HHS Public Access
Author manuscript
Endoplasmic Reticulum Stress Dis. Author manuscript; available in PMC 2014 November 20.

Published in final edited form as:
Endoplasmic Reticulum Stress Dis. 2014 January 1; 1(1): 27–39. doi:10.2478/ersc-2014-0002.

Approaches to imaging unfolded secretory protein stress in living cells

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Abstract

The endoplasmic reticulum (ER) is the point of entry of proteins into the secretory pathway. Nascent peptides interact with the ER quality control machinery that ensures correct folding of the nascent proteins. Failure to properly fold proteins can lead to loss of protein function and cytotoxic aggregation of misfolded proteins that can lead to cell death. To cope with increases in the ER unfolded secretory protein burden, cells have evolved the Unfolded Protein Response (UPR). The UPR is the primary signaling pathway that monitors the state of the ER folding environment. When the unfolded protein burden overwhelms the capacity of the ER quality control machinery, a state termed ER stress, sensor proteins detect accumulation of misfolded peptides and trigger the UPR transcriptional response. The UPR, which is conserved from yeast to mammals, consists of an ensemble of complex signaling pathways that aims at adapting the ER to the new misfolded protein load. To determine how different factors impact the ER folding environment, various tools and assays have been developed. In this review, we discuss recent advances in live cell imaging reporters and model systems that enable researchers to monitor changes in the unfolded secretory protein burden and activation of the UPR and its associated signaling pathways.

Keywords

Green Fluorescent Protein; Photobleaching; FRAP; Transcriptional Reporter; BiP; Kar2; IRE1; yeast; UPRE; Quality Control

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Introduction

Proper folding and quality control (QC) of secretory proteins are crucial to cell viability. Accumulation of misfolded proteins can lead to loss of protein function and cell death. To cope with aberrant misfolded protein accumulation in the endoplasmic reticulum (ER), cells evolved the protective unfolded protein response (**UPR**) [1].

Secretory proteins enter the lumen of the ER where the QC machinery, including the chaperone BiP/GRP78 (Kar2 in yeast, *S. cerevisiae*) ensures proper folding of nascent peptides. Correctly folded proteins are then exported into the secretory pathway. Disruption of the ER folding environment can cause unfolded secretory proteins to accumulate and aggregate in the ER, activating the UPR signaling pathway [1]. The UPR coordinates the transcriptional up-regulation of ER chaperones, degradative machinery, and trafficking machinery [2]. Another arm of the UPR transiently attenuates global protein translation to decrease the nascent protein burden and prevent aberrant accumulation of unfolded proteins [3]. Unresolved ER stress and constitutive UPR activation in metazoans can both lead to cell death via caspase activation and apoptosis [4-6]. Apoptotic induction appears to be a consequence of prolonged UPR activation [6, 7].

The number of functional UPR sensors/transducers expanded during the transition from single celled organisms to metazoans. For example, yeast have one sensor/effector (Ire1) and metazoans have at least three (IRE1, PERK, and ATF6) (**Figure 1**), with mammals also encoding two isoforms of IRE1 (α and β) and ATF6 (α and β) [8]. In *S. cerevisiae*, Ire1 cleaves *HAC1* mRNA as part of a splicing reaction [9] to enable correct translation of the transcription factor Hac1 and upregulation of ~400 UPR target genes **(Figure 1)** [10]. Targets include ER chaperones, degradation machinery and genes involved in lipid synthesis [10]. Attenuation of Ire1 signaling is critical for yeast cell adaptation to ER stress and Ire1 mutants unable to deactivate following UPR induction are hypersensitive to ER stressors [11, 12].

