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Endoplasmic Reticulum Area Expands Upon Onset of the Unfolded Protein Response

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Abstract

The endoplasmic reticulum (ER) is a dynamic organelle responsible for essential cell functions such as protein folding, calcium storage, and lipid synthesis. It also directly interacts with and regulates other organelles in the cell, such as endosomes or mitochondria, and is the site of bulk protein synthesis for both secretion and the cellular endomembrane system. However, cellular stressors such as heat shock, chemical imbalance, or calcium depletion can disrupt the protein-folding capacity of the ER, causing activation of the ER-mediated unfolded protein response (UPR) in order to either restore homeostasis or initiate apoptosis. Chronic ER stress is a hallmark of viral and bacterial infections, neurodegenerative disorders, cancers, and diseases such as retinis pigmentosa, yet little is known about the morphology changes of mammalian ER upon activation of the UPR. This change is important to characterize because recent evidence shows that the shape of the ER is directly related to its many functions. Here we use a system of live-cell tracking to show a marked morphology change in mammalian ER upon the onset of UPR. In the future we hope to characterize the purpose of UPR-mediated membrane expansion in concert with effects on ER dynamics and organelle contacts.
Introduction

The endoplasmic reticulum (ER) is a dynamic, membrane-bound organelle formed by a complex network of sheets and tubules that extend throughout the cytoplasm. The ER serves many essential purposes in the cell including protein scaffolding, folding, and overall quality control, lipid synthesis, and calcium storage. The ER also directly contacts other organelles such as mitochondria, endosomes, and the plasma membrane to participate in processes such as lipid transfer, calcium exchange, and recruitment of fission factors.\textsuperscript{1,2}

ER morphology is intimately linked to each of its functions. For example, cell types that specialize in protein excretion, such as liver or fibroblast cells, are often rich in ER sheets, while cells that require extensive calcium deposits or motile function, such as muscle or neuron cells, contain mostly ER tubules. The ER is comprised of a lipid bilayer surrounding an inner luminal space. Tubules are regions of high membrane curvature and a low lumen-to-membrane ratio, a shape mediated by the reticulon (Rtn) family of proteins.\textsuperscript{3,4} They are the primary sites of lipid synthesis and are very dynamic, capable of sliding along microtubules to contact other organelles in order to mediate processes such as mitochondrial fission, lipid exchange, or endosomal budding.\textsuperscript{1,5,6} Sheets, or cisternae, are large membrane areas with a high lumen-to-membrane ratio usually located in the perinuclear region. They provide a scaffold for translational machinery and therefore are highly associated with ribosomes, leading to use of the term “rough ER.”\textsuperscript{4} While sheets are less dynamic than tubules, it is important to note that ER membrane in general constantly reorganizes in response to cellular needs.

In addition, the ER is the primary mediator of cell homeostasis via a process known as the Unfolded Protein Response (UPR). Stressors such as heat shock, chemical imbalance, or calcium depletion compromise the protein-folding capacity of the ER by preventing proteins from taking their proper fold. Unfolded proteins tend to form aggregates in the lumen and cannot be secreted, disrupting ER function and other cellular processes that require proteins synthesized in the ER. The ER senses and responds to increase in unfolded proteins via UPR signaling pathways, initiating a massive reprogramming of the cell’s genome.

UPR signaling takes a dual and time-sensitive approach to restoring cell homeostasis. Initially, signaling will work towards remedying the build-up of aggregates by increasing protein folding capacity. This process includes upregulation of ER-resident chaperones, quality-control proteins, and translational machinery and downregulation of bulk protein synthesis.\textsuperscript{7} However, the UPR will initiate apoptosis if homeostasis is not restored quickly enough.
In yeast, UPR signaling is entirely mediated by the IRE1 pathway, which is conserved in mammalian cells. Mammalian UPR also involves the ATF6 and PERK pathways; the ATF6 pathway primarily works towards increasing the ER protein folding capacity, the PERK pathway signals the cell to push toward apoptosis, and the IRE1 pathway overlaps with both.\textsuperscript{7,8} The master transcription factors activated by the IRE1, ATF6, and PERK pathways are XBP1, ATF6α, and ATF4, respectively.

