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A close connection between the PERK and IRE arms of the UPR and the transcriptional regulation of autophagy

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ABSTRACT

Endoplasmic reticulum (ER) stress is known to lead to activation of both the unfolded protein response (UPR) and autophagy. Although regulatory connections have been identified between the UPR and autophagy, it is still unclear to what extent the UPR regulates the genes involved at the different stages of the autophagy pathway. Here, we carried out a microarray analysis of HCT116 cells subjected to ER stress and observed the transcriptional upregulation of a large cohort of autophagy-related genes. Of particular interest, we identified the transcriptional upregulation of the autophagy receptor genes SQSTM1/p62, NBR1 and BNIP3L/NIX in response to ER stress and show that the inhibition of the UPR transmembrane receptors, PERK and IRE1, abrogates this upregulation.

1. Introduction

The endoplasmic reticulum (ER) is essential for the folding and post-translational processing of membrane bound and secreted proteins. Environmental factors or intracellular conditions that disturb the homeostasis of this organelle cripple the ER's protein folding machinery resulting in a condition referred to as ER stress [1].

Upon ER stress, unfolded proteins accumulate in the ER lumen triggering the unfolded protein response (UPR). The UPR is orchestrated by the three ER transmembrane receptors, protein kinase (RNA)-like endoplasmic reticulum kinase (PERK), Inositol requiring protein 1 (IRE1) and activating transcription factor 6 (ATF6), initiating a cascade of events which function to restore homeostasis to the ER. The UPR promotes survival of the cell by attenuating general protein synthesis and inducing the expression of genes encoding for chaperones and protein degradation systems. If these events fail to resolve the stress and reinstate the ER homeostasis, the UPR will switch from pro-survival signaling to a pro-death response, initiating apoptosis signaling [2].

An important component of the pro-survival signaling activated by the UPR is the removal of unfolded and aggregated proteins. The UPR utilizes two cellular degradation systems to achieve this, which include the ER-associated degradation (ERAD) pathway and autophagy. The ERAD pathway is well established as an integral component of the UPR's pro-survival response, utilizing the proteasome for the removal and degradation of soluble unfolded proteins which accumulate in the ER lumen upon conditions of ER stress [3].

However, ERAD is limited in its ability to degrade large substrates such as protein aggregates and damaged organelles, which often occur as a result of acute or prolonged ER stress. In order to remove these undesirable components the cell requires a more robust degradation system, namely autophagy. Autophagy is an evolutionarily-conserved, lysosomal-mediated system for bulk degradation of intracellular components [4].

It is characterized by the induction of an isolation membrane, also known as a phagophore, which elongates into a double membraned vacuole, capable of engulfing large amounts of cytosolic components. Autophagy, frequently aids in the relief of cellular stress by degrading unfolded proteins, large protein aggregates

Abbreviations: ATG, autophagy related gene; BFA, brefeldin A; DDIT3, DNA-damage inducible-transcript 3; DNA, deoxyribonucleic acid; DTT, dithiothreitol; ER, endoplasmic reticulum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HSPA5, heat shock protein A5; IRE1, inositol requiring protein 1; LC3, microtubule-associated protein 1 light chain 3; MEF, mouse embryonic fibroblasts; MOMP, mitochondrial outer membrane permeabilization; PARP, poly (ADP-ribose) polymerase; PERK, protein kinase-like endoplasmic reticulum kinase; PBS, phosphate buffered saline; PI, propidium iodide; Tm, tunicamycin; XBP1, X-box binding protein-1.

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and damaged organelles such as mitochondria [5,6]. As noted earlier, ER stress can lead to accumulation of all of these unwanted conditions, thus providing multiple substrates for autophagic degradation.

Although it is well established that autophagy is induced in response to ER stress [7–9], and that it contributes to reinstating homeostasis through removal of protein aggregates and damaged organelles, there is a lack of data describing how autophagy is specifically regulated by ER stress and the UPR. Although much of the research literature has regarded ER stress-induced autophagy as a side show to the UPR, there are many connections linking ER stress and autophagy [4].

In this study we performed a microarray analysis of HCT116 cells treated with brefeldin A (BFA) or tunicamycin (Tm). The screen revealed transcriptional upregulation of a number of autophagy-related genes involved in many aspects of the autophagy pathway. Of these genes we noted the upregulation of the recently characterized autophagy receptors, SQSTM1/p62, NBR1, and BNIP3L/NIX, which specifically target damaged organelles, ubiquitinated proteins and protein aggregates to the autophagosome [10]. The data presented here clearly show that in response to ER stress a massive transcriptional cascade occurs of genes encoding major players in the autophagy pathway; furthermore, the upregulation of the autophagy receptor genes suggests that ER stress-induced autophagy is a selective process.

