protein is destined for secretion, it will be released by the chaperones and packaged for travel through the Golgi on to a final destination (such as the plasma membrane or secreted) or move into peroxisomes. Additionally, the cytosolic regions of the transmembrane protein may interact with cytosolic proteins or chaperones to properly fold these domains. On the other hand, even with several proteins and complexes dedicated to folding proteins properly, a fraction of proteins do not achieve native and functional form and are either misfolded or aggregated. These proteins can either remain in the ER or enter the ER-associated degradation (ERAD) pathway mediated by the proteasome, assuring that aberrant polypeptides do not inadvertently enter the secretory pathway. Recognition of misfolded proteins, followed by clearing of these aggregates through the ERAD pathway, needs to be tightly controlled so as not to affect cellular function. Interestingly, there are several connections to activation of ER stress response pathways and pathological human conditions. Several neurodegenerative protein misfolding diseases, such as Alzheimer's disease, activate ER stress response pathways. Additionally, activation of the ER stress response pathway is observed in diabetes, inflammatory bowel disease, and various cancers. How ER stress response pathways play a role in these pathologies is an active area of research and various components of the stress response pathways are being investigated as potential therapeutic targets. [5]

Because protein folding is a complex and error-prone process, the protein-folding capacity of the ER can easily be saturated under a number of physiological and pathological insults such as glucose deprivation, aberrant Ca^{2+} regulation, viral infection, envi-

ronmental toxins, hypoxia, oxidative injury, hypoglycemia, mutant protein expression, aging, and simple increases in secretory protein synthesis. To buffer ER stress and orchestrate the recovery of ER function, cells use 4 different strategies. First, cells focus on translation attenuation for a few hours, thereby lessening the freshly prepared protein load into the ER until mRNAs encoding unfolded protein response (UPR) proteins are processed. In a second attempt, the UPR upregulates the folding machinery by inducing ER chaperone genes. Third, the ER compartment proliferates to accommodate the high protein load and then begins ER-associated degradation (ERAD) of unfolded or misfolded proteins. ERAD mainly consists of 2 mechanisms: ubiquitin-proteasome-dependent ERAD (type I) and autophagy- lysosome dependent ERAD (type II). Type II ERAD represents an autophagic pathway in which both soluble and insoluble forms of misfolded proteins are targeted, whereas type I ERAD targets only soluble misfolded proteins.4 Initially, it was thought that ER stress initiates autophagy only when aggregated proteins become excessive enough to overwhelm the canonical ubiquitin- proteasome-dependent ERAD. Current findings suggest, how- ever, that ERAD-mediated partially processed proteins are also a target of autophagy, which includes all other unfolded proteins.5 Finally, programmed cell execution is initiated when stress exceeds a given threshold and the ER is so severely impaired that compensatory mechanisms are no longer able to sustain its function. [6]

The Unfolded Protein Response

The unfolded protein response (UPR) is a highly conserved pathway that allows the cell to manage endoplasmic reticulum (ER) stress that is imposed by the secretory demands associated with environmental forces. In this role, the UPR has increasingly been shown to have crucial functions in immunity and inflammation. [7]

In metazoans, the UPR is activated by the coordinated action of three ER transmembrane stress sensors: inositol-requiring enzyme 1α (IRE1α; also known as ERN1), PKR-like ER kinase (PERK; also known as EIF2AK3) and activating transcription factor 6α (ATF6α). Under homeostatic conditions, the luminal domains of these ER stress sensors are retained in an inactive state through association with binding immunoglobulin protein (BiP; also known as GRP78 and HSPA5). However, owing to the higher affinity of BiP for misfolded proteins, BiP dissociates from the ER stress sensors as misfolded proteins accumulate in the ER lumen, thereby releasing the stress sensors to permit downstream signaling (Figure 2). In addition, it has been elegantly shown that, at least in *Saccharomyces cerevisiae*, basic and hydrophobic residues on unfolded proteins can directly bind to a putative peptide groove of IRE1α, and direct binding of unfolded proteins is sufficient to induce the UPR. It is currently unclear whether direct binding of unfolded proteins as a second mechanism of UPR activation is limited to IRE1α or whether it can also be observed with PERK and ATF6α. These mechanisms require further characterization. [7]

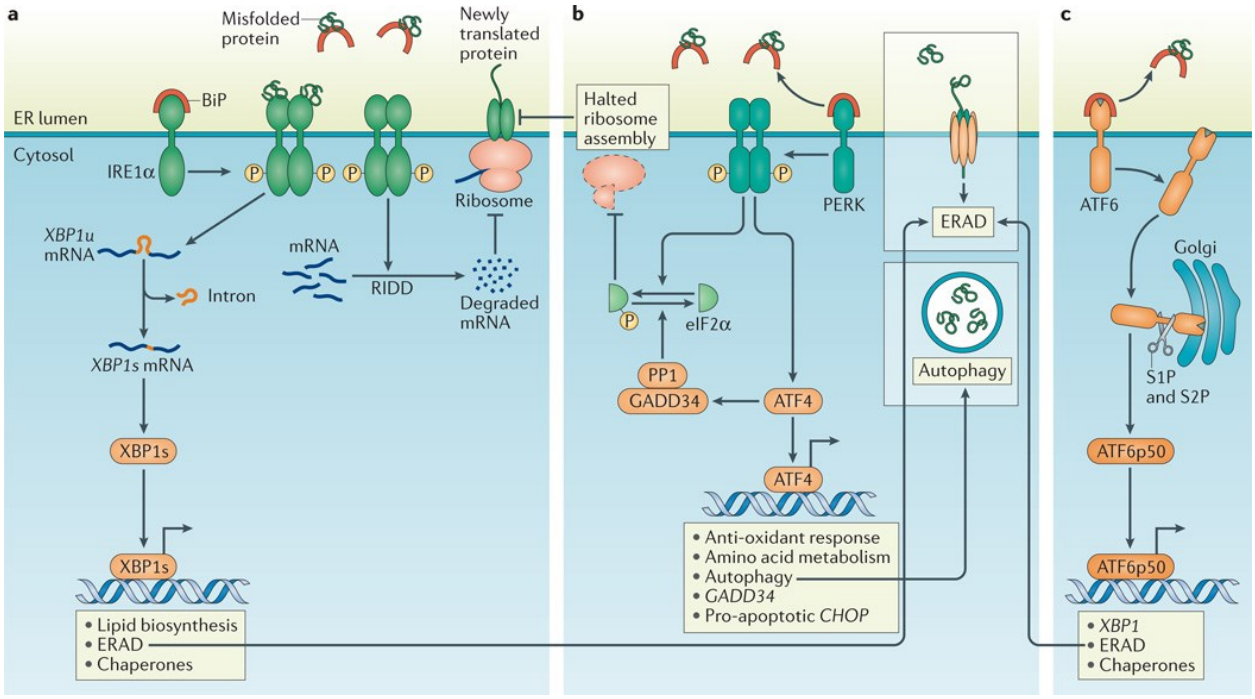

Figure 2: The three arms of the UPR: a) Mechanism of activation and downstream regulation of IRE1α b) Mechanism of activation and downstream regulation of PERK c) Mechanism of activation and downstream regulation of ATF6, taken from [7]

Ribosome stalling

In eukaryotic cells, a major source of erroneous translation that causes ribosome stalling comes from naturally occurring faulty mRNAs. For instance, non-stop mRNAs bearing polyadenine (poly- A) stretch are generated in as much as 5%–10% transcripts in eukaryotic cells due to premature polyadenylation. Translation of poly-A stretches that are normally outside of open reading frames (ORFs) can also cause ribosome stalling or prolonged pausing, resulting in arrested products (AP) that are prone to misfolding and aggregation. (Figure 3) These aberrant translation products can interfere with other functional proteins and are therefore deleterious to cells. In the cytosol, a ribosome-associated quality control (RQC) pathway effectively eliminates APs upon ribosome stalling. This RQC pathway employs a collection of factors that coordinately sense translation stalling, rescue stalled ribosomes, and degrade aberrant nascent polypeptides. Specifically, ubiquitination of 40S ribosomal subunits by the ribosome-associated ubiquitin ligase (E3) ZNF598 serves as a signal to trigger ribosome splitting by a ribosome recycling factor (e.g., PELO and ABCE1). Subsequently, the 60S ribosome-associated ubiquitin ligase Listerin and its binding partner NEMF are recruited, resulting in AP ubiquitination and degradation by proteasome. RQC deficiency can cause abnormal development and neurodegeneration in animals, underscoring the importance of this quality control pathway in protein homeostasis regulation. In sharp contrast to the cytosolic RQC pathway, little is known about the quality control mechanism for endoplasmic reticulum (ER)-bound ribosomes when they encounter translation arrest. Unlike ribosome stalling in the cytosol, translation arrest during co-translational protein translocation at the ER creates a unique “crisis” because APs are situated inside the Sec61 translocon with their N-termini in the ER lumen, which may have participated in protein biogenesis-associated events such as folding, glycosylation, and disulfide

bond formation. Thus, the removal of translation-arrested ER proteins might engage a mechanism more akin to ERAD than cytosolic RQC. Failure to degrade nascent chains stalled in the translocon is expected to cause accumulation of APs in the ER, which could interfere with ER protein biogenesis, leading to a secretion defect and ER stress.[8]