Due to the interplay between ER morphology and each of its functions, it is important to characterize changes in ER structure during the UPR. If ER morphology is changing in response to UPR signaling it would imply that its other functions are changing in tandem. Because recent work has painted the ER as a master regulator of many cell functions, it is crucial to understand how these functions are entwined, especially during such a time-sensitive and essential process such as the UPR. Additionally, active UPR is a hallmark of diseases such as retinis pigmentosa, cancers, neurodegenerative disorders, and both viral and bacterial infections.\textsuperscript{9} A greater understanding of UPR function beyond genome regulation is a necessary step in furthering possible therapies and overall realization of basic cell processes.

ER morphology during stress has been extensively studied in yeast but only minimally examined in mammalian cells. It has been shown that ER membrane in yeast expands in the form of sheets during stress via UPR signaling.\textsuperscript{10} Furthermore, lipid biogenesis activated by the yeast Ino2/4 transcriptional regulator complex was directly linked to XBP1-mediated UPR signaling and determined to be both necessary and sufficient for ER membrane expansion. In mammalian cells it has been shown that active ATF6α is capable of inducing ER membrane expansion in the absence of XBP1 signaling.\textsuperscript{11} However, to date there is no comprehensive study that links UPR effects on ER morphology with its other dynamic functions in mammalian cells.

Use of live-cell confocal microscopy has allowed us to show that ER membrane expands upon onset of UPR in mammalian cells. By use of a specific cell stress reporter and live-cell imaging system we were able to measure and quantify the membrane expansion, which generally manifests in the form peripheral sheets. We also repeated these results in a human cell type in order to increase translational relevance. In addition, we explored the requirements of specific ER shape during the stress response by performing the same stress experiments in Rtn4a over-expressing cells, which have a mostly tubular ER network. These results imply that expansion of the ER membrane is a primary function of UPR signaling during stress in mammalian cells. In the future we hope to characterize the purpose of membrane expansion and how it affects ER dynamics in concert with organelle contact.
Results

A System for Live-Cell Tracking UPR-Activated Cells

In order to reliably characterize ER morphology change during the onset of UPR, we wanted to formulate a system that would allow us to select and monitor specific cells that begin unstressed and become stressed over time. To do so we took advantage of the IRE1 UPR-signaling pathway by cloning XBP1-ΔDBD-GFP. When IRE1 is activated by the presence of unfolded proteins, it alternatively splices 26 nucleotides from XBP1 mRNA, causing a frame-shift that results in activation of its transcriptional activity. Therefore, fusion of GFP to the end of the XBP1 gene results in expression of GFP only after UPR activation, giving us a visual indicator of cellular stress levels (Figure 1B). We also removed the DNA-binding domain (DBD) of XBP1 in our construct to prevent the GFP-fusion protein from interfering with endogenous XBP1 function in the cell (Figure 1A). Overall, the XBP1-ΔDBD-GFP expression system was designed in order to select against stress-resistant or chronically stressed cells during analysis and focus solely on cells that undergo pharmacologically induced stress.

To complement the XBP1-ΔDBD-GFP system, we needed a reliable method to induce the UPR during live-cell imaging. The drug Tunicamycin (Tm) has been canonically used to induce UPR in both mammalian and yeast cells. Tm was identified as a viable stress-inducing drug because it blocks N-linked glycosylation of proteins by inhibiting GlcNAc phosphotransferase (GPT), causing proteins translated in the ER to be inherently misfolded. Additionally, To verify Tm stress induction in mammalian cells, whole cell lysates of COS-7 cells were collected after treatment with Tm for 2, 4, 6, or 8 hours to visualize the progression of stress by Western blotting for BiP content; BiP is an ER-resident chaperone directly upregulated by UPR signaling. At each 2 hour increment of Tm-treatment BiP levels increased (Figure 1C), identifying Tm as a reliable UPR-inducing drug for our experiments.