2. Materials and methods

2.1. Cell culture and treatments

HCT116 colon cancer cells were cultured in McCOYs 5A medium modified from Sigma (M9309) supplemented with 10% heat inactivated FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. MDA-MB-468 breast cancer cells were cultured in DMEM from Sigma (D6429) supplemented with 10% heat inactivated FBS, 100 U/ml penicillin and 100 mg/ml streptomycin and 10 μg/ml of insulin. All cells were cultured at 37 °C, 5% CO_2 in a humidified incubator. Cells were seeded at a 60–70% density 24 h prior to treatment. To induce ER stress, cells were treated with tunicamycin (Tm) or brefeldin A (BFA) at the indicated concentrations for the indicated time. To inhibit autophagosome degradation, cells were treated with 60 μM of chloroquine. To inhibit IRE1 endoribonuclease activity, cells were treated with 10 μM of IRE1 inhibitor MKC8866 (Mannkind Corp., USA). To inhibit PERK activation cells were treated with 300 nM of GSK PERK inhibitor (Toronto Research Chemicals Limited, Cat # G797800). All reagents were purchased from Sigma–Aldrich unless otherwise stated.

2.2. Western blotting

Western blotting was carried out as previously described [9]. The membranes were incubated with the primary antibody against LC3-B (Sigma, L7543), DDIT3 (Santa Cruz, sc-793), SQSTM1/p62 (ENZO, PW9869), PERK (CST, 3192), Ubiquitin (Santa Cruz, sc-8017), XBP1s (Biolegend, 619502), EIF2S1 (Cell Signaling Technology, 9722), phospho-EIF2S1 (Cell Signaling Technology, 9721S) or β-actin (Sigma–Aldrich, A-5060) for 2 h at room temperature or overnight at 4 °C. All the secondary antibodies were purchased from Jackson. Membranes were developed using Western Lightning ECL substrates (Perkin Elmer, NEL102001EA).

2.3. Microarray sample preparation

HCT116 cells were seeded at 60–70% density in a 75 cm^2 culture flask and treated with Tm or BFA for 24 h. Following treatment, cells were harvested and centrifuged at 1000 g for 5 min at 4 °C to pellet the cells. The media was removed from the cell pellet and 1 ml of TRI reagent (Invitrogen; Cat No. #9738) was added. RNA extraction was carried out as described below. Samples were sent to DNA Vision on dry ice. DNA Vision carried out the microarray experiments using Affymetrix HG-U133_Plus-2 arrays. Analysis of microarray data was carried out using BRB ArrayTools. All chips were normalized using robust multichip analysis algorithm and analysed using the BRB-ArrayTools developed by Dr. Richard Simon and BRB ArrayTools Development Team from the Biometric Research Branch at the US National Cancer Institute [11]. Gene lists were generated using class comparison analysis, using a random variance *t*-test including a random permutation *t*-test and a calculated false discovery rate (FDR) for the differentially expressed genes using the Class Comparison Tool in BRBarray tools. The microarray data was uploaded to NCBI, Gene Expression Omnibus (Accession GSE63252).

2.4. RNA extraction and cDNA synthesis

After experimental treatment cells were scraped into the media and transferred to a 2 ml microfuge tube. Samples were centrifuged at 1000 g for 5 min at 4 °C to pellet the cells. Cells were resuspended and lysed in 1 ml of TRI reagent and incubated for 5 min at room temperature. RNA was extracted from the whole cell lysate in accordance with manufacturers work instructions (Invitrogen; Cat No. #9738). The cDNA was synthesized from 2 μg of RNA and Oligo dT (Invitrogen) using 20 U Superscript II Reverse Transcriptase (Invitrogen).

2.5. Real time PCR

Assays for BNIP3L/NIX, SQSTM1/p62, NBR1, HSPA5, PERK, DDIT3, ATF4 and GAPDH were supplied from Integrated DNA technologies (Table 1). cDNA was diluted 1:5 ratio in ddH_2O, the assay probes (0.5 μl/well) were diluted in the supplied 2× master mix (5 μl/well) and ddH20 (2 μl/well). Next, 2.5 μl of cDNA was pipetted into each well of the 96 well reaction plate (Applied Biosystems; cat No. N8010560). To this, 7.5 μl of the master mix containing the probe of interest was added to each well. The plate was sealed and centrifuged 400 g for 1 min to mix the reaction. The plate was placed into the thermocycler and subjected to 40 cycles of PCR. Relative expression was evaluated with ΔΔCT method and GAPDH was used as the house-keeping gene to normalize gene expression.