When levels of unfolded proteins increase significantly in the ER, UPR sensors are activated following titration of free Kar2/BiP by unfolded proteins and depletion of BiP from the sensors [13]. While BiP release is not necessarily sufficient to activate UPR sensors, the bound chaperone appears to inhibit oligomerization of PERK and IRE1 or secretion of ATF6. Upon activation, the sensors trigger signaling pathways including transiently attenuating translation through phosphorylation of eIF2α by PERK while simultaneously upregulating specific luminal chaperones (e.g., BiP and GRP94)[14, 15] and ER– associated degradation (ERAD) components [7, 16-21]. Upon BiP release, PERK and IRE1 can each homodimerize, autophosphorylate, and then modify their effectors [13, 18]. Direct binding of unfolded peptides is an additional component required for acute Ire1 activation in yeast [22-24]. Alternative activation pathways have been reported in which no peptide binding by Ire1 is necessary [25]. PERK phosphorylates eIF2α to attenuate global translation and also dramatically enhances translation of ATF4, which then upregulates transcription of ER chaperones **(Figure 1)** [26, 27]. IRE1 cleaves *XBP1* mRNA as part of a splicing reaction to generate an in frame form to generate a transcription factor that upregulates chaperones, ERAD components, and XBP1 **(Figure 1)** [15, 18]. Upon release from BiP, ATF6 enters the secretory pathway, undergoes proteolytic processing, releasing a transcription factor **(Figure 1)** [18, 28]. Similar to XBP1, the ATF6 transcription factor also upregulates ER QC machinery [29]. Excessive activation of UPR pathways has been associated with important human diseases including heart disease, cancer, diabetes, fatty liver, and various neurodegenerative diseases including Alzheimer's disease and Huntington's disease [30-33]. Thus, establishing how cells respond and cope with accumulation of misfolded secretory protein is critical for our understanding of the etiology of these pathologies. To this end, various reporters and assays have been developed to enable detection and monitoring of the UPR in living cells. In this review, we provide an overview of the expanding toolbox available to researchers for imaging unfolded secretory protein stress in live cells.

2 Approaches for Imaging ER Stress and UPR Activity in Living Cells

The UPR has been studied extensively using biochemical and molecular biology tools. The standard assays for UPR activation and attenuation in terminal assays (i.e. fixed or dead cells) have been described elsewhere [34-38] and are a valuable complimentary approach to live cell assays. Given the availability of robust assays, what can be learned with live cell assays?

Live cell studies provide two major opportunities for researchers. First, the spatial and temporal resolution of cellular processes in live cells is unmatched. Few assays that involve fixing or lysing cells can distinguish time points less than 30 s to 1 min apart, while live cell imaging can readily achieve sub-second to even millisecond temporal resolution. Furthermore, fixed samples only provide snapshots of the distribution of labeled molecules/ structures in cells. A distribution could be static, a dynamic steady state or a step in a progression. In contrast, live cell assays capture and reveal the dynamics of molecular distributions. Second, standard biochemical and molecular biology approaches are ensemble measurements. Such measurements miss cell-to-cell variability, which can be considerable [39]. In the simplest example, a population of cells expresses a protein in a binary manner. An immunoblot of cell lysates would indicate that cells, on average, have a specific amount of protein, even though only half of the cells actually express the protein. In fairness, a fixed cell assay would be able to reveal this heterogeneity. However, only live single cell assays enable investigation of how a range of protein expression levels influences the fates of cells. It is our hope that this review will stimulate interest in studies of the fates and outcomes of cells coping with ER stress.

2.1 Fluorescent Proteins in the ER

Proteins and mRNA cannot be visibly detected in cells. However, molecular tags, especially fluorescent proteins (FPs) that provide high contrast against the background of the cell, enable robust real time detection of changes in transcriptional activity, protein expression levels, and protein localization. More advanced imaging techniques including photobleaching and Förster Resonance Energy Transfer (FRET) [40, 41] further exploit FPs and provide information on protein interactions and dynamics. The availability of tens of thousands of papers and a Nobel Prize testify to the power and popularity of FPs. Yet, the use of FPs requires careful experimental design and an appreciation of potential limitations and caveats [42]. In this section, we describe some general issues with FPs and then issues specifically relevant to the ER.

First, it is useful to consider how FPs function. The majority of FPs form β barrel structures that enable a three amino acid stretch inside the b barrel to undergo an autocatalytic reaction converting the amino acids into a fluorophore. It is essential that the β barrel forms and only then can the fluorophore form [43]. The fluorophore will not fluoresce if the β barrel is disrupted or unfolded [43]. Therefore, any conditions that prevent β barrel formation will impair fluorescent signal generation. In addition, FPs exhibit pH sensitivity. This is termed the pKa of the FP. This is the pH value at which the FP produces half of its maximum potential fluorescence intensity. As some compartments of the secretory pathway are acidic, the choice of FP can impact the ability to detect a fluorescent signal.