Next we evaluated XBP1-ΔDBD-GFP function by measuring GFP protein levels during stress induction. COS-7 cells transfected with XBP1-ΔDBD-GFP were treated with Tm and harvested for Western blot analysis at pre-treatment, 2.5, and 5.5 hours. Staining for BiP and GFP in the same samples showed GFP expression increased in tandem with levels of BiP (Figure 1D). In addition, COS-7 cells were again transfected with XBP1-ΔDBD-GFP and analyzed via live-cell confocal microscopy before and during Tm treatment. Individual cells initially expressing no GFP were selected and imaged pre-treatment and at intervals for 6.5 hours post-treatment, during which time GFP expression had a clear visual increase (Figure 1E). This data proved the concept of XBP1-ΔDBD-GFP function and showed that the construct could be reliably used as a visual tracker of UPR. It is important to note here that all future experiments
were carried out at 2.5 and 5.5 hour increments because XBP1-ΔDBD-GFP visual expression seemed to reach a threshold after 5.5 hours for most cells.

Figure 1. (A) Diagrams depicting domains in normal human XBP1 mRNA versus our XBP1-ΔDBD-GFP construct. (B) Schematic showing differences in XBP1-ΔDBD-GFP translation under normal versus stressed conditions. GFP is only translated when the first stop codon is put out of frame by IRE1 splicing of a 26-nucleotide piece of XBP1 mRNA. (C) Western blot showing COS-7 whole cell lysates of untreated and 0.025μg/mL Tunicamycin (Tm) treated cells at 2, 4, 6, and 8 hours. BiP levels increase as time in stressed conditions increases. GAPDH is used as a loading control. (D) Western blot showing COS-7 whole cell lysates of cells transfected with 0.25μg/mL XBP1-ΔDBD-GFP and subjected to untreated or Tm treatment for 2.5 and 5.5 hours. GFP expression increases with BiP expression during Tm treatment. GAPDH is used as a loading control. (E) Confocal microscopy images of a single cell transfected with XBP1-ΔDBD-GFP during Tm treatment. GFP expression is absent at 0 hours, or pre-treatment, and clearly present at later time-points. Scale bar represents 10μM.
We began to apply this system of live-cell stress tracking to mammalian COS-7 cells (C. aethiops kidney fibroblast cells) also expressing a fluorescent ER membrane marker, mCh-Sec61β. Individual COS-7 cells were imaged before Tm treatment, when no XBP1-ΔDBD-GFP signal was noticeable, and at 2.5 and 5.5 hours post-Tm treatment in order to visualize ER morphology in concert with progression of stress.

It became apparent that as the XBP1-ΔDBD-GFP stress signal increased a marked ER membrane expansion occurred (Figure 2). This expansion was especially noticeable in the periphery of the cell where sheets formed in areas previously occupied solely by tubules, unusual due to the fact that sheets are usually found in the perinuclear region and excluded from the far periphery of the cell (Figure 2A).3,4 We also imaged mock-treated COS-7 cells expressing XBP1-ΔDBD-GFP and mCh-Sec61β to verify ER membrane expansion was a product of UPR induction via Tm-treatment and not due to prolonged imaging; to do this we analyzed mock-treated cells that did not show a significant increase in XBP1-ΔDBD-GFP expression, and conversely analyzed all Tm-treated cells that displayed a significant increase in XBP1-ΔDBD-GFP expression.

In order to get a quantitative representation of our observations during live-cell stress treatments we developed a system to measure the ER expansion phenotype. It proved to be a challenge to numerically represent ER membrane area in mammalian cells in a way that accurately portrayed the correct dimensions of sheets and tubules. The method we ended up choosing allowed us to measure overall ER area in a region of interest (ROI) from the confocal image as a ratio of black to white pixels; white pixels represent positive regions, or ER membrane, while black pixels represent background (Figure 3B). Three ROIs per time-point were selected in the periphery of the cell in order to measure the most easily resolvable region of ER membrane, or the area in which most of the ER was present in one focal plane and entirely represented in the confocal image (Figure 3A). Area values for each binary ROI were averaged to get an overall area value for the cell, then a percent area change was calculated by equalizing the value at each time point to the pre-treatment value. This system allowed us to assign raw numerical values to ER membrane area for each image in order to get a quantitative output that represented change in ER membrane during progression of the UPR.