2.6. Propidium iodide staining

Following treatment, cells were trypsinized and transferred to a 2 ml microfuge tube. Cells were allowed to recover from trypsinization for 10 min at 37 °C. The cells were collected by centrifugation at 1000g for 5 min at 4 °C and resuspended in 300 μl of PBS buffer containing 4 μl of propidium iodide (50 μg/ml). The cells were immediately analyzed using FACSCalibur flow cytometer (Becton Dickinson).

2.7. Statistical analysis

Cell death data are expressed as mean ± SD for three independent experiments. Real time data is expressed as mean ± SEM for three independent experiments unless stated otherwise. Differences between the treatment groups were assessed using GraphPad's two-tailed unpaired Student's *t*-tests. Values of $p < 0.05$ are considered statistically significant and values with $p < 0.001$ are considered very statistically significant. *p*-value ($^{*}pV < 0.05$, $^{**}pV < 0.01$, $^{***}pV < 0.001$).

Table 1
Integrated DNA technologies (IDT) probes.

Gene	Primer1	Primer2	Probe	Species
PERK	5′-GAACCAGACGATGAGACAGAG-3′	5′-GGATGACACCAAGGAACCG-3′	5′-/56-FAM/AGCAGTGGG/ZEN/ATTTGGATGTGCGAT/31ABkFQ/-3′	Human
NBR1	5′-AGTAGATCCTTTCCCCTCCG-3′	5′-TGCTCAGTCACTTCACCAAG-3′	5′-/56-FAM/AGTTTCCTC/ZEN/TTGCTGCAGGTGA/31ABkFQ/-3′	Human
SQSTM1/p62	5′-AAGAACTATGACATCGGAGCG-3′	5′-AGAGGTGGGCAAAAGTGG-3′	5′-/56-FAM/ACACCATCC/ZEN/AGTATTCAAAGCATCCCC/31ABkFQ/-3′	Human
BNIP3L/Nix	5′-CTCTTCCTTTCTCATGTTTTGGC-3′	5′-ACTTCACAGGTCACACGC-3′	5′-/56-FAM/TGCTCAGTC/ZEN/GCTTTCCAATATAGATGCC/31ABkFQ/-3′	Human
GAPDH	5′-TCTTCCTCTTGTGCTCTTGC-3′	5′-CTTTGTCAAGCTCATTTCCTGG-3′	5′-/56-FAM/CACCCTGTT/ZEN/GCTGTAGCCAAATTCG/31ABkFQ/-3′	Human
HSPA5	5′-GTG CCT ACC AAG AAGTCT CAG-3′	5′-TCC AAGTCG AACTCC TTC AAAT-3′	5′-/56-FAM/TTG TCT TTT/ZEN/GTC AGG GGT CTT TCA CCT/31ABkFQ/-3′	Human
DDIT3	5′-AGC GAC AGA GCC AAA ATC AG-3′	5′-CAG GTGTGGTGA TGT ATG AAG A-3′	5′-/56-FAM/ACCTGA GGA/ZEN/GAG AGT GTT CAA GAA GGA/31ABkFQ/-3′	Human
ATF4	5′-TCC AAGTCG AACTCC TTC AAAT-3′	5′-GTG GAT GGGTTG GTC AGT-3′	5′-/56-FAM/ACA ACA GCA/ZEN/AGG AGG ATG CCT TCT/31ABkFQ/-3′	Human

3. Results

3.1. Response of HCT116 cells to ER stress

Treatment of HCT116 cells with Tm (2 μg/ml) or BFA (0.5 μg/ml) activated a canonical UPR response, shown by immunoblotting for typical markers of the UPR, including EIF2S1, p-EIF2S1, XBP1 spliced (XBP1s), and DDIT3 (also known as CHOP) (Fig. 1A).

To confirm that ER stress activated a functional autophagy response in HCT116 cells, we treated cells with BFA or Tm in combination with the lysosomotropic agent chloroquine (60 μM CQ) over an extensive time course. Cells were harvested, and whole cell lysates were assessed by immunoblotting for LC3I to LC3II conversion, SQSTM1/p62 and ubiquitin accumulation (Fig. 1B). Our results show that autophagy is induced in response to BFA and Tm treatment; furthermore, the addition of CQ to BFA and Tm dramatically increases the levels of LC3I and LC3II, confirming that autophagosomes are in a constant flux of biogenesis and degradation (Fig. 1B). This was supported by the accumulation of the autophagy substrate SQSTM1/p62. In the presence of CQ, SQSTM1/p62 and ubiquitinated protein levels accumulated indicating a requirement for the autophagy machinery in the degradation of ubiquitinated protein aggregates which accumulate during conditions of ER stress.

To confirm that HCT116 cells were susceptible to ER stress-induced death, we treated cells with BFA and Tm for 24 and 36 h and determined cell viability using propidium iodide followed by FACS analysis. Both drugs induced a loss of viability by 24–36 h (Fig. 1C).