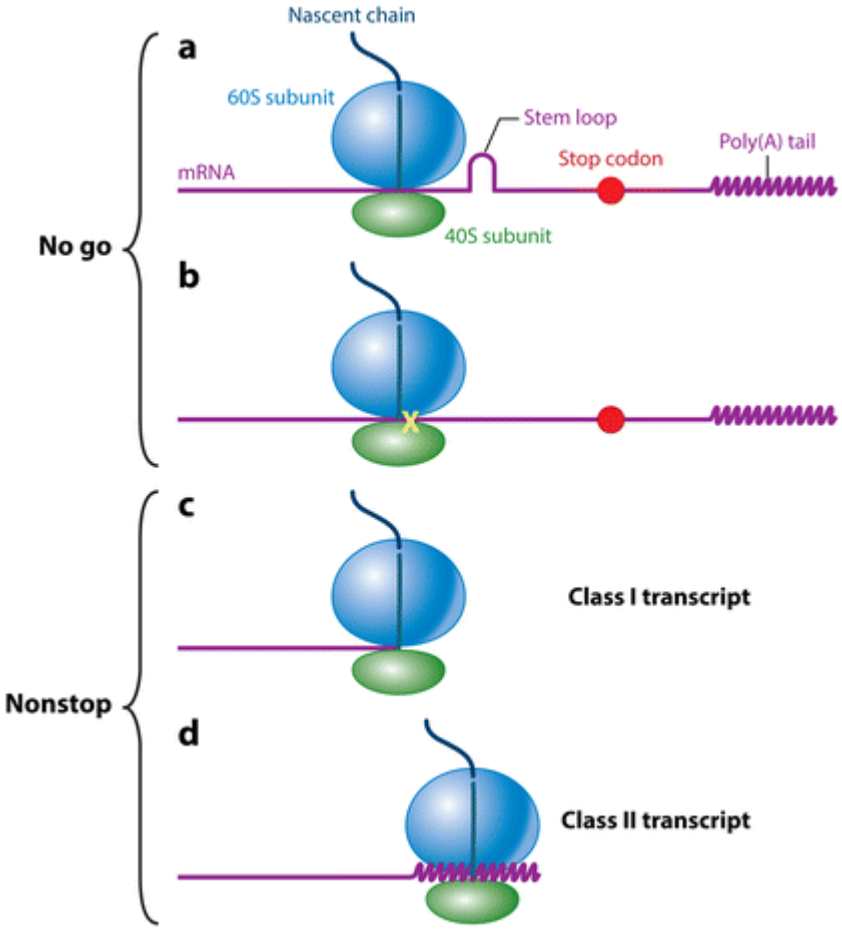

Figure 3: Different mechanisms of ribosome stalling, taken from [29]

UFMylation

Ubiquitin and ubiquitin-like molecules (UBLs) constitute the third most common post-translational modification after phosphorylation and glycosylation. This family of small proteins can be covalently attached to their targets following an E1–E2–E3 reaction, altering either their stability, structure, or interactome. Although the ubiquitin 'code' has been extensively studied over the course of decades, the functions of many other UBLs are still poorly understood. One such modifier is ubiquitin-fold modifier 1 (UFM1). UFM1 and its conjugating machinery are highly conserved among most eukaryotes except for yeasts and other fungi. Interestingly, UFM1 substrates appear to have been insulated from other UBLs because they share a high percentage of exclusivity for UFMylation. The UFM1 system is also tightly related to the function of the endoplasmic reticulum (ER), and the expression of most components of the UFM1 pathway are modulated by ER stress. Given that ER stress is a hallmark of many pathologies, recent studies pointed to the UFM1 system as a contributor to tissue homeostasis and as a possible key

player in the progression of different diseases such as cancer, heart failure, gut inflammation, and neurodevelopmental disease.[9]

Establishing conditions for further experiments

Inducing ER Stress in HeLa cells

As the observation of autophagic processes in the cell during ER stress is the main objective of this work, the first staple was to establish conditions to induce ER stress in tissue culture conditions in a reproducible manner. Since ongoing ER stress may tip the cellular scales of fate from autophagy to apoptosis[10] the screening for both, the right concentration of stressor and the timeframe of induced stress was of the essence to keep the cells alive. From the possibilities to induce ER stress[11] the drug Tunicamycin was chosen, as it yielded the most reproducible reaction. In an experiment the conditions were set to 16 hours of 2,5ug/ml Tunicamycin and an optional subsequent recovery phase of 2 hours, in which, to accumulate autophagic markers, a treatment with 100nM Bafilomycin A1 can be performed. The reasoning behind these choices is more on the side of cell viability than actual ER stress, as with prolonged incubation times with Tunicamycin the intensities of Vinculin bands on a western blot are reduced. (Figure 4) As Vinculin is acting as a loading control a decrease of the band intensities indicates fewer surviving cells, as for sample preparation dead cell are washed away, since they don't stick to the tissue culture plate anymore. As an ER stress marker on western blots the chaperone BIP3 was used, as it is a key player in the UPR and gets enriched upon prolonged ER stress.[7]

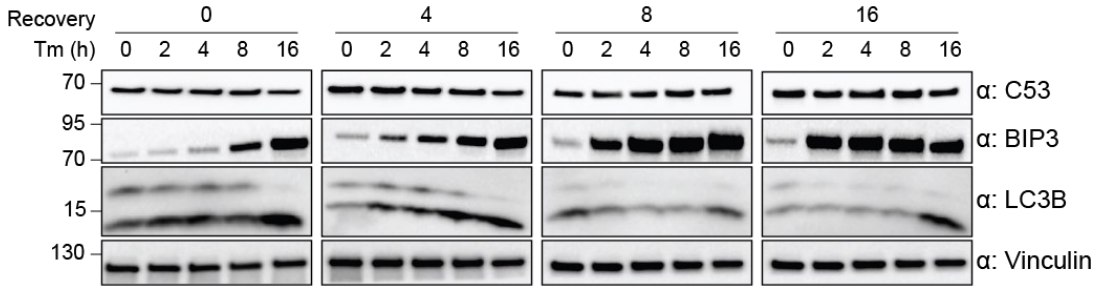

Figure 4: Establishing ER stress conditions: Western blot analysis of HeLa whole cell lysates. Cells were either left untreated or treated with 2,5 µg/ml Tunicamycin (Tm) and subsequently given an indicated recovery period. C53 and BIP3 blots were run on 4-20% gradient gels and transferred to nitrocellulose membranes, LC3B blot was run on 15% gels and transferred on PVDF membranes.

Determining the best conditions to blot LC3B-I and LC3B-II

An established marker for analyzing autophagy in mammalian cells is the immunoblotting of LC3B (Microtubule-associated protein 1A/1B-light chain 3). This ATG8 family protein is ubiquitously present in all mammalian tissues and cell and is cytosolic (also called LC3-I. When autophagy is induced the autophagic machinery conjugates LC3B to phosphatidylethanolamine (PE) to form PE-LC3B or LC3B-II[12]. This form of LC3B is anchored into phagophores/autophagosomes and therefore a turnover of this protein can be used as an indicator for autophagic processes. To monitor this process the drug Bafilomycin A1 (BAF) can be utilized. BAF has two separate mechanisms of action to block autophagic degradation. For once it blocks the lysosomal proton pump V-ATPase

and therefore reduces acidity of the lysosomes, so autophagic cargo can accumulate. Secondly BAF also blocks the ER calcium pump Ca-P60A/SERCA and therefore inhibits the fusion auf autophagosomes with lysosomes and leads to further slow down of the degradation of autophagosomal cargo.[13] (Figure 5)

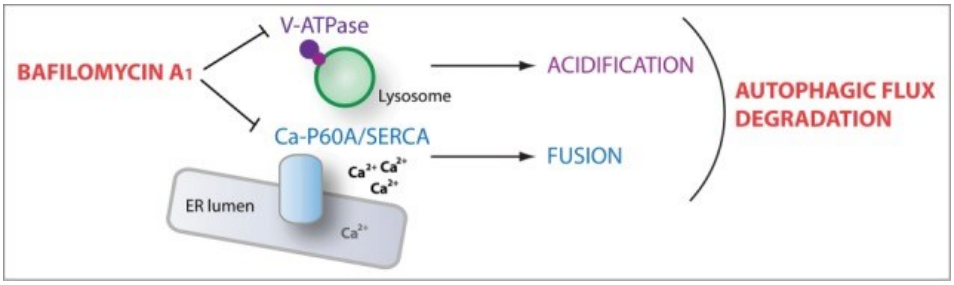

Figure 5: Schematic mechanism of action of Bafilomycin A1, taken from [13]

Unfortunately, it proved difficult to yield a blot of satisfying quality. Multiple improvement and refining steps were necessary, distributed over the whole course of the experimental set-up of a western blot experiment and in the end almost every parameter of the initial set-up, which by then was an established procedure in the research group, had to be altered (Table 1). The main problems of blotting LC3B were that the separation of LC3B-I and LC3B-II was not good enough on the 4-20% gradient gels, the LC3B antibody was not detecting enough signal on nitrocellulose membranes and the transfer system had trouble transferring one of the LC3B forms. (Figure 6)

Experimental Parameter	Established	Altered for LC3B
Gel	4-20% precast gradient gel	15% self-made gel
Electrophoresis	150V for 1,5h	70-100V for 2h
Transfer	Trans Blot Turbo (Biorad)	Classical wet transfer (200mA for 70 minutes)
Membrane	Nitrocellulose	PVDF (methanol activated)

Table 1: Necessary alterations of the standard western blot protocol for blotting LC3B

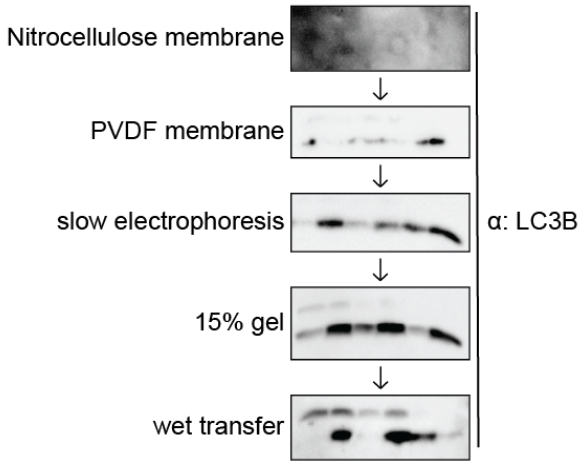

Figure 6: Overview of the quality improvements made with changing parameters of the experiments

Results

C53 is undergoing autophagic flux

The first question that arises when a protein shows hints of being an autophagy receptor is if said protein is undergoing autophagic flux. Autophagic flux meaning that the protein of interest is located inside of autophagosomes and therefore has a turnover which can be measured via immunoblot. To further prove the turnover Bafilomycin A1 is used to stop the degradation of autophagic cargo[13] and therefore increase the signal on the blot. At the same time LC3B-I/II blots are performed as a positive control auf autophagy in general[12]. Two ways were chosen to induce autophagy. Firstly ER stress induced by Tunicamycin[11] and secondly via inhibition of mTOR by Torin.[14]
Results show that indeed C53 is undergoing autophagic flux during ER stress conditions, as its levels are reduced upon induction of autophagy and subsequently increased when the cells have been treated with Bafilomycin A1 to inhibit degradartion. BIP3 chaperone was used as a positive control for ER stress, and as expected there are only bands visible in Tunicamycin treated samples. The LC3B blots clearly indicate that there are autophagic processes happening in the samples. To further prove that C53 is indeed inside autophagosomes a microscopic observation was made necessary.