Quantification of peripheral ER area revealed that Tm-treated COS-7 cells on average saw a membrane increase of about 36% after 5.5 hours of Tm treatment, significantly different from about 7% in mock-treated cells (n=22). This directly correlated with XBP1-ΔDBD-GFP expression fold-change, which was about 5 fold in Tm-treated as opposed to 1.5 fold in mock-treated cells.
Figure 2. (A) Model example of a single COS-7 cell time course expressing mCh-Sec61ß and XBP1-ΔDBD-GFP during Tm treatment. The blue boxes indicate a specific peripheral region that sees clear increase in sheets in an area previously occupied by mainly tubules. However, notice that the rest of the cell sees increase in ER area as well, including large cisternae extending from the perinuclear region across the diameter of the cell. Also note that the GFP channel dramatically increases during Tm treatment in tandem with the amount of ER membrane expansion. Scale bar represents 10 μM. (B) Tm-treated COS-7 cells showed an average membrane area increase of about 24% at 2.5 hours and 35% at 5.5 hours, significantly different from mock-treated cells at 4% and 7%, respectively. Area values for each time point were equalized to the pre-treatment value to show a percent increase. Statistical significance (*) compared with mock-treated at each time point was P < 10^{-4}. Error bars represent standard SEM values. (C) Tm-treated COS-7 cells saw an increase in GFP intensity of about 2.1 and 5.0 fold at 2.5 and 5.5 hours, respectively, significantly different from mock-treated cells. Intensity values were equalized to the pre-treatment value to show a fold change. Statistical significance (*) compared with mock-treated at each time point was P < 10^{-2}.
Figure 3. (A) Example of analysis of ER area. Three ROIs are selected in the cell periphery, cropped, and converted to binary. Percent area of each ROI is calculated as a ratio of white to black pixels and averaged to give a percent ER area for the cell periphery. (B) Equation used to calculate the percent change in ER area for an individual cell for each time point, $x$ hours, during treatment.
ER Area Expands in U2-OS Cells during UPR

To increase translational relevance we desired to repeat the live-cell stress imaging experiment in a human cell line, U2-OS cells (human bone osteosarcoma epithelial cells). We treated U2-OS cells transfected with XBP1-ΔDBD-GFP and mCh-Sec61β with the same concentration of Tm used for COS-7 cells and live-cell imaged them for 5.5 hours. As stress visually progressed in U2-OS cells we observed a very similar phenotype: ER membrane area expanded upon activation of UPR (Figure 4).

ER membrane expansion occurred throughout the entire cell but especially in the form of peripheral sheets, similar to COS-7 cells. Tm-treated U2-OS cells displayed about a 5% and 13% ER area change at 2.5 and 5.5 hours, respectively. This was in concert with an XBP1-ΔDBD-GFP fold change of 1.9 and 3.1, once again tying ER membrane expansion with UPR signaling.
Figure 4. (A) Model example of a single U2-OS cell time course expressing mCh-Sec61ß and XBP1-ΔDBD-GFP during Tm treatment. The blue boxes indicate a specific peripheral region that sees clear increase in sheets in an area previously occupied by mainly tubules. However, notice that the rest of the cell sees similar increase in ER area as well. Also note that the GFP channel dramatically increases during Tm treatment in tandem with the amount of ER membrane expansion. Scale bar represents 10μM. (B) Tm-treated U2-OS cells showed an average membrane area increase of about 5% at 2.5 hours and 13% at 5.5 hours. Area values for each time point were equalized to the pre-treatment value to show a percent increase. Error bars represent standard SEM values. (C) Tm-treated U2-OS cells saw an increase in GFP intensity of about 1.8 and 3.1 fold at 2.5 and 5.5 hours, respectively. Intensity values were equalized to the pre-treatment value to show a fold change.
**ER Size Rather than Shape is Essential during UPR**