Together the results demonstrated a functional UPR response and also showed that in response to ER stress, ubiquitinated protein levels did not increase over time; however, blockade of the autophagosome degradation with CQ resulted in the accumulation of ubiquitinated proteins indicating that autophagy mediates the degradation of ubiquitinated proteins in response to ER stress.

3.2. Microarray analysis of HCT116 cells reveals several autophagy-related genes are transcriptionally upregulated in response to ER stress

It is well established that the autophagy pathway is upregulated in response to ER stress. However it remains unclear to what extent the UPR regulates genes involved at the different stages of the autophagy pathway. Therefore, microarray analysis of HCT116 cells treated with ER stress-inducing agents was performed to identify autophagy-related genes transcriptionally upregulated in response to ER stress. The microarray experiment was carried out using Affymetrix HG-U133_Plus-2 arrays.

Analysis of the microarray data identified a large number of autophagy-related genes differentially regulated in response to ER stress (Fig. 2 and Supplementary Tables 1 and 2). These genes were seen to be involved in all stages of the autophagy pathway. Both BFA and Tm were found to upregulate a large number of autophagy-related genes, and to downregulate a smaller number of autophagy-related genes (Fig. 2). While the majority of the genes were commonly regulated by the two ER stress-inducing drugs, some genes showed unique regulation by the two ER stressors (see Venn diagrams in Fig. 2). Considering that both treatments have different modes of action (i.e., Tm inhibits N-linked glycosylation and BFA inhibits ER to Golgi translocation) and different levels of toxicity and that only one time-point was investigated, it is not unexpected that differences between treatments are observed. Interestingly, the upregulation of the autophagy receptor genes SQSTM1/p62, NBR1 and BNIP3L/NIX suggests that the UPR may regulate these receptors in order to selectively target substrates in response to ER stress. Of note, although NBR1 and BNIP3L were