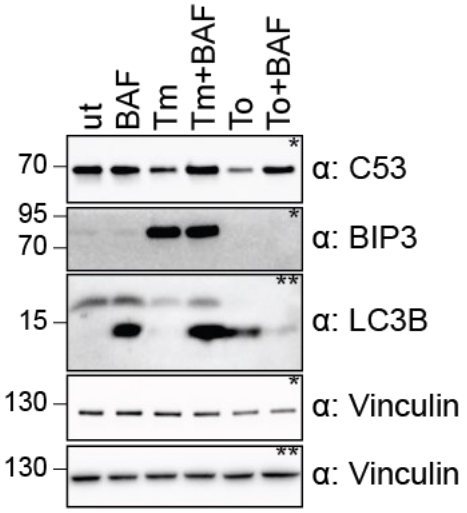

Figure 7: Western blot analysis of HeLa whole cell lysates. Cells were either left untreated or treated for 16 h with 2,5 µg/ml Tunicamycin (Tm) or 1,5 µM Torin (Tor) and subsequently given a recovery period of 2 h in presence or absence of 100 nM Bafilomycin A1 (BAF). C53 and BIP3 blots were run on 4-20% gradient gels and transferred to nitrocellulose membranes, LC3B blot was run on 15% gels and transferred on PVDF membranes. * indicate corresponding membranes.

C53 colocalizes with autophagosomes

Confocal microscopy techniques were used to further prove that C53 is located inside of autophagosomes. As a proxy for autophagosomes the ATG8 family protein GABARAP[15] was used, as Lorenzo Picchianti showed that C53 is mostly interacting with this ATG8 isoform in an recombinant experiment[1]. To visualize the interaction of both proteins in vivo/in cellulo C53 was tagged with green fluorescent protein (GFP) on its N terminus and GABARAP with the red fluorescing protein mCherry on its N terminus. It is important to tag ATG8 family proteins on their N terminus as for their activation a peptide from their C terminus need so be truncated to expose a C terminal Glycin to which PE can be conjugated[12]. Microscopically the GABARAP signal should be visible in the form of foci, so called puncta, as it indicates the location of autophagosomes in the cell. If C53 indeed interacts with GABARAP it should present a similar signal upon induction of autophagy via ER stress. If both these puncta are in the same position both signals are present in the same pixel and this pixel presents itself in a different color, according to the additive mixing of the colors of the singular channels. This is called colocalization and indicates a biological interaction in a cell.[16] The experimental results show that, upon induction of ER stress, C53 indeed is forming puncta and that these puncta colocalize with GABARAP (Figure 8). Therefore, this is further strengthening the claims made in the previous experiments, that C53 is an autophagic receptor linked to perturbations of the ER homeostasis. Interestingly, when the cells do not experience ER stress the C53 signal is diffuse and mainly nuclear and forms close to none punctate structures. This is explainable by considering the fact that C53 is repeatedly reported as a transcription (co-)factor.[17,18] To further increase the significance and validity of the experiment the cells were also treated with Bafilomycin A1 and observe an increase in colocalizing puncta[13]. Even though the colocalization was increased, Bafilomycin A1 alone was not sufficient to significantly increase it, strengthening the claim that C53 is an autophagic receptor that is only active during specific conditions. The degree of colocalization was quantified to present the results in a more graspable way. To achieve this the software Fiji[19] was used, more specifically its plug-in JACoP[20], to calculate the Pearson's correlation coefficient of the colocalization[16]. As mentioned only after inducing ER stress colocalization increases significantly. Bafilomycin A1 nevertheless has a synergistic effect. (Figure 9)

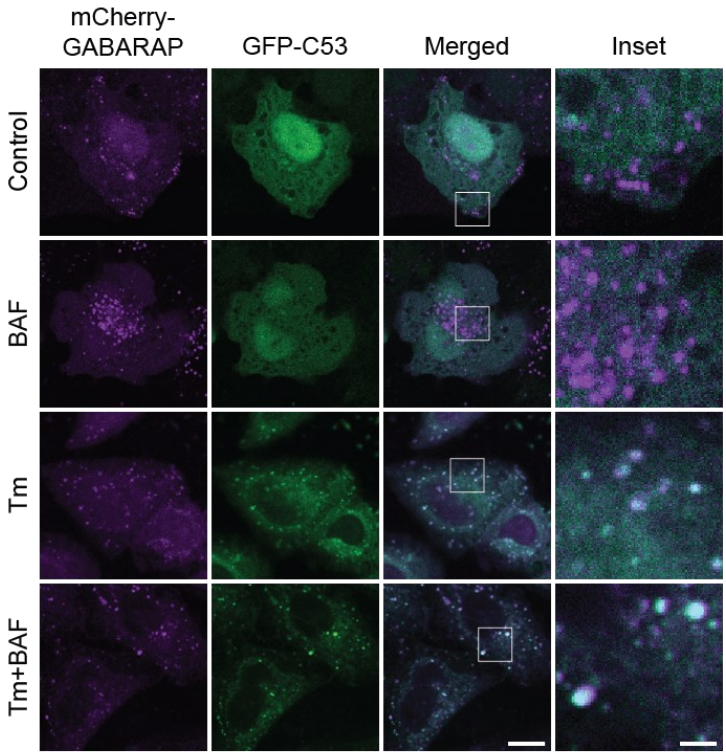

Figure 8: C53 puncta colocalize with mCherry-GABARAP labelled autophagosomes during ER stress. Confocal images of PFA fixed HeLa cells transiently expressing C53-GFP (green) and mCherry-GABARAP (magenta). Cells were either left untreated (Control) or treated with Tunicamycin (Tm) or Tm + Bafilomycin (BAF). Scale bar, 10µm. Inset scale bar, 2 µm.

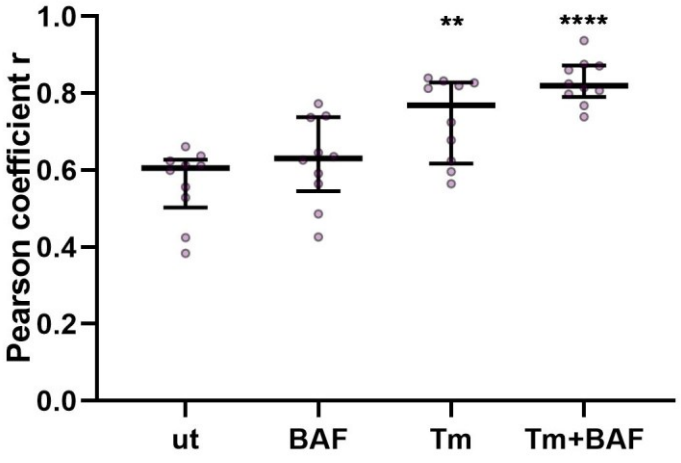

Figure 9: Pearson's Coefficient analysis of the colocalization of GFP-C53 with mCherry-GABARAP under Control and Tm treated conditions. Bars represent the mean (± SD) of at least 5 biological replicates. Significant differences are indicated with * when p value ≤ 0.05, ** when p value ≤ 0.01, and *** when p value ≤ 0.001.

Generating a C53 Knockdown Line

To facilitate further characterization of C53, its role in cellular homeostasis and even detect a possible cargo, a cell line with C53 knocked down was deemed necessary. Gene knockdown can be performed by RNA interference (RNAi), mediated through small interfering RNA (siRNA). siRNA interacts with the host cells mRNA to prevent it from being translated.[21] As cells have mechanisms to cope with this kind of interference siRNA mediated knockdowns are limited to only transient experiments. For a more stable knockdown small hairpin RNA (shRNA) can be used, as the host cell generates siRNA from it. The downside of this method of knockdown is that shRNA is not transfecable.[21] Therefore it has to be delivered into the host cell via viral transduction[22]. The lentiviral pLKO.1 system[23] was chosen to perform the shRNA delivery. To achieve the knockdown first the virus containing the shRNA had to be produced in HEK293T cells, by transfecting plasmids coding for the viral particles and the pLKO.1 plasmid. The virus was secreted from the cells into the medium. This medium was harvested and transferred to the target HeLa cells, where the virus could infect the cells and deliver the shRNA. The pLKO.1 system provides puromycin resistance as a selection marker, so it is guaranteed that all cells are successfully C53 knocked down cell. (Figure 10)

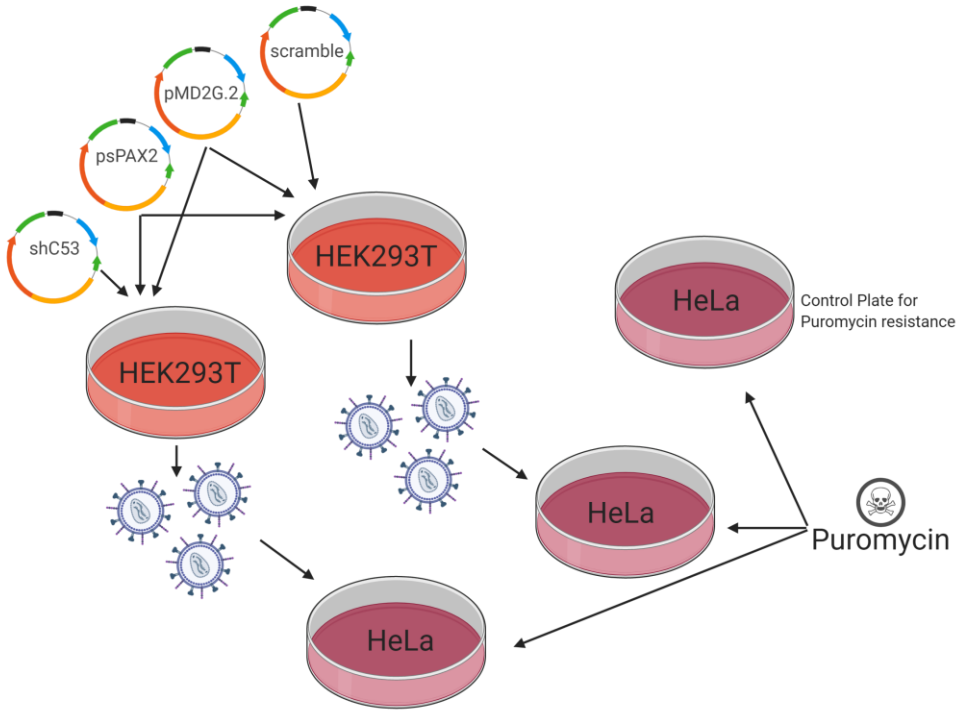

Figure 10: Schematic of the experimental procedure of a lentivirus transduced shRNA mediated knockdown. psPAX2 and pMD2G.2 encode for the lentiviral particles. Scramble acts as a negative control.