Generation of sheets in the peripheral ER seemed to be the major phenotype of membrane expansion during UPR. To verify this we probed Tm-treated cells for endogenous levels of the ER tubule-shaping protein reticulon 4a (Rtn4a). ER cisternae can generally be characterized by exclusion of Rtn4a due to the high lumen-to-membrane ratio. COS-7 cells transfected with XBP1-ΔDBD-GFP and BFP-Sec61β were fixed after 5.5 hours of Tm-treatment and immunostained for Rtn4a (αNogoA). While this prevented us from tracking live-cell stress induction in individual cells over time, it did allow us to visualize an overall picture of ER morphology in a large population of cells expressing high XBP1-ΔDBD-GFP stress signal. Peripheral ER cisternae devoid of Rtn4a were detected in the majority of cells expressing high levels of XBP1-ΔDBD-GFP, characteristic of what we’d observed in live-cell stress treatments (Figure 5A).

To follow up this observation we wanted to ask if specific ER morphology – in the form of sheets – was required to alleviate ER stress during UPR. Therefore, we overexpressed Rtn4a in COS-7 cells to tubulate the ER and eliminate all ER cisternae (except the nuclear envelope). We then treated Rtn4a-overexpressing cells with Tm and performed the same live-cell stress experiment as in Figure 2. Analysis of these images revealed a similar increase in ER membrane area, except regions of increased ER membrane now contained Rtn4a (Figure 5B), implying that these regions were actually masses of tubules instead of cisternae and are morphologically distinct from the peripheral sheets seen in the normal COS-7 stress response.

Quantification of live-cell images showed that ER area increased by about 25% and 30% at 2.5 and 5.5 hours of Tm-treatment, respectively, in Rtn4a-overexpressing COS-7 cells (Figure 5C). Once again, this data was compared to mock-treated cells at about 12% and 15%, respectively, and shown to be significantly different. However, it is important to note that Rtn4a-overexpressing mock-treated cells showed higher levels of ER area increase than in normal COS-7 mock-treated cells (Figure 5B). This is likely because overexpression of ER tubules makes cells more susceptible to stresses from long-term imaging.
Figure 5. (A) Endogenous Rtn4a is excluded from peripheral sheets in a representative UPR-induced COS-7 cell. BFP-Sec61ß is a transfected fluorescent ER membrane marker, αNogoA is immunostained Rtn4, and Overlay displays BFP-Sec61ß in red, Rtn4a in green, and overlapping regions in yellow. XBP1-ΔDBD-GFP channel displays a stress signal induced by 5.5 hours of Tm treatment. Arrows indicate characteristic peripheral ER membrane expansion devoid of Rtn4 protein. Scale bar represents 10μM. (B) An individual COS-7 cell overexpressing Rtn4a still undergoes ER membrane expansion during Tm treatment. BFP-Sec61ß is a transfected fluorescent ER membrane marker, Rtn4a-mCh is transfected Rtn4 overexpressed at a level to prevent sheet formation, and Overlay displays BFP-Sec61ß in red, Rtn4 in green, and overlapping regions in yellow. XBP1-ΔDBD-GFP channel displays a stress signal induced by 5.5 hours of Tm treatment. Arrows indicate regions of peripheral high ER membrane area that are expressing Rtn4a, indicating they are masses of tubules instead of sheets. Scale bar represents 10μM. (C) Tm-treated COS-7 cells overexpressing Rtn4a showed an average membrane area increase of about 24% at 2.5 hours and 31% at 5.5 hours, significantly different from mock-treated cells at 12% and 14%, respectively. Area values for each time point were equalized to the pre-treatment value to show a percent increase. Statistical significance (*) compared with mock-treated at each time point was P < 0.05. Error bars represent standard SEM values. (D) Tm-treated COS-7 cells overexpressing Rtn4a saw an increase in GFP intensity of about 1.7 and 2.6 fold at 2.5 and 5.5 hours, respectively, significantly different from mock-treated cells. Intensity values were equalized to the pre-treatment value to show a fold change. Statistical significance (*) compared with mock-treated at each time point was P < 10^{-2}. 
**Discussion**

Characterizing the link between ER morphology and its many specialized functions is an essential step in furthering understanding of the ER as a master cellular regulator. The ER must rearrange its dynamic shape in response to cellular needs, which in turn affects its ability to synthesize lipids, mediate organelle fission, act as a translational scaffold, and transfer Ca\(^{2+}\).