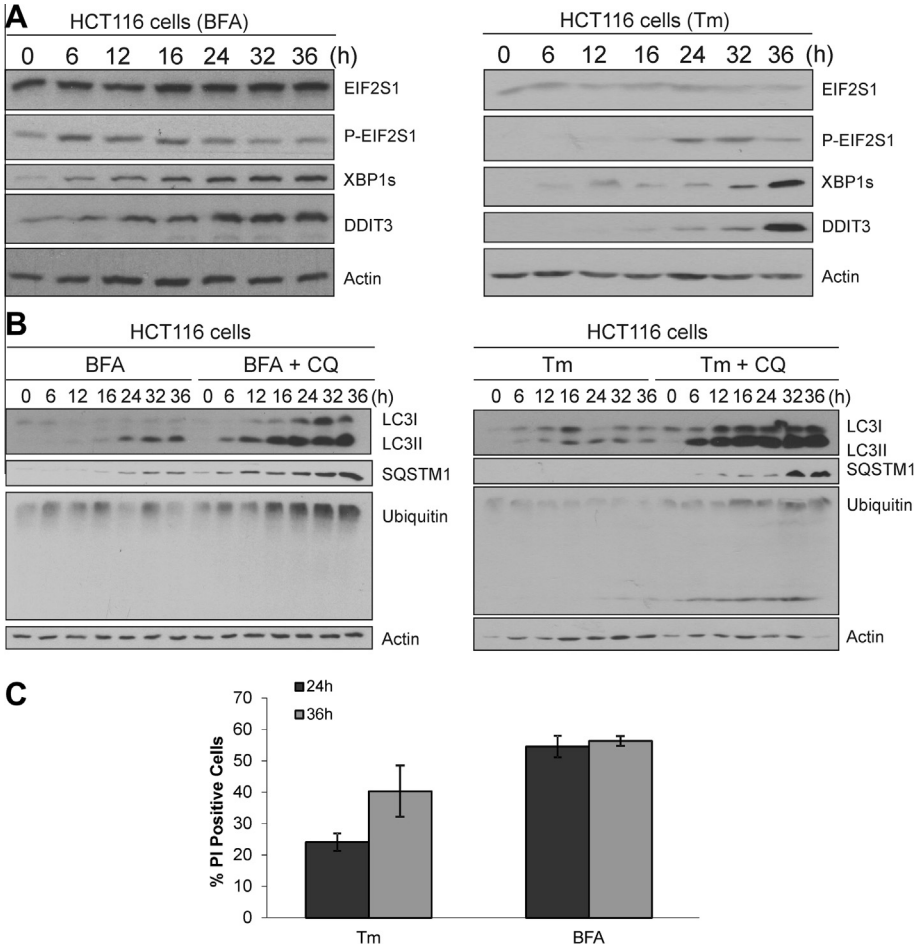


Fig. 1. HCT116 cells exhibit a functional UPR, autophagy and cell death response to ER stress. (A) HCT116 cells were treated with 0.5 μg/ml of BFA or 2 μg/ml of Tm for the indicated time points. Protein extracts were subjected to SDS–PAGE followed by immunoblotting. Total protein was assessed using antibodies against EIF2S1, p-EIF2S1, XBP1 spliced (XBP1s), and DDIT3. (B) HCT116 cells were treated with 0.5 μg/ml of BFA or 2 μg/ml of Tm, in the presence or absence of 60 μM of chloroquine (CQ) to monitor autophagy flux at the indicated time points. Following treatment, cells were harvested in 1× sample buffer. Whole cell lysates were subjected to SDS–PAGE followed by immunoblotting. Cell lysates were assessed using antibodies against actin, LC3, p62/SQSTM1 and ubiquitin. (C) HCT116 cells were treated with 0.5 μg/ml of BFA or 2 μg/ml of Tm for 24 and 36 h. Following treatment, cells were stained with propidium iodide (PI) and assessed by FACS analysis for cell viability.

commonly upregulated with Tm and BFA, SQSTM1/p62 displayed a differential response depending on the treatment and the probe set used.

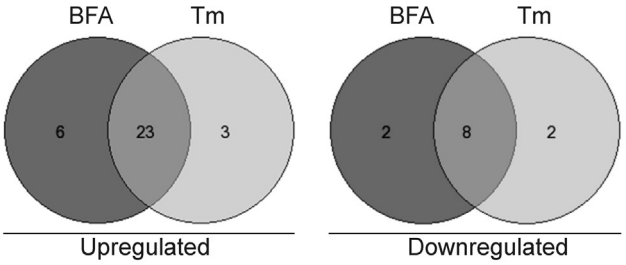
3.3. ER stress results in the transcriptional upregulation of the autophagy receptor genes SQSTM1/p62, NBR1 and BNIP3L/NIX

The treatments, Tm and BFA, resulted in different severities of stress, such that Tm caused only 20% cell death while BFA resulted in around 60% cell death (Fig. 1C). This allowed us to determine whether the transcriptional upregulation of the genes was an early or late event in the ER stress response and also whether it was a drug specific response or a general ER stress response. The microarray analysis identified the transcriptional upregulation of the three autophagy receptors, SQSTM1/p62, NBR1 and BNIP3L/NIX. As mentioned earlier, NBR1 and BNIP3L/NIX were commonly upregulated with Tm and BFA treatment, however SQSTM1/p62 was uniquely up with BFA treatment for 3 of the SQSTM1/p62 probe sets and commonly down for 1 of the probe sets. The conflicting results observed for SQSTM1/p62 regulation could be due to a number of factors; however, considering microarray experiments are high throughput screening processes it is important to validate the results using qPCR. To investigate the regulation by ER stress of autophagy receptors in more detail, we performed a detailed time course by treating HCT116 cells with 0.5 μg/ml of BFA for up to 36 h and analysed expression of the genes by qPCR (Fig. 3A). We also confirmed by qPCR the induction of the autophagy receptors genes by 2 μg/ml Tm (Fig. 3B). These data confirmed the transcriptional upregulation of the autophagy receptor genes, SQSTM1/p62, NBR1 and BNIP3L/NIX, in response to these ER stressors.

To confirm that this was not a cell-type specific effect we treated MDA-MB-468 breast cancer cells with 0.5 μg/ml of BFA and monitored the transcriptional upregulation of the autophagy receptor genes at the indicated time points (Fig. 3C). Although the MDA-MB-468 cells clearly showed increased transcript levels of the autophagy receptor genes statistical significance was not achieved.

3.4. The PERK and IRE1 arms of the UPR play a role in the transcriptional regulation of the autophagy receptor genes

Next we investigated the role of PERK and IRE1 arms of the UPR in the regulation of the autophagy receptor genes. To do this we utilized two chemical compounds; the PERK inhibitor (GSK PERK inhibitor) and the IRE1 inhibitor (MKC8866). HCT116 cells were treated with 0.5 μg/ml BFA, 2 μg/ml Tm or 60 μM of CQ alone or in combination with the GSK PERK inhibitor or MKC8866 for 12



Commonly Up		Uniquely up in BFA	Commonly Down	Uniquely Down in BFA	Opposite effects
ATG12	LAMP2	ATG13	ATG16L1	VTI1A	SQSTM1
ATG2B	LAMP3	ATG2A	ATG4B	VTI1A	VTI1A
ATG3	MAP1LC3B	SESN2	ATG4D	-	-
ATG4A	NBR1	SQSTM1	ATG7	**Uniquely Down in Tm**	-
ATG5	PIK3C3	SQSTM1	ATG9A	C12orf44	-
BECN1	RB1CC1	SQSTM1	MAP1LC3A	ULK1	-
BNIP3	SH3GLB1	-	SQSTM1	-	-
BNIP3L	STX17	**Uniquely up in Tm**	VAMP8	-	-
DCTN4	VAMP7	ULK2	-	-	-
DDIT4	WDFY3	ULK2	-	-	-
DRAM1	WIPI1	VTI1A	-	-	-
GABARAPL1	-	-	-	-	-