The actual knockdown of C53 was checked by western blot. (Figure 11) After the successful knockdown the cells were split and bulked up to generate a small stock of the knockdown line, as even with shRNA a knockdown is not permanent.[24] During further experiments with this line the knockdown was tested regularly by western blot and if C53 was reappearing on the blots, the cells were trashed and a new vial from the generated stock was taken in culture. All repetitions of a specific experiment were performed during one culture cycle to reduce variability that freeze-thawing cells could have introduced.

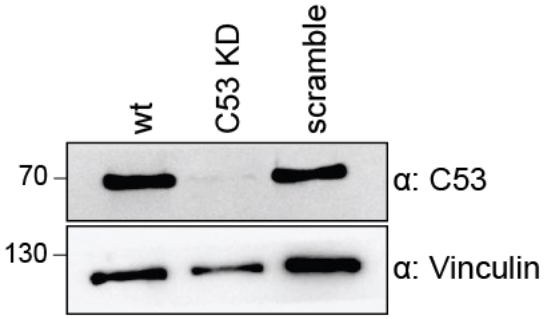

Figure 11: Successful knockdown of C53. Western blot analysis of HeLa whole cell lysates. Cells were either left untreated or treated with lentiviral particles containing either shRNA for C53 or scrambled shRNA.

Phenotypes of C53 Knockdown Cells

As to be expected, the absence of C53 altered the cells and they show various signs of an imbalance in cellular homeostasis. For once it was observed that the C53 KD line had a significantly slower pace of growth and cellular division. Furthermore, when the cells were growing not in groups, they hat an elongated and slimmer shape. This may indicate some faults in the cytoskeleton. The fault in morphology was rescuable, by transfecting the cells with C53. In fact, not just the human isoform of C53 was capable of this but also the isoform from Arabidopsis thaliana (AtC53). Another variant of C53 that has its AIMs mutated in a way that it cannot bind to ATGs anymore ($HsC53^{sAIM}$) was not able to rescue this phenotype. (Figure 12). As this phenotype vanishes when the cells group up (and therefore the majority of the cells appear to be normal), and is therefore highly specific, it was just noted, but not investigated further.

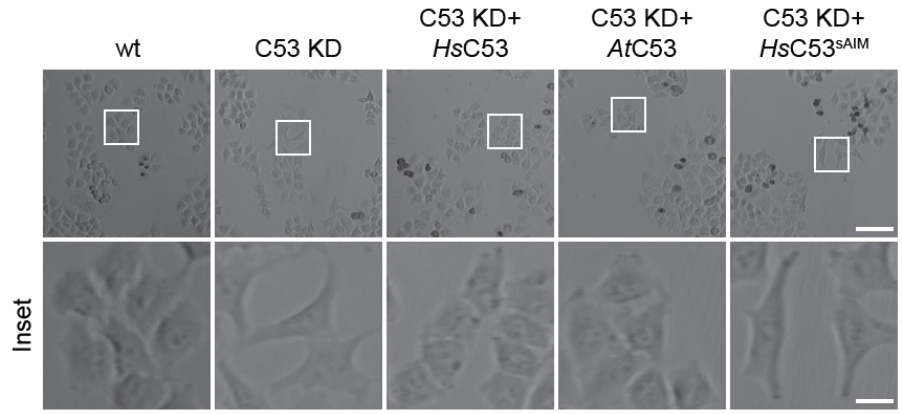

Figure 12: DIC images of live HeLa cells of different genotypes. The cells were left untreated after transfection. Bar equals 100um. Inset bar 10um.

Another reaction of the cell to the lack of C53 is the probability of a raised sensitivity to ER stress. This can be measured by immunoblotting for the BIP3 chaperone as discussed earlier in this work. Interestingly the mutated C53 HsC53^{sAIM} could not be detected anymore by the C53 antibody, which was used in former experiments, apparently the mutations of the AIMs aberrates the binding to the antibody as it does to ATG8s. To circumvent this GFP tagged constructs were used in further experiments to be able to detect the construct indirectly via its tag. The results of the experiment show that in fact a knockdown of C53 leads to elevated BIP3 levels, compared to the wild type, even without a treatment. A complementation with C53 dampens the expression of BIP3 again, even to lower levels than the wild type. This may be due to the overexpression of C53 in the complemented cells, indicating that C53 may be a protective agent for ER stress. Interestingly though, the mutant C53 HsC53^{sAIM} did also dampen BIP3 expression. Therefore, it can be assumed that the protective impact of C53 is not linked to autophagy. This phenomenon would need thorough investigation and could be linked to UFMylation.

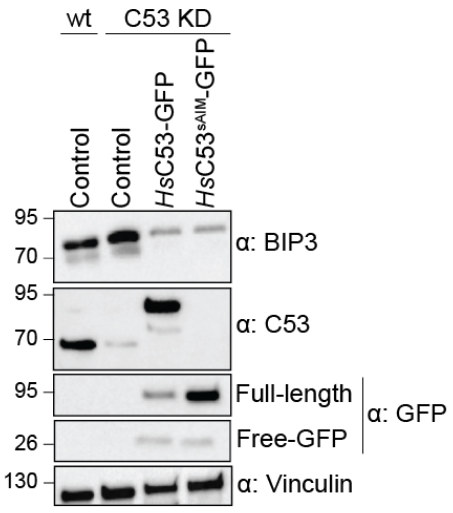

Figure 13: Western blot analysis of HeLa whole cell lysates. Cells were left untreated or treated after transfection.

C53 is cross kingdom conserved, but not functionally

Another question that was arising is if C53 is cross kingdom conserved, as previous work in the group had indicated (cite c53 paper). In fact, the AIM motives of C53 are conserved through most of species (c53 paper or image). An experiment to check if the isoform of C53 from Arabidopsis thaliana AtC53 behaves the same as human C53 was deemed necessary. To achieve this a confocal study of colocalization with GABARAP, analogously to the experiment discussed earlier, was performed. GABARAP was N terminally tagged with mCherry and (Hs)C53, AtC53 and the mutant HsC53^{sAIM} were all tagged N terminally with GFP. The results of the experiment showed that HsC53^{sAIM} in´s a true mutant and does not form puncta during ER stress conditions. On the other hand, AtC53 is forming puncta but these puncta are not colocalizing with autophagosomes. This may indicate that AtC53 can still be activated by the human activation machinery, but it is not truly functionally conserved. (Figure 14)

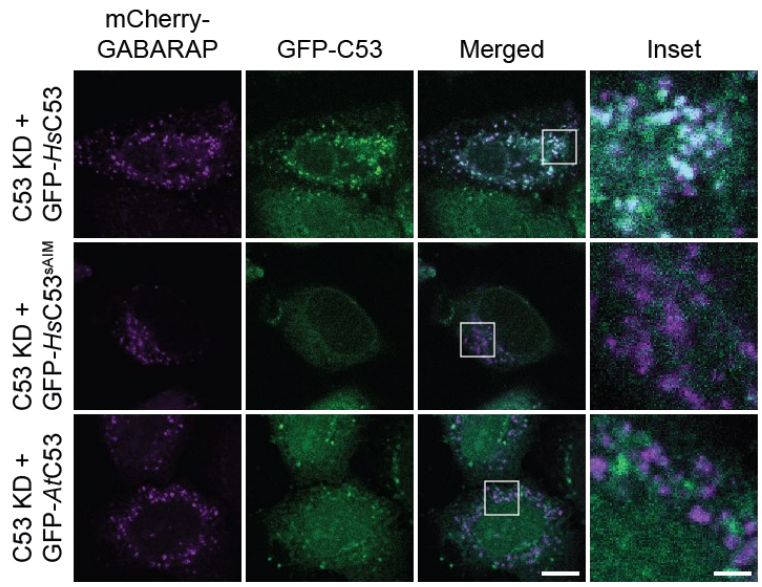

Figure 14: Confocal images of PFA fixed C53 knockdown HeLa cells transiently expressing GFP-HsC53, GFP-HsC53^{sAIM} or GFP-AtC53 (green) and mCherry-GABARAP (magenta). Cells were treated for 16h with 2,5 µg/ml Tunicamycin (Tm). Scale Bar, 10 µm. Inset scale bar, 2 µm. Representative images are shown.

Inhibition of the UPR leads to activation of C53

When studying an autophagic receptor, the question of its cargo and activation mechanism always arises at some point. For C53 it was proven in this work that its activation is connected to ER stress. Therefore, the possibility of a connection to the UPR needed investigation. This can be achieved by blocking the UPR. A blockage of UPR pathways should cause ER stress and if C53 activation is linked to the UPR it should not be activated by this stress. This activation, or lack thereof, is easily observed via confocal microscopy of GFP-C53, as it evacuates the nucleus upon activation, as discussed before. As the UPR consists of three pathways[7] all of them need to be blocked. The most interesting pathway though is IRE1 as it is the only one conserved to plants[25] (Arabidopsis

thaliana specifically) and conservation of C53 activation was already discussed. To execute the tests multiple drugs to block the UPR on different points were used (Table 2)

Drug	Mechanism of action
4u8c	Inhibition of IRE1 RNAse activity
KIRA6	Inhibition of IRE1 kinase activity
APY29	Inhibition of IRE1 oligomerization
Ceapin	Inhibition of ATF6 golgi localization
GSK 2606414	Inhibition of PERK kinase activity

Table 2: Specifications of the UPR inhibitors used.