Mediation of cell stress by the UPR is a cellular process that seems to require large-scale reorganization of ER membrane in many mammalian cell types. Our data here shows that COS-7 ER membrane undergoes a significant expansion of over 36% in tandem with UPR signaling, as verified by XBP1-ΔDBD-GFP expression increase. However, the percent area change in human U2-OS cells was less than observed in COS-7 cells at about 13%. This could be due to the fact that U2-OS cells are more three-dimensional than COS-7 cells and a percentage of ER expansion occurred on a different focal plane, preventing quantification with our method. It is also possible that different cell types have varying responses to ER stress and some may see more membrane expansion than others. In this case, COS-7 cells are kidney fibroblast cells and in normal conditions produce greater quantities of protein for excretion than U2-OS cells; therefore, it would make sense that COS-7 cells respond more dramatically to UPR-induction in order to restore normal levels of protein excretion.

While the ER morphology change we observed occurs across the entire ER and usually manifests in the form of peripheral sheets, devoid of tubule-shaping proteins such as Rtn4a, we have shown that converting ER membrane into an entirely tubular network does not prevent UPR-induced membrane expansion. Instead, ER membrane seems to expand in masses of tubules, or “tubule bundles” rich in Rtn4a, marking them as morphologically distinct from the expanded membrane in normal cells. This implies that overall membrane size, rather than a particular shape, is the main goal of ER membrane expansion during UPR-signaling.

While these findings agree with previous work in both yeast and mammalian cells, it leaves unanswered the purpose of such a dramatic ER membrane expansion or how the morphology change is specifically affecting its other cellular roles. We have two main hypotheses to explain the purpose of UPR-induced ER membrane expansion. First, it seems likely that the attempt to attenuate protein misfolding by UPR-signaling would require increased scaffolding for translational machinery. Expansion of the ER membrane may increase the efficiency of the ER to restore homeostasis by allowing for additional translational and quality control complexes to be assembled on the ER membrane. While it has already been shown that chaperones, quality control proteins, and translational complexes are directly upregulated by UPR signaling, it would be insightful to look for ribosome accumulation on
regions of expanded ER membrane to show that it is indeed being used for increased translational scaffolding.

On the other hand, it is possible that ER membrane increases to accommodate the large increase in aggregates formed under stress conditions. This could either be to prevent them from escaping the ER or from interfering with other localized ER functions. Unfolded proteins could be sequestered to regions of new ER membrane to await proper folding or degradation, possibly by signaling or tethering methods not yet known. There is growing evidence for a process known as “ER-phagy” in which entire portions of ER membrane are degraded by direct invagination in the lysosomal membrane. This has been best studied in yeast and directly implicated as a downstream event of ER stress; it would be beneficial to look for these same signs in mammalian cells with a similar system to what we have developed here. The concept of ER-phagy also complements the idea that increased ER size rather than specific shape is the primary goal of UPR signaling as tubule bundles could likely facilitate sequestering of aggregates but would be unlikely to support ribosome accumulation.

Such a massive ER membrane expansion phenotype implies that there must be a large upregulation in the activity of some lipid biogenesis pathways. In yeast, the Ino2/4 complex was found to be activated by UPR signaling and in turn activated Opi3-mediated phosphatidylcholine synthesis in order to facilitate ER membrane expansion. It seems necessary to look for similar regulation of lipid biogenesis pathways in mammalian cells to deduce what types of lipids are being synthesized, which in turn would give insight into the functional properties of the expanded ER membrane. There could be a shift in ER lipid composition that drives certain organelle contacts, lipid transfers, or favors a particular type of morphology.