Fig. 2. Microarray analysis of HCT116 identifies the transcriptional upregulation of several autophagy-related genes in response to ER stress. HCT116 cells were treated with 0.5 μg/ml of BFA or 2 μg/ml of Tm for 24 h. Following treatment, cells were harvested and lysed in TRI reagent and RNA was extracted. Microarray analysis was carried out using Affymetrix HG-U133_Plus-2 arrays. Analysis of microarray data was carried out using BRB array tools. A list of known genes involved in the autophagy pathway was selectively extracted. Multiple probe sets were used for some genes. Venn diagrams showing the numbers of genes that were commonly or differentially altered were generated from the extracted data. Supplementary Table 1 and 2 details the extracted data for each gene used to construct the Venn diagrams in Fig. 2.

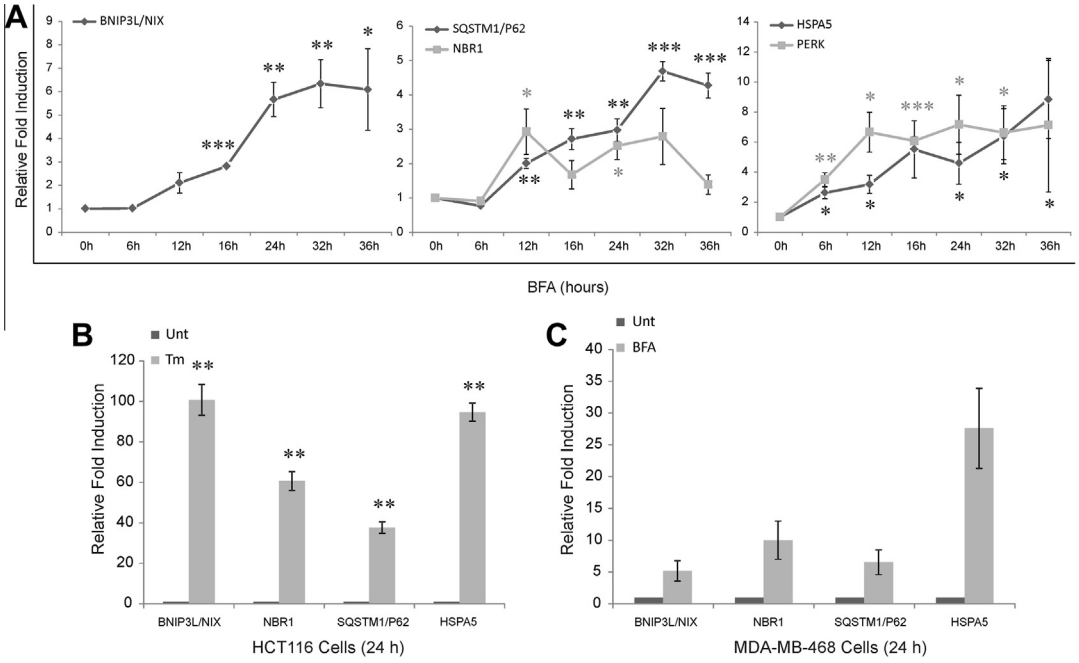


Fig. 3. Real-Time PCR confirms the transcriptional upregulation of the autophagy receptor genes. HCT116 cells were treated with either, (A) 0.5 μg/ml of BFA for 0–36 h or (B) treated with 2 μg/ml Tm for 24 h, and samples were subjected to real-time PCR to monitor the gene expression kinetics of SQSTM1/p62, NBR1 and BNIP3L/NIX. (C) MDA-MB-468 cells were treated with 0.5 μg/ml BFA for 24 h. Samples were subjected to real-time PCR to monitor the expression of SQSTM1/p62, NBR1 and BNIP3L/NIX. Real time data for (B) and (C) are expressed as mean ± SEM for three measurements of an independent experiment. The data are a representative of 3 independent experiments.

or 24 h. Cells were harvested in 1X sample buffer and whole cell lysates were assessed by immunoblotting for PERK, XBP1s and LC3 (Fig. 4A). The results confirmed that both the PERK and IRE1 inhibitors were blocking their targets.