The results show clearly that C53 does not stay inactive during inhibition of the UPR but contrary even gets activated when IRE1 is inhibited. On the images without activation of C53 black gaps in the cytosol indicate ER perturbations and can therefore be a sign that the drug treatment influenced the cell itself, just not C53. These results indicate that the activation of C53 is not driven by the UPR and that the UPR receptors are not a specific cargo of C53. (Figure 15)

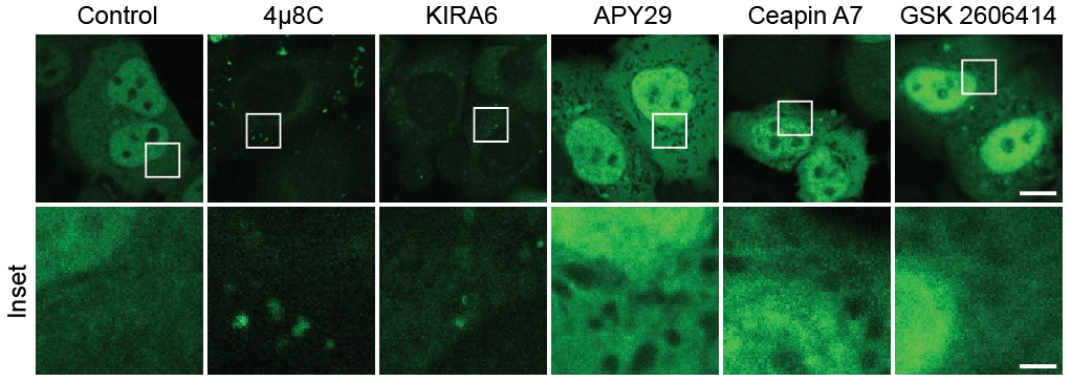

Figure 15: Confocal images of PFA fixed HeLa cells transiently expressing GFP-C53 (green). Cells were treated with indicated compounds. Scale Bar, 10 μm. Inset scale bar, 2 μm. Representative images are shown.

Inhibition of Protein Synthesis activates C53

Another process linked to ER perturbation is a faulty protein synthesis[6] and especially the degradation of inhibited ribosomes which translate proteins at the ER translocon[8]. For a general check if C53 is activated by this kind of perturbation a similar essay as the one used for Figure 15 was performed by Alibek Abrakhmanov. The drug Anisomycin was utilized to inhibit protein synthesis by inhibiting the peptidyl transferase on the 80S ribosome in GFP-C53 transfected cells. Results suggested an increase of C53 puncta upon even short treatments with Anisomycin (Figure 16). Again, Bafilomycin A1 was used as a co-treatment to indicate that the structures observed are autophagosomes, by increasing their numbers compared to the not Bafilomycin treated samples. (Figure 17)

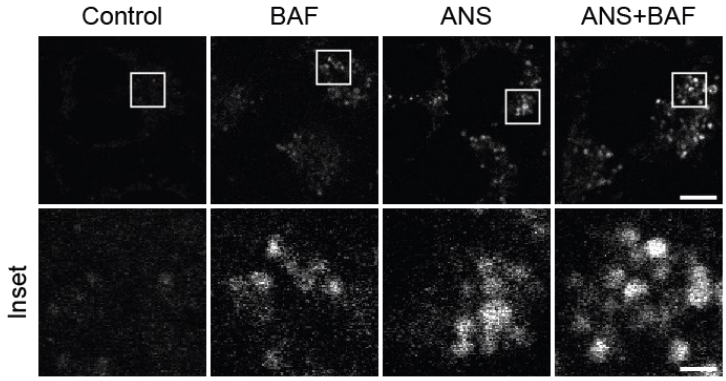

Figure 16: Confocal images of PFA fixed HeLa cells transiently expressing GFP-C53 (grey). Cells were treated for 15 min with the indicated compounds. Scale Bar, 10 µm. Inset scale bar, 2 µm. Representative images are shown.

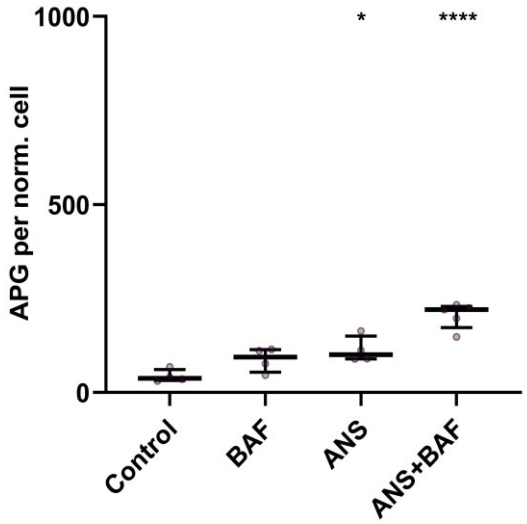

Figure 17: Quantification of Autophagosomes (APG) per normalized cell from images in Figure 16. Bars represent the mean (± SD) of at least 5 biological replicates. Significant differences are indicated with * when p value ≤ 0.05, ** when p value ≤ 0.01, and *** when p value ≤ 0.001.

C53 gets activated when ribosomes are stalled but not when translocons are clogged

To investigate the findings from Figure 16 and 17 further, the Ribosome-Translocon-Complex was inhibited separately in different experiments to pinpoint the error in homeostasis which activates C53. To achieve selective inhibition of either the translating ribosome or the translocon two different constructs were utilized. For blocking the ribosome a so called staller construct, ER-K20[8], was used, which stalls ribosomes on the ER surface. Secondly a construct, pcDNA3-Erdj3-GFP-3Gly[26], that while being translocated gets stuck in the translocon and therefore clogs it. As both of these constructs were obtained from other studies and were GFP tagged, C53 had to be retagged with mCherry to make a confocal analysis of colocalization feasible. Experimental results show that ribosome stalling leads to clear activation of C53 and even to colocalization of C53 puncta and ER-K20 foci, indicating that C53 plays a role in the degradation of the stalled ribosome. On the other hand, C53 stays completely unaffected by clogging of translocons which is easily visible as C53 does not evacuate the nucleus (Figure 18).

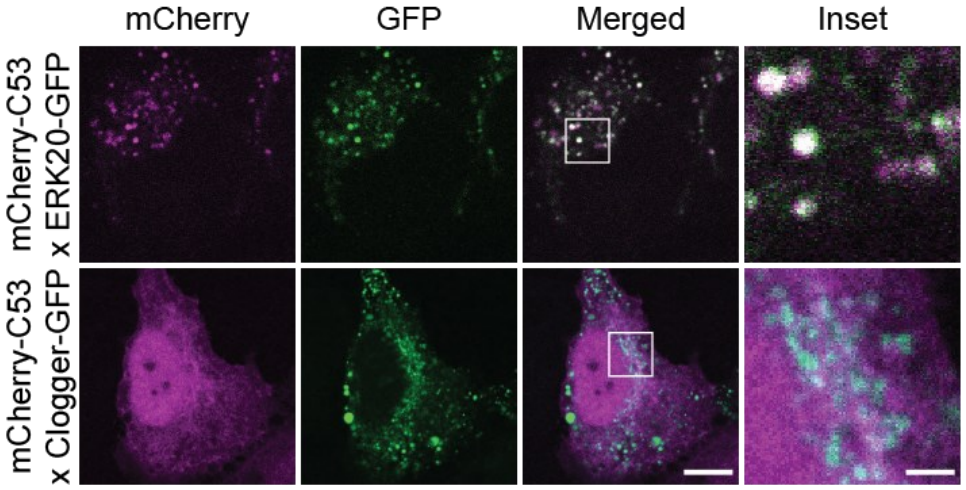

Figure 18: Confocal images of PFA fixed HeLa cells transiently expressing C53-mCherry (magenta) and either ERK20-GFP or GFP tagged clogger construct (green). Cells were left untreated post transfection. Scale bar, 10µm. Inset scale bar, 2 µm.

In absence of C53 ERK20 does not colocalize with lysosomes

So far in this work it was only proven that stalled ribosomes activate C53. To check if indeed C53 is also involved in their degradation an assay was performed by Alibek Abdrakhmanov to see if ERK20 colocalizes with ribosomes in a C53 dependent manner. The assay was performed using the C53-Knockdown line created in this study. Compared to the wildtype the GFP tagged staller construct should colocalize significantly less with miRFP-LAMTOR tagged Lysosomes. To further strengthen the assay a reconstitution of HsC53wt and AtC53wt was performed as a control that the fewer amount of colocalizing puncta is not due to any other homeostatic changes in the knockdown cell line. Furthermore, this is a way to see if human and Arabidopsis C53 are functionally conserved. As this was an assay of lysosomal degradation, BafilomycinA1 was used in the assay to raise the pH inside of lysosomes13 to reduce quenching of GFP signal27. Results of the experiment strengthened the theory that C53 is involved in stalled ribosome degradation, as ERK20 signal and LAMTOR signal are colocalizing in wild type and in both reconstituted cells but not so in cells that lack C53. In the latter case puncta are observed close to each other but very seldomly on top of each other. Also if the Bafilomycin treated and untreated samples are compared, there is a clear raise in colocalization in the functional samples compared to the dysfunctional sample (due to the fact that more green puncta are present).

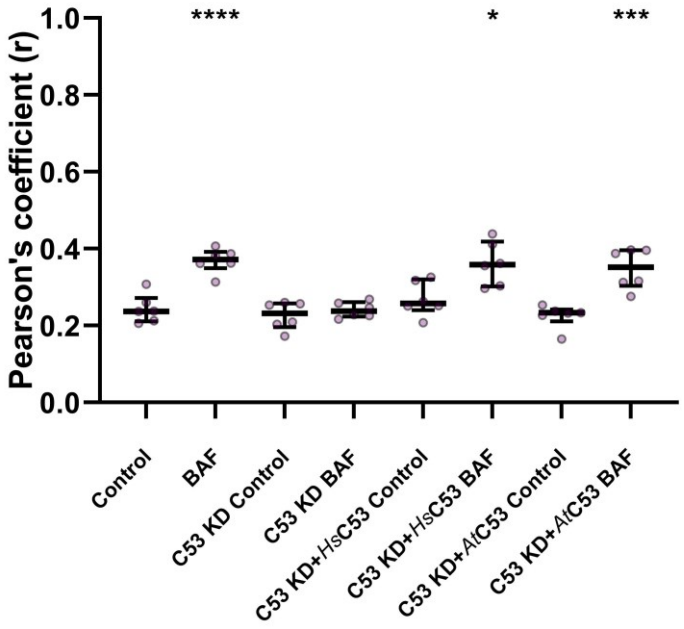

Figure 19: Pearson's Coefficient analysis of the colocalization of ER-K20 with miRFP-LAMTOR under Control and Bafilomycin treated conditions. Bars represent the mean (± SD) of at least 5 biological replicates. Significant differences are indicated with * when p value ≤ 0.05, ** when p value ≤ 0.01, and *** when p value ≤ 0.001.