UPR-induced reorganization and expansion of the ER membrane must directly affect its ability to perform other functions besides protein synthesis and quality control. As a master regulator of other organelles, the ER is inherently dynamic and can constantly reorganize its diverse network to contact endosomes, mitochondria, the plasma membrane, etc. in order to regulate their function by transfer of lipids, recruitment of fission and fusion factors, and secretion of Ca^{2+}. An expanded ER membrane could inhibit the dynamic properties of the ER as a product of simple overcrowding or by generation of cisternae, which are intrinsically less dynamic than tubules. This could disrupt proper vesicle trafficking, for example, if the ER cannot properly localize to endosomal budding sites or focus ER exit-sites (ERES) to Golgi.

Alternatively, increased ER membrane could offer more opportunities for ER-organelle contacts and result in increased regulation of organelle dynamics. An interesting candidate for this idea would be
ER-mitochondrial contacts, since recent work has shown that the ER directly regulates mitochondrial fission and mediates the formation of specialized mitochondrial apoptotic membrane domains.\textsuperscript{13,14} Mitochondria undergo dramatic fragmentation when releasing apoptotic factors and apoptosis can be triggered by UPR signaling; therefore, it would follow that ER-mitochondrial contacts are increased or even stabilized during the UPR. Also, we have seen that plasma membrane contact seems to increase during ER stress (data not shown), which could be to maximize lipid or Ca\textsuperscript{2+} exchange in order to facilitate the increase in ER membrane. If the UPR is causing ER-phagy it would be expected to see increased lysosomal contact as well.

Though the UPR has classically been viewed as a venue to simply restore proper protein folding capability to the cell, the role of the ER as a master regulator of a plethora of cell functions makes this single-minded approach very unlikely. It is essential to characterize how UPR-induced ER membrane expansion affects ER-organelle contacts in order to truly understand the full picture of the ER stress response. In turn, this will further the scientific community’s understanding of how prion diseases, viral infections, and neurodegenerative disorders are affecting tissues at the cellular level and allow for investigation of novel treatment methods.
Materials and Methods

Cell Culture and Transfection of COS-7 and U-2 OS Cells
COS-7 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. U-2 OS cells were grown in McCoy’s 5A Medium also supplemented with 10% FBS and 1% penicillin/streptomycin. The day before transfection cells were seeded in a 2mL well at a concentration of 0.9x10^5 cells/mL. Transfections were performed in 2mL of OPTI-MEM (Invitrogen) with 5µl of Lipfectamine 2000 (Invitrogen) and vector DNA for 5 hours at 37°C incubation, then split to a 2mL, 35mm glass-bottom microscope dish or a 2mL well in supplemented DMEM or McCoy’s at 0.9x10^5 cells/mL. Cells were allowed to recover overnight before additional experiments were carried out (live-cell imaging, collection of whole cell lysates, fixation, etc).
DNA constructs were used at the following levels for all 2mL transfections: 0.5µg mCherry-Sec61ß, 0.25µg XBP1-ΔDBD-GFP, 1.0µg BFP-Sec61ß, and 0.4µg Rtn4a-mCherry (for overexpression).

Drug Treatments
COS-7 and U-2 OS cells were treated with either Tunicamycin (Tm) for UPR stress experiments or dimethyl sulfoxide (DMSO) for mock-treated experiments. For Tm-treated experiments, 5µl of a 2mg/mL stock of Tm in DMSO was added to 2mL of fresh cell media for a final concentration of 5µg/mL. For cell-untreated experiments, 5µl of DMSO was added to 2mL of fresh cell media.

Generation of Target DNA Constructs
mCherry-Sec61ß, BFP-Sec61ß, and Rtn4a-mCherry constructs were generated as previously described in Shibata et al. 2008. XBP1-ΔDBD (nucleotides 409-632 of human xbp1 gene) was PCR amplified from HeLa cDNA then inserted into the pAc-GFP-N1 vector with Xho1 and BamH1 restriction sites. Primers were designed so that stress splicing of 26 nucleotides in XBP-1 would put GFP in frame. Site directed mutagenesis PCR was used to remove the GFP start codon from the pAc-GFP-N1 vector to eliminate any possible transient translation of GFP without stress splicing.