Second, native FPs evolved in the cytoplasm and their derivatives were evolved in the cytoplasm of bacteria. Practically, this means that FPs were not optimized for use in the secretory pathway. The ER is distinguished by being more oxidizing relative to the cytoplasm, which then favors disulfide bond formation between cysteines. In addition, tripeptide sequences of N-X-S/T are potential sites for glycosylation. Numerous FPs contain these consensus N-linked glycosylation sequences. As N-linked glycosylation increases protein size can affect regulation of secretory protein turnover by ER-associated degradation (ERAD) [44, 45], and depending on location, N-linked glycosylation can impair FP folding, such modifications are generally not desirable. Therefore, FPs need to be resistant to disulfide bond formation and engineered to remove N-linked glycosylation sites and this has been done for some FPs [46-48].

Third, FPs are not necessarily inert. Several popular FPs, including EGFP, have a propensity to oligomerize [49, 50] and proteins advertised as “monomeric,” may still oligomerize in cells [47]. This is especially true when the FPs are attached to integral membrane proteins, which confines the FPs to a two-dimensional plane and increases the effective concentration leading to a higher probability of oligomerizing. Monomerizing mutations and variants have been reported and these should be used in the design of any fusion proteins [49, 50].

Fourth, FPs are not small molecules. Rather, FPs typically have a 5 nm diameter [51]. This is comparable in size to several 30-80 kDa proteins, which form 3-6 nm structures. FPs can potentially sterically hinder interactions with clients of the fusion protein [52]. Therefore, it is important to develop robust assays for protein function to ensure that the FP fusion protein retains the desired relevant properties of the non-FP tagged parent protein.

Finally, even if the experiment will not involve fusion proteins and instead uses FPs as transcriptional reporters, there are FP properties that will affect interpretation of experiments. FPs vary in terms of maturation times from less than 10 minutes to several hours [53]. The choice of FPs in multi-color experiments can lead to different outcomes depending on which FP is used for a reporter. Thus, use of rapid maturing FP variants is highly desirable. Another relevant reporter parameter is the slow turnover rate. In general FPs are long lived (half-lives > 24 h). A major consequence of this property is that a fluorescent signal indicates that reporter activation has occurred, but that the stimulus is not necessarily still active. Reporter fluorescence levels are best assayed over multiple time points to determine the time of activation and if and when a plateau in FP levels occurs, which may indicate inactivation. The long half-life of FPs does have one major advantage. A weak, but physiologically significant, signal may become detectable as FPs accumulate. For example, deletion of the gene *SCS3* does not cause detectable splicing of *HAC1* mRNA [54], but analysis of *scs3*Δ with a GFP driven by the UPR element reveals a low, but above background, level of fluorescence consistent with low level constitutive activation [55].

The list of available FPs is ever expanding. At this time, we recommend superfolder GFP [56, 57], secBFP2 [47], and FusionRed [58] for protein fusions. For transcriptional reporters, mNeonGreen matures rapidly and produces an intense signal [59] and TagRFP has similar properties for a red reporter [60].

2.2 Transcriptional reporters for UPR activation

UPR activation triggers a transcriptional program that upregulates expression of ~400 genes in the *S. cerevisiae* [10]. Many features of the UPR are conserved in metazoans with additional components that increase the complexity of the stress response. Several fluorescent reporters have been developed to enable quantification of the transcriptional activity of the UPR following induction of misfolded protein stress in the ER. In yeast, these reporters consist of promoters containing UPR elements (UPRE) fused to FPs such as GFP or mCherry [61] (**Figure 2**). Alternatively, FPs containing an appropriate ER retrieval motif (HDEL) can be chromosomally inserted to tag UPR targets, such as Kar2 [62, 63]. When Kar2-sfGFP-HDEL is expressed under its endogenous promoter, fluorescence levels will also reflect activation of the UPR. However, as mentioned in the FP section, high levels of Kar2-sfGFPHDEL do not necessarily indicate simultaneous UPR activity. Many ER chaperones are exceptionally long-lived proteins and will thus persist long after inactivation of a UPR response.