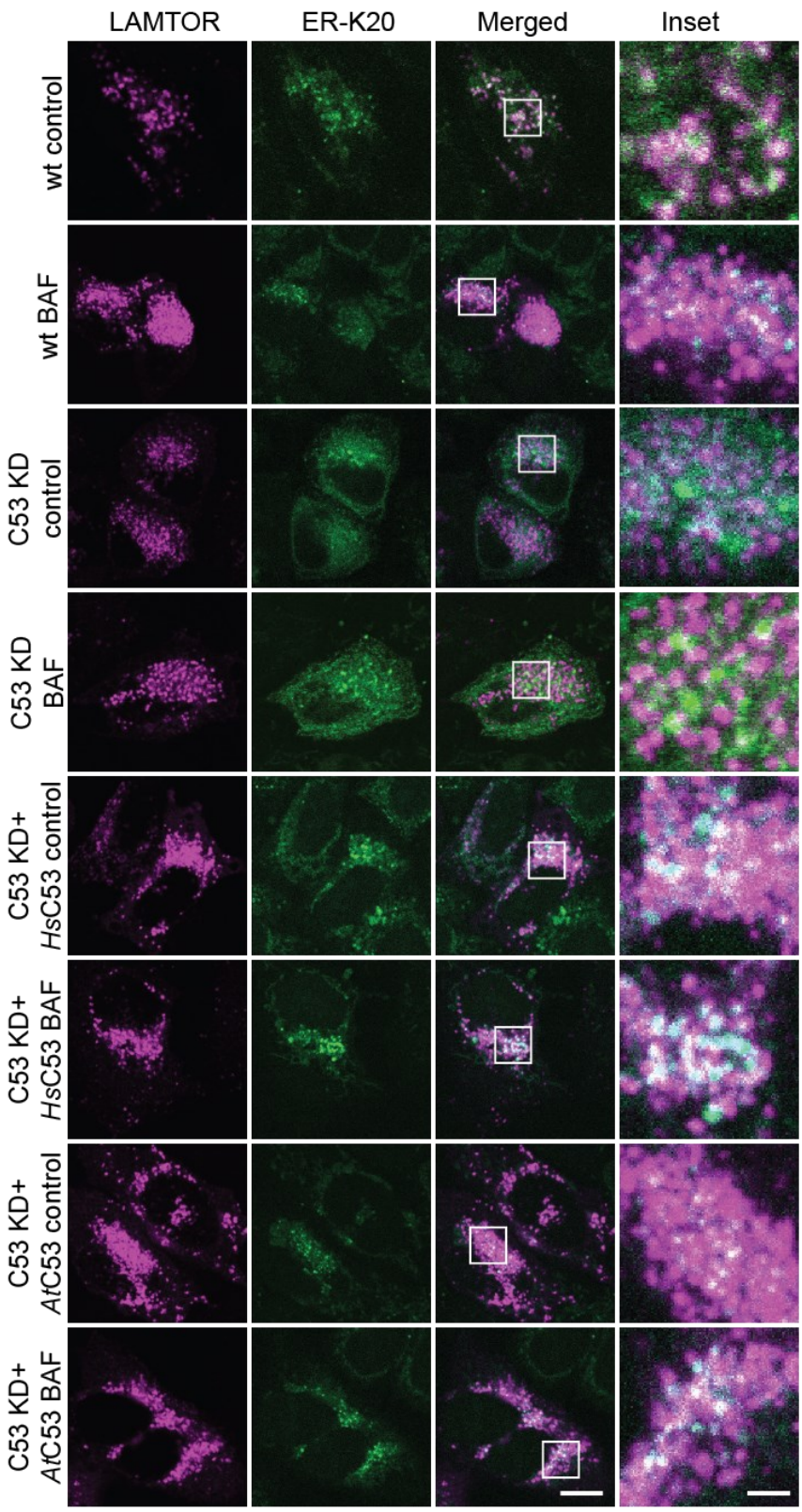

Figure 20: Confocal images of PFA fixed HeLa (wt) or C53-Knockdown HeLa (C53 KD) cells transiently expressing miRFP-LAMTOR (magenta) and ERK20-GFP (green). Cells were either left untreated or treated with 100nM Bafilomycin A1 for 2h. Scale bar, 10µm. Inset scale bar, 2 µm.

C53 colocalizes with model ERAD substrates

Another reaction of the ER to proteotoxic stress is ERAD[5] and therefore C53 might also play a role in this process. To find out if this is true, model ERAD substrates[28] were subdued to a confocal colocalization analysis with C53. Differently to previous experiments, the ERAD substrates did not form clear loci in confocal images, as they are spread out over the ER in tubular shapes. Therefore, for this assay a microscope with a higher resolution and airy scan (Zeiss LSM 980 with Airy Scan) was indicated and utilized to achieve images of puncta instead of tubular structures. The ERAD substrate set consists out of three separate substrates with ERAD inducing substructures that are either ER luminal (ERAD-L), cytosolic (ERAD-C) or inside the ER membrane (ERAD-M) that are all GFP tagged. The experimental procedure was analogue to the previous experiments. Results show that C53 is colocalizing with all ERAD substrates. Though when analyzed numerically it shows that ERAD-C has the most stable colocalization with C53, while the other two tend to colocalize less on a cell to cell comparison basis. These results acknowledge that C53 is a cytosolic protein but also that there are mechanisms that can activate C53 even from the ER-lumen. During this study first hints were gathered that these mechanisms might be connected to UFM1 and UFMylation, but this need further investigation.

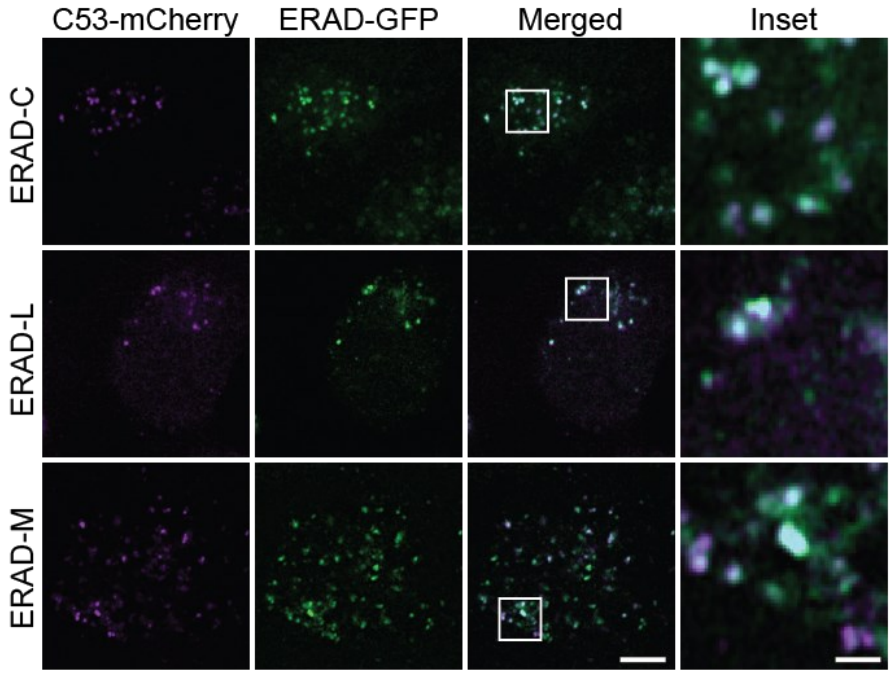

Figure 21: Airy Scan images of PFA fixed HeLa cells transiently expressing C53-mCherry (magenta) and one of the model ERAD substrates tagged with GFP (green). Cells were left untreated post transfection. Scale bar, 5µm. Inset scale bar, 1 µm.

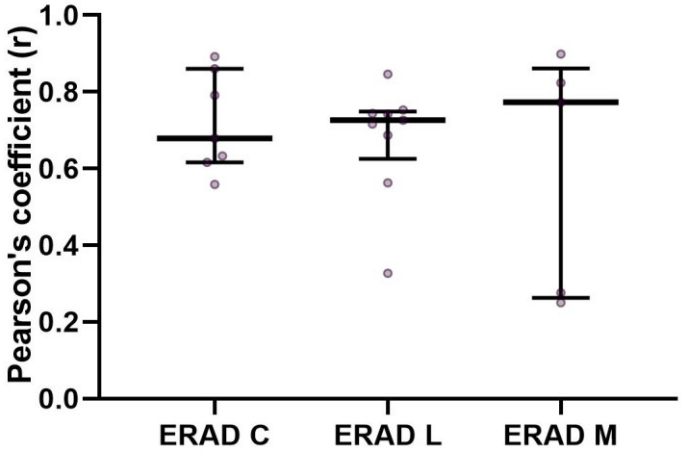

Figure 22: Pearson's Coefficient analysis of the colocalization of mCherry-C53 with model ERAD substrates in untreated conditions. Bars represent the mean (± SD) of at least 5 biological replicates. Significant differences are indicated with * when p value ≤ 0.05, ** when p value ≤ 0.01, and *** when p value ≤ 0.001.

Conclusion

In conclusion the data shows that C53 plays a crucial role in ER and protein homeostasis and in tolerance for ER perturbations via autophagy. It is clearly activated when various forms of ER stress are put upon the cell and localizes to autophagosomes. Furthermore, there are results that show that an excess of C53 raises the ability of the cell to withstand proteotoxic stress. This study also provides a claim of C53 cargoes, which are stalled ribosomes and structures marked for ERAD, though the latter still needs further investigation. The study that this work is a part of[1] further shows that C53 is activated via UFMylation and provides further insight to the nature of interactions between C53 and the autophagic machinery.

Results also suggest that C53 is conserved between humans and Arabidopsis to a certain degree, but not in a fully functional way as experimentally the isoforms do not react in the exact same way.

The lack of C53 is disrupting cellular homeostasis in an unknown way, it may be either due to permanent ER stress or due to the missing transcription factor function of C53[17,18] or due to processes and functions that need to be discovered.