To investigate the role of PERK and IRE1 in the regulation of the autophagy receptor genes, we treated HCT116 cells with Tm alone and in combination with either the PERK or IRE1 inhibitor. Real time PCR was performed on the samples for both UPR marker genes (HSPA5, DDIT3 and ATF4) and autophagy receptor genes (SQSTM1/p62, NBR1 and BNIP3L/NIIX) (Fig. 4B). Our results show that the transcriptional upregulation of the autophagy receptor genes is affected by both PERK and IRE1 inhibition.

Consistent with a previous report by B'Chir et al. [12], PERK inhibition strongly blocked the upregulation of the autophagy receptor genes; however it is clear that IRE1 inhibition also significantly reduced the upregulation of the autophagy receptor genes.

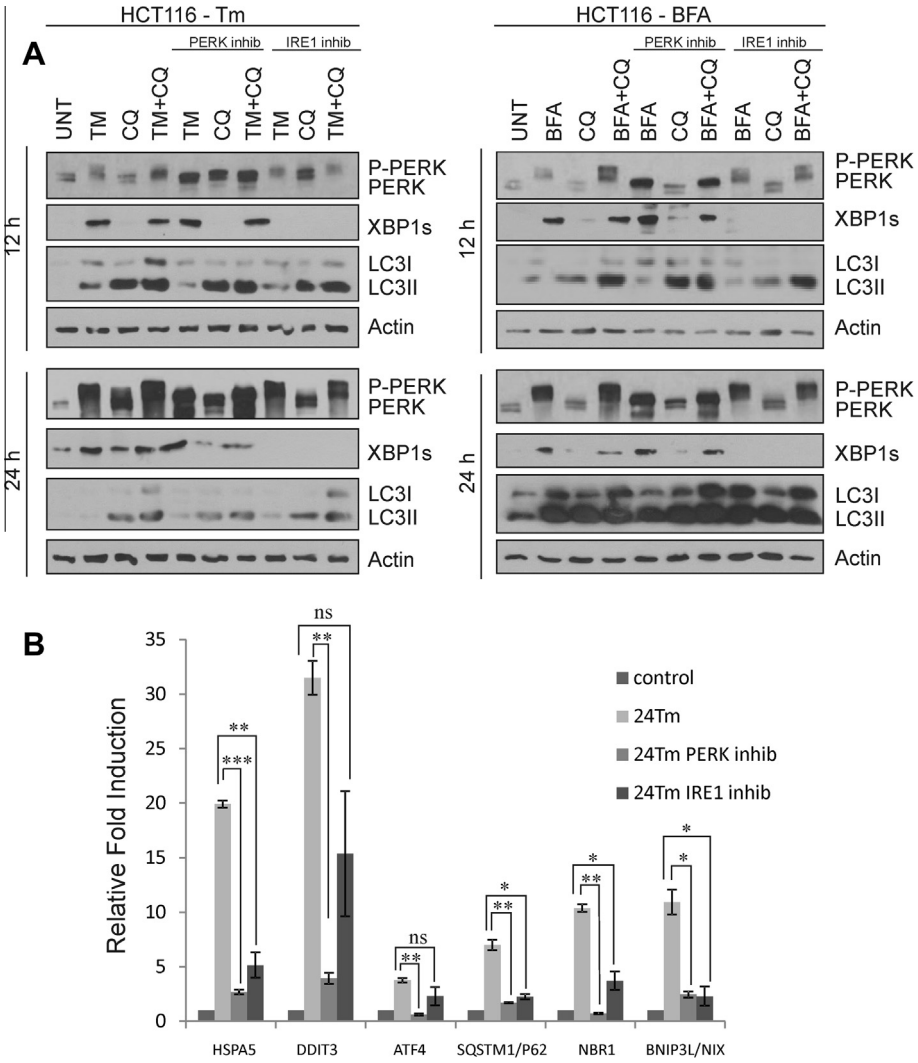


Fig. 4. Inhibition of PERK or IRE1 abrogates ER stress-induced expression of the autophagy receptor genes. (A) HCT116 cells were treated with 0.5 μg/ml BFA, 2 μg/ml Tm or 60 μM of CQ alone or in combination with the PERK inhibitor (GSK) or the IRE1 inhibitor (MK) for the indicated time points. Whole cell lysates were subjected to SDS–PAGE followed by immunoblotting. Total protein was assessed using antibodies against DDIT3, XBP1 spliced, PERK, LC3 and actin. (B) HCT116 cells were treated with 0.5 μg/ml of BFA or 2 μg/ml Tm alone or in combination with the GSK PERK inhibitor (PERK inhib) or the IRE1 inhibitor MKC8866 (IRE1 inhib) for 24 h. Samples were subjected to real-time PCR analysis to monitor the expression of SQSTM1/p62, NBR1, BNIP3L/NIX, PERK and HSPA5.