Western Blots
Whole cell lysates were collected by treatment with 1.5mL of 0.25% Trypsin + EDTA for 4-5 minutes, then centrifuged in a 15mL conical tube at 200rcf for 2 minutes to pellet. Supernatant was aspirated off and pellets were washed and resuspended in 1mL of Phosphate Buffered Saline (PBS), transferred to a
1.5mL Eppendorf tube, and centrifuged at 400Xg for 3 minutes to re-pellet. Pellets were then denatured by resuspension in 2X Protein Sample Buffer (PSB) + 0.05% β-mercaptoethanol (BME) and boiled at 95°C for 5-10 minutes. Samples were loaded into a Criterion TGX 4-20% gradient polyacrylamide gel and ran for 30-40 minutes at 200 Volts.

Samples were transferred to a polyvinylidene difluoride (PVDF) membrane and blocked in 5% non-fat milk + TTBS for 1 hour. Membranes were stained with primary antibodies in 5% non-fat milk + TTBS and secondary antibodies in 1% non-fat milk + TTBS. The following primary antibodies were used: α-BiP Rabbit mAb (Cell Signaling C50B12) at 1:1000, α-GAPDH Rabbit mAb (Sigma Aldrich G9545) at 1:20,000, and α-GFP Rabbit mAb (Abcam ab6556) at 1:4000.

**Live-Cell Confocal Microscopy**

After transfection and recovery period (described above), cells were imaged using an inverted fluorescence microscope (Nikon TE2000-U) equipped with a Yokagowa Spinning disc confocal (CSU-Xm2) and Photometrics Cascade II EM-CCD camera. Cells were put in 2mL of OPTI-MEM immediately prior to imaging and subsequently kept in a live-cell imaging chamber at 37°C and 5% CO₂ for the duration of imaging. Images were collected via MetaMorph v.7.0 at pre-treatment, 2.5, and 5.5 hour increments after drug addition.

**Measuring Percent ER Area Change**

ImageJ software was used for image analysis. For all experiments, the Sec61ß channel was analyzed for ER area measurement. Three 6µm diameter circle ROIs were selected in the periphery of the cell for each time point image. We chose to analyze the cell periphery because it is difficult to resolve one plane of ER in the perinuclear region. Each ROI was cropped and individually converted to binary via the adaptive threshold plugin for ImageJ (Qingzong Tseng). Percent areas were calculated as a ratio of white to black pixels and averaged. Percent ER area change per cell was calculated by equalizing each time point average value to the pre-treatment average. ROIs for future time points of the same cell were kept in the same general area in the cell periphery after compensating for cell movement and growth.

**Measuring XBP1 Intensity Fold Change**

ImageJ software was used for image analysis. For all experiments, the XBP1-ΔDBD-GFP channel was analyzed to determine stress level. Average background fluorescence was subtracted from each image and the same ROIs used in ER area measurement were used to measure mean fluorescence intensity.
The three ROI values were averaged and divided by the pre-treatment average to calculate fold change. It is important to note that if any Tm-treated cell did not show an XBP1 intensity fold change of at least 2.0 it was thrown out of analysis; conversely, if a mock-treated cell showed an XBP1 intensity fold change of more than 2.1 it was not used for further analysis.

**Fixation and Immunofluorescence**

After transfection with mCherry-Sec61ß and XBP1-ΔDBD-GFP as described above cells were fixed in glass-bottom microscope dishes in 4% paraformaldehyde (PFA) + 0.5% glutaraldehyde in PBS for 15 minutes, then in 0.1% Triton X100 in PBS for 5 minutes. Dishes were blocked in 10% Donkey Serum + 0.1% Triton X100 in PBS for 15 minutes, then stained with αNogoA Goat mAb antibody (Invitrogen #36-660) at a 1:200 concentration.
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References


12. Schuck, S., Gallagher, C. M. & Walter, P. ER-phagy mediates selective degradation of endoplasmic reticulum independently of the core autophagy machinery.