Further work may provide more insight to the activation processes of C53 via the UFMylation pathway and specify the players in the C53 receptor complex.

Methods

Tissue Culture Methods

General Cell Maintenance

Cells were maintained in 10ml DMEM supplemented with 10% FBS, 1% L-Glutamine and 1% Penicillin/Streptopmycin in an 37°C/5% CO_2 atmosphere in 10cm tissue culture dishes. Passaging of cells was performed at a confluency of 70-80%. After removing the media, cells were washed with ½-2/3 culture volume PBS and after removal of the same treated for 5-7min with 2ml of Trypsin. Inactivation of Trypsin was performed by adding 1,5 Volumes of full media. Cells were split according to necessity in fractions of 1:5, 1:10 or 1:20.

Seeding Procedure

For Experiments cells were counted after trypsinization to normalize the number of cells between wells. Cell counting was performed by the CASY system, according to the manufacturers protocols. Cells were diluted 1:1000 in CASY-Ton and placed in the Counter. Depending on the duration of the experiment cells were seeded at a density of 150000-250000 cells per well of a 6-well tissue culture plate, to stop cells from becoming overconfluent.

Transfection

Transfection of plasmids into the cells was performed utilizing the GeneJuice system, according to the manufacturers protocols. 1ug of DNA was mixed in a mixture of 100uL empty DMEM and 3uL of GeneJuice and incubated for 20 minutes. Afterwards this mixture was added dropwise to the cells. No further treatments of procedures have been performed for at least 24 hours post transfection to give the cells time to adapt and express the construct.

Cell Lysis

Cell lysis was performed by scraping the cells, after washing them with iced PBS, with a lysis buffer containing 50mM HEPES, 150mM NaCl, 1mM EDTA, 1mM EGTA, 25mM NaF, 10uM ZnCl2, 1% Triton X-100 and 10% Glycerol. After scraping, lysates were spun down at 15000G and supernatant obtained for further analysis. For western blotting experiments the lysates were mixed 1:1 with 2x Laemmli Sample Buffer.

Lentiviral Knockdown

The knockdown was performed in S2 conditions. HEK293T cells were seeded 24 h prior to transfection in DMEM without antibiotics. At 50-60 % confluency cells were transfected with 1 µg shRNA, 750 ng psPAX2 and 250 ng pMD2.G utilizing 6 µL of GeneJuice in 250 µL of empty DMEM. After 48 h of incubation the virus containing media was har-

vested and mixed 1:1 with full media. This mixture was applied to HeLa cells that were seeded 24 h prior. To this Polybrene with a final concentration of 4 µg/ml was added. After 24 h of incubation the medium on target cells was exchanged with full media. After 24h, selection with 2 µg/ml Puromycin was started. No living cells were observed in a control plate after 24 h. After splitting cells in S2 conditions, cells were transferred into S1 conditions.

Treatments

For treatments with specific drugs, cells were seeded at least 24h prior and treated at a confluency of 50-60%, if they were not transfected prior to drug treatments. Just before starting a treatment pre-warmed drug containing media was prepared. The non-drug media was removed from the cells and the cells washed with ½-2/3 culture volume PBS and after removal of the same the drug containing medium carefully put onto the cells from the side of the well. The same procedure was repeated if there was subsequent treatments with different drugs or a drug recovery was performed.

Wet Lab Methods

Cloning

The constructs used in this study were obtained by classical restriction site cloning. The coding sequence of interest was amplified by PCR with appropriate primers. The PCR product was purified utilizing the Mini-PEx Kit provided by in-house facilities. Both the vector and the purified PCR product were digested with the same restriction enzymes, fit to insert the PCR product into the vector in a senseful manner. Ligation was performed with T4 Ligase overnight. Final products were transformed into DH5α E.Coli and plated out on LB-plates containing the appropriate antibiotic. Picked colonies were grown in LB media for 16h. After 16h the culture was spun down and the pellet lysed and the plasmids purified with the Mini-PEx Kit. The plasmids were sequenced in-house by a facility.

Plasmid Propagation

For the propagation of a plasmid it was transformed into DH5α E.Coli. Transformation was performed using 1uL of Plasmid and 50uL of Bacterial suspension. Both were mixed gently in an 1,5ml Eppendorf tube and incubated on ice for 20 minutes. Afterwards the mixture was subject to a heat shock of 42C for 30-40 seconds. The tubes were placed back on ice for 2 minutes and afterwards 200uL of SOC media was added and the tubes incubated at 37C for 45 minutes while shaking. 50uL of the bacterial suspension was plated out on a LB plate containing the appropriate antibiotic and was incubated at 37C overnight. Single colonies were picked the next day and grown in 5ml of LB media at 37C for 16 hours while shaking. Afterwards the culture was spun down and the pellet lysed, and the plasmids purified with the Mini-PEx kit provided by the in-house facilities. The eluate was measured on a nanodrop to work out the concentraton of DNA.

SDS-PAGE and Western Blot

SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) was performed either on gradient 4-20% Mini-PROTEAN® TGX Precast Protein Gels (Biorad) or on self-made 15% polyacrylamide gels with 4% stacking gel. The electrophoresis was run at 100V for 1,5 hours. Western Blot was performed onto Nitrocellulose membranes utilizing the semi-dry Turbo transfer blot system (BioRad) or onto (methanol activated) PVDF membranes with a classical wet transfer at 200mA for 70 minutes. The membranes were blocked with 5% BSA in TBS and 0,1% Tween-20 (TBST) for 1h at room temperature or overnight at 4C. Afterwards primary antibodies, stored in the same 5% BSA-TBST buffer, were incubated in the same manner. After 3 washes with TBST for 10 minutes each a subsequent incubation with an appropriate HRP conjugated secondary antibody was incubated in 5% skimmed milk in TBST for 1 hour. After 3 washes with TBST for 10 minutes each the immune-reaction was developed using ECL Super-Pico Plus (Thermo) and detected with ChemiDoc Touch Imaging System (Biorad).

Imaging Methods

Slide Preparation for microscopy

In accordance of all the above-mentioned procedures in tissue culture, cells were grown in a 6 well tissue culture plate, while each well contains a cover slip, which was sterilized by UV radiation for 30 minutes on each side. After transfection with fluorophore tagged constructs and eventual treatments the slips were washed with iced PBS and fixed with a 0,4% Paraformaldehyde solution in PBS for 30 minutes and subsequently washed 5 times with iced PBS. The slips were mounted in Vectashield mounting medium on microscopy slides and sealed with clear nail polish.

Confocal Imaging

Samples were imaged at an upright ZEISS LSM800 or LSM 980 confocal microscope (Zeiss) with an Apochromat 40x objective lens at 1x magnification. LSM 980 was utilized with Airy Scan.
Excitation/detection parameters for GFP and mCherry were 488 nm/463 nm and 510 nm and 561 nm/569 to 635 nm, respectively and sequential scanning mode was used for colocalization of both fluorophores. Identical settings, including optical section thickness of 1 µm per z-stack, were used during the acquisition for sample comparison, and the images processed using identical parameters. Confocal images were processed with ZEN (version 2011) and ImageJ (version 1.48v) software.

DIC Imaging

Samples were imaged at an upright ZEISS AXIO Vert A1 with SNAP camera and software. On this microscope it was possible to image live cells, therefore samples were grown in 6 well tissue culture plates without further measures taken besides the experimental procedures.

Analytical Methods

Confocal Image Analysis

Autophagic puncta were counted using ImageJ. Several (at least five) z-stack merged images were manually background subtracted, thresholded and the same threshold value was applied to all the images and replicates of the same experiment. The image was converted to eight-bit grayscale and then counted for puncta by the Particle Analyzer function of ImageJ. The average number of autophagosomes per z-stack was averaged between 10 or more different samples.
Colocalization analysis was performed by calculating Pearson's correlation coefficient as previously described using ImageJ software with the plug-in JACoP (S. Bolte & F. P. Cordelières, A guided tour into subcellular colocalization analysis in light microscopy, Journal of Microscopy, Volume 224, Issue 3: 213-232.). Values near 1 represent almost perfect correlation, whereas values near 0 reflect no correlation and values nearing -1 indicate anticorrelation. The average Pearson's correlation coefficient was determined in 5 or more different samples.

Western Blot Image Analysis

Protein bands intensities were quantified with Image Lab 6 (Biorad). Equal rectangles were drawn around the loading control lane and the band of interest. Then, the protein band of interest was normalized for the total protein level of the loading control. Average relative intensities and standard error of at least 3 independent experiments were calculated.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 8 software. For all the quantifications described above, statistical analysis was performed. Statistical significance of differences between two experimental groups was assessed wherever applicable by either a two-tailed Student's t-test if the variances were not significantly different according to the F test, or using a non-parametric test (Mann-Whitney or Kruskal-Wallis with Dunn's test for multiple comparisons) if the variances were significantly different ($p < 0.05$). Differences between two data sets were considered significant at $p < 0.05$ (*) ($p < 0.01$ (**); $p < 0.001$ (***); $p < 0.0001$ (****); n.s., not significant.