4. Discussion

The UPR and autophagy are two robust cellular processes that are activated in response to stress conditions. However, although autophagy can be activated independent of UPR, it is clear that they can both be activated in response to ER stress. Both direct and indirect links between the UPR and the autophagy pathway have been demonstrated suggesting a high level of cross-talk between these processes (for recent review see [4]).

The microarray analysis performed here further confirms this connection, with the identification of over 20 genes involved in the regulation of autophagy whose expression was altered in response to ER stress. Most of these genes have not yet been demonstrated to be regulated downstream of the UPR. We further characterized three of these genes that function as autophagy receptors, namely, SQSTM1/p62, NBR1 and BNIP3L/NIX, demonstrating that these are upregulated in response to ER stress.

Autophagy is well known as a macro response of cells to conditions of starvation, and is also implicated in the removal of protein aggregates and damaged organelles from cells. In the last few years the identification of the autophagy receptor proteins has established the importance of autophagy in the selective removal of unwanted substrates such as invading pathogens, protein aggregates and old or damaged organelles [10]. The current observation of an upregulation by ER stress of autophagy receptor genes indicates that selective autophagy is likely to form part of the cellular response to ER stress. The best characterized autophagy receptor is SQSTM1/p62 and more recently NBR1, which has been shown to function in a similar way to SQSTM1/p62 [13]. Both SQSTM1/p62 and NBR1 were upregulated by ER stress and both are involved in the selective degradation of ubiquitinated substrates and protein aggregates [13]. SQSTM1/p62 and NBR1 both contain a ubiquitin-associated (UBA) domain which facilitates binding of ubiquitinated substrates, and a LC3 interacting region (LIR) which targets ubiquitinated substrates to the autophagosome via direct interaction with LC3 family members [13–15]. SQSTM1/p62 and NBR1 are capable of forming hetero-oligomers via an N-terminal Phox and Bem1p (PB1) domain resulting in the formation of unfolded protein aggregates in the cytosol. These aggregates have been clearly shown to co-localize with autophagosomal structures [16]. We show that both SQSTM1/p62 and NBR1 are transcriptionally upregulated in response to multiple ER stress inducing agents

in both HCT116 colon cancer cells and MDA-MB-468 breast cancer cells. Inhibition of autophagy degradation with the addition of CQ resulted in the accumulation of ubiquitinated substrates in response to ER stress. These data indicate that ubiquitinated substrates require autophagy for their removal in response to ER stress.

Damaged mitochondria are known to be targeted for autophagic degradation in a process known as "mitophagy". BNIP3L/NIX was first identified as an important adaptor for the removal of mitochondria in reticulocyte development [17]. Further studies showed that damaged or depolarized mitochondria are also removed by autophagy in a BNIP3L/NIX-dependent manner [6]. Here we show that BNIP3L/NIX is transcriptionally upregulated in response to ER stress in both HCT116 cells and MDA-MB-468 cells, indicating a role for mitophagy in protecting cells against ER stress. It is likely that this would help to limit apoptosis during ER stress.

Using the pharmacological inhibitors of PERK and IRE1 we demonstrated that the PERK arm of the UPR is essential for the transcriptional upregulation of SQSTM1/p62, NBR1 and BNIP3L/NIX genes. Although IRE1 inhibition also reduced the upregulation of the autophagy receptor genes, this was found to also reduce the upregulation of HSPA5 and DDIT3, known downstream targets of PERK. This suggests that IRE1 inhibition may exert negative feedback on PERK signaling, thus reducing its activation and dampening the upregulation of its downstream targets such as HSPA5 and DDIT3. It follows that such an indirect dampening of PERK activity through IRE1 inhibition would also reduce the upregulation of the autophagy receptor genes. Alternatively IRE1 may directly regulate the autophagy receptor genes but to a lesser extent than PERK. The UPR is quite a dynamic system and thus inhibiting one arm of the UPR can have a knock-on effect to other arms of the UPR in order to compensate for the deficit.

This study showed that the UPR transcriptionally induced the autophagy receptor genes, SQSTM1/p62, NBR1 and BNIP3L/NIX, which have been shown, in different contexts, to play a crucial role in the selective degradation of autophagy substrates. Our data also show an accumulation of ubiquitinated proteins when cells were exposed to ER stress in the presence of CQ. Together, these findings implicate selective autophagy in the degradation of misfolded proteins due to ER stress, and specifically implicate autophagy in the removal of ubiquitinated proteins. Furthermore, our data strongly implicate the PERK arm of the UPR in the transcriptional regulation of SQSTM1/p62, NBR1 and BNIP3L/NIX in response to ER stress, and suggest that the IRE1 arm may play a direct role or may indirectly affect the PERK arm.

Conflict of interest

AS is a co-founder and director of Aquila Bioscience Ltd.

Financial disclosure statement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.076.

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