Materials

Cell lines

Organism	Background	Supplier
Human Tumor Cell Line	HeLa-Kyoto	Fumiyo Ikeda
Human Embryonic Kidney Cell Line	HEK293T	Fumiyo Ikeda

Bacterial Strains

Organism	Strain	Supplier
Escherichia Coli	DH5α	In House Facility

Antibodies

Antibody	Host	Supplier	Cat.Number	Working concentration
Anti-Rabbit IgG HRP-Conjugate	goat	Biorad	1706515	1:10000
Anti-Mouse IgG-HRP Conjugate	goat	Biorad	1706516	1:10000
GFP	mouse	Roche	11814460001	1:3000
C53	mouse	SCBT	sc271671	1:1000
LC3B	mouse	nanoTools	0260-100/LC3-2G6	1:100
BIP3	rabbit	CST	3177	1:1000
Vinculin	mouse	Sigma Aldrich	V9131	1:1000
UFM1	rabbit	Abcam	ab108062	1:2000

Plasmids

Name	Supplier	Cat.Number	Reference
psPAX2	Addgene	12260	Didier Trono
pMD2.G	Addgene	12259	Didier Trono
C53 shRNA in pLKO1	Honglin Li	Gift	Wu *et al. Cell Res* (2013).
peGFP(N2)-HsC53-GFP	This study		
peGFP(N2)-AtC53-GFP	This study		
peGFP(N2)-HsC53^{sAIM}-GFP	This study		
peGFP(N2)-AtC53	This study		
pmCherry(N2)-HsC53-mCherry	This study		
pmCherry-GABARAP-mCherry	Fumiyo Ikeda	Gift	

mRFP-LAMTOR1	Sascha Martens	Gift	
ER-K20	Addgene	133861	Wang *et al.* Cell Res. (2020)
ERAD-C (pEGFP-GFP:CFTR$_{\Delta F508}$)	Ron R. Kopito	Gift	Leto *et al.* Mol. Cell (2019)
ERAD-L (pcDNA3-NHK-GFP)	Ron R. Kopito	Gift	Leto *et al.* Mol. Cell (2019)
ERAD-M (pMCB497-pTRE-INSIG1-GFP)	Ron R. Kopito	Gift	Leto *et al.* Mol. Cell (2019)
pcDNA3-Erdj3-GFP-3Gly	Maya Schuldiner	Gift	Ast *et al.* Cell (2016)

General Chemicals

Name	Supplier	Cat. #
Vectashield	Vector	H-1000-10
GeneJuice	Sigma Aldrich Aldrich	70967
Phenolred	Sigma Aldrich Aldrich	P3252
Polybrene	Gift from Fumiyo Ikeda	
5x Transfer Buffer	BioRad	
Glycerol (87%)	Applichem	A0970
Acrilamide/Bis-acrylamide, 30% solution, 37.5:1	Sigma Aldrich	
Bovine Serum Albumine	Sigma Aldrich	
EDTA-free cOmplete Protease inhibitor Cocktail	Roche	4693159001
Tween-20	Sigma Aldrich	
TBS	In-house facility	
DMSO	Sigma Aldrich	
Isopropanol	Sigma Aldrich	
10x Transfer Buffer (wet)	In-house facility	
pH Stripes	In-house facility	
NaN3	In-house facility	
NaOH	In-house facility	
Ethanol	In-house facility	
Naphthol Blue (Amidoblack)	Sigma Aldrich	N3393-25G
Acetic Acid	In-house facility	
Methanol	In-house facility	
$ZnCl_2$	Gift from Fumiyo Ikeda	
Triton X-100	In-house facility	
NaF	In-house facility	
EGTA	In-house facility	
HEPES	In-house facility	
EDTA	Sigma Aldrich	E6760-100G
NaCl (sol.)	In-house facility	
Tris Buffers	In-house facility	
Bromophenolblue	In-house facility	
DTT	Sigma Aldrich	43815
SDS	In-house facility	
APS	In-house facility	
TEMED	In-house facility	
Paraformaldehyd	In-house facility	

Drugs and Inhibitors

Name	Supplier	Cat. #
Tunicamycin	SCBT	sc-3506
Torin	SCBT	sc-396760
Bafilomycin A1	Abcam	ab120497
4μ8C	Sigma Aldrich	SML0949
KIRA6	MedChemExpress	HY-19708
APY29	MedChemExpress	HY-17537
GSK 2606414	MedChemExpress	HY-18072
Ceapin	Sigma Aldrich	SML2330
Anisomycin (ANS)	Sigma Aldrich	A5862-0.5ml
DTT	Sigma Aldrich	43815

Media and Supplements

Name	Supplier	Cat. #
FBS	Sigma Aldrich	F7524
PBS/PBS Tissue Culture	In-house facility	
LB	In-house facility	
DMEM	In-house facility	
SOC	In-house facility	
Puromycin	Sigma Aldrich	P8833
Ampicillin	In-house facility	
Kanamycin	In-house facility	
Penicillin-Streptomycin	Sigma Aldrich	P4333
Trypsin	Thermo Fisher	25300054
L-Glutamine	Sigma Aldrich	G7513

Software

Name	Company	Application
CLC main work bench 7	Qiagen	Cloning
Zen Software	Carl Zeiss	Microscopy
Image J (Fiji)	NIH	Image Quantification
Prism 8	Graph Pad	Statistics
Image Lab	BioRad	Western Blot Analysis
Adobe Illustrator 2020	Adobe Inc.	Graphics editing

Literature

1. Stephani M, Picchianti L, Gajic A, et al. A cross-kingdom conserved ER-phagy receptor maintains endoplasmic reticulum homeostasis during stress. eLife 2020;9:e58396 DOI: 10.7554/eLife.58396
2. Glick D, Barth S, Macleod KF. Autophagy: Cellular and molecular mechanisms. J. Pathol. 2010;221(1):3–12.
3. Dikic I, Elazar Z. Mechanism and medical implications of mammalian autophagy. Nat. Rev. Mol. Cell Biol. 2018;19(6):349–64.
4. Saha S, Panigrahi DP, Patil S, Bhutia SK. Autophagy in health and disease: A comprehensive review. Biomed Pharmacother [Internet] 2018;104(February):485–95. Available from: Autophagy disease
5. Schwarz DS, Blower MD. The endoplasmic reticulum: Structure, function and response to cellular signaling. Cell. Mol. Life Sci. 2016;73(1):79–94.
6. Rashid HO, Yadav RK, Kim HR, Chae HJ. ER stress: Autophagy induction, inhibition and selection. Autophagy 2015;11(11):1956–77.
7. Grootjans J, Kaser A, Kaufman RJ, Blumberg RS. The unfolded protein response in immunity and inflammation [Internet]. Nat. Rev. Immunol. 2016 [cited 2020 Mar 11];16(8):469–84. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27346803
8. Wang L, Xu Y, Rogers H, et al. UFMylation of RPL26 links translocation-associated quality control to endoplasmic reticulum protein homeostasis. Cell Res [Internet] 2020 [cited 2020 Mar 12];30(1):5–20. Available from: http://www.ncbi.nlm.nih.gov/pubmed/31595041
9. Gerakis Y, Quintero M, Li H, Hetz C. The UFMylation System in Proteostasis and Beyond [Internet]. Trends Cell Biol. 2019 [cited 2020 Mar 12];29(12):974–86. Available from: http://www.ncbi.nlm.nih.gov/pubmed/31703843
10. Song S, Tan J, Miao Y, Li M, Zhang Q. Crosstalk of autophagy and apoptosis: Involvement of the dual role of autophagy under ER stress [Internet]. J. Cell. Physiol. 2017 [cited 2020 Mar 13];232(11):2977–84. Available from: http://doi.wiley.com/10.1002/jcp.25785
11. Abdullahi A, Stanojcic M, Parousis A, Patsouris D, Jeschke MG. MODELING ACUTE ER STRESS in VIVO and in VITRO. Shock [Internet] 2017 [cited 2020 Mar 13];47(4):506–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27755507
12. Tanida I, Ueno T, Kominami E. LC3 and autophagy. Methods Mol Biol 2008;445:77–88.
13. Mauvezin C, Neufeld TP. Bafilomycin A1 disrupts autophagic flux by inhibiting both V-ATPase-dependent acidification and Ca-P60A/SERCA-dependent autophagosome-lysosome fusion. Autophagy [Internet] 2015 [cited 2020 Mar 13];11(8):1437–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26156798
14. Wang C, Wang X, Su Z, Fei H, Liu X, Pan Q. The novel mTOR inhibitor Torin-2 induces autophagy and downregulates the expression of UHRF1 to suppress hepatocarcinoma cell growth. Oncol Rep [Internet] 2015 [cited 2020 Mar 13];34(4):1708–16. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26239364
15. Johansen T, Lamark T. Selective Autophagy: ATG8 Family Proteins, LIR Motifs and Cargo Receptors. J. Mol. Biol. 2020;432(1):80–103.
16. Dunn KW, Kamocka MM, McDonald JH. A practical guide to evaluating

colocalization in biological microscopy. Am. J. Physiol. - Cell Physiol. 2011;300(4):C723.
17. Mak GWY, Lai WL, Zhou Y, Li M, Ng IOL, Ching YP. CDK5RAP3 is a novel repressor of p14ARF in Hepatocellular carcinoma cells. PLoS One 2012;7(7).
18. Egusquiaguirre SP, Liu S, Tošić I, et al. CDK5RAP3 is a co-factor for the oncogenic transcription factor STAT3. Neoplasia (United States) 2020;22(1):47–59.
19. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods. 2012;9(7):671–5.
20. Bolte S, Cordelières FP. A guided tour into subcellular colocalization analysis in light microscopy [Internet]. J. Microsc. 2006 [cited 2020 Mar 14];224(3):213–32. Available from: http://doi.wiley.com/10.1111/j.1365-2818.2006.01706.x
21. Rao DD, Vorhies JS, Senzer N, Nemunaitis J. siRNA vs. shRNA: Similarities and differences [Internet]. Adv. Drug Deliv. Rev. 2009 [cited 2020 Mar 14];61(9):746–59. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19389436
22. Zufferey R, Dull T, Mandel RJ, et al. Self-Inactivating Lentivirus Vector for Safe and Efficient In Vivo Gene Delivery. J Virol 1998;72(12):9873–80.
23. Moffat J, Grueneberg DA, Yang X, et al. A Lentiviral RNAi Library for Human and Mouse Genes Applied to an Arrayed Viral High-Content Screen. Cell 2006;124(6):1283–98.
24. Moore CB, Guthrie EH, Huang MTH, Taxman DJ. Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown. Methods Mol Biol [Internet] 2010 [cited 2020 Mar 14];629:141–58. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20387148
25. Chen Y, Brandizzi F. IRE1: ER stress sensor and cell fate executor. Trends Cell Biol. 2013;23(11):547–55.
26. Ast T, Michaelis S, Schuldiner M. The Protease Ste24 Clears Clogged Translocons. Cell 2016;164(1–2):103–14.
27. Riccio V, Vissa M, McQuibban A, Kim P. Lysosome Targeting RedGreen-assay: Selective Autophagy Sensing Assay for Mammalian Cells. BIO-PROTOCOL 2019;9(24).
28. Leto DE, Morgens DW, Zhang L, et al. Genome-wide CRISPR Analysis Identifies Substrate-Specific Conjugation Modules in ER-Associated Degradation. Mol Cell 2019;73(2):377-389.e11.
29. Joazeiro CAP. Ribosomal Stalling During Translation: Providing Substrates for Ribosome-Associated Protein Quality Control. Annu Rev Cell Dev Biol 2017;33(1):343–68.

Register of images