Expression of transcriptional reporters can be monitored by fluorescence microscopy and flow cytometry, allowing high content monitoring of UPR activation under various conditions (pharmacological stressors, deletion mutants) [62]. A similar approach can be used in model organisms. For example, expression of Bip1-GFP in *C. elegans* [64] has been successfully used to quantitatively monitor UPR activation in these organisms. In mammalian cells, our group generated a fluorescent UPR reporter by fusing a portion of the BiP promoter containing ER stress response elements (-169 ERSE) [65] to the red FP tdTomato [66]. This reporter exhibits significant upregulation following treatment of cells with ER stressors such as tunicamycin (Tm) or DTT or upon expression of mutant proteins known to induce the UPR, such as exon1 of the Huntington's disease-associated mutant polyQ protein, huntingtin [66]. Activation of other mammalian UPR targets can also be followed in living cells. For example, the protein CHOP is upregulated during ER stress [26]. CHOP is a transcription factor that activates expression of genes involved in protection of cells from stress [67] and induction of apoptosis [5]. CHOP expression is regulated by the UPR via increased translation of ATF4 [26]. The fluorescence intensity of a GFP reporter, consisting of an FP under the control of the CHOP promoter, parallels the expression of the endogenous CHOP and can be successfully detected in cells upon UPR activation [68]. A translational reporter for ATF4 (whose levels are regulated post-transcriptionally) has also been described [69]. Therefore, multiple fluorescent reporters, using different FPs, can be co-expressed in cells to report on activation of distinct UPR signaling pathways in living cells.

2.3 Measuring Ire1 activity in live cells

UPR signaling in yeast depends on the ability of Ire1 to oligomerize upon activation. An Ire1 mutant unable to dimerize fails to activate the UPR [70]. Ire1 oligomers in yeast cells can be detected by immunofluorescence [25, 71]. To visualize Ire1 oligomers in living intact cells, it is possible to fluorescently tag Ire1 with FPs, such as GFP [24, 71]. However, to conserve all of the Ire1 protein functions, the FP needs to be inserted into the Ire1 linker domain. Indeed, N or C terminus-GFP-tagged Ire1 reportedly cannot activate the UPR [71]. Upon treatment with ER stressors, Ire1-GFP displays oligomer formation that can be quantified in live cells using fluorescence microscopy [24, 71, 72]. The same approach has been described for mammalian IRE1. A HEK-293 cell line expressing a tetracycline-inducible version of IRE1-GFP displays robust IRE1-GFP clustering upon treatment with ER stressors such as Tm or DTT [73] (**Figure 3**). However, the formation of these high order oligomers in both yeast and mammalian cells relates to amplitude of UPR signaling is unclear. Further investigation will be required to determine the minimal size of IRE1 oligomers required for sufficient UPR signaling for a given stress. If IRE1 dimerization is sufficient for *HAC1* splicing, then detection of IRE1-GFP clusters may only reflect exceptionally strong UPR activation, as dimers are unlikely to generate a signal distinct from monomers. It is important to note that the reported GFP fusions use GFP variants prone to oligomerization and, thus, any future studies should be performed with monomerized GFP or better yet, with monomeric sfGFP.

Similarly, fluorescently tagged Ire1 can be used to monitor Ire1 activation using another live cell microscopy technique called FRET. This method relies on the photon-independent exchange of energy between two chromophores. When excited, the donor chromophore (in this case EGFP) can transfer energy to the acceptor (here RFP) when the two proteins are in close proximity (1-10 nm) leading to increased fluorescence of the acceptor [74, 75]. In this method, Ire1 tagged with either GFP or mCherry (a red FP) are co-expressed in yeast cells. It was shown that upon pharmacological induction of the UPR, FRET could be detected in cells expressing FP-tagged Ire1 proteins [24]. Importantly, the intensity of the FRET signal is proportional to the amount of *HAC1* splicing observed by northern blot.

There is an additional way to assess Ire1/IRE1 activation in live cells. Both mammalian and yeast Ire1 can cleave the mRNA of a transcription factor as part of a splicing reaction: *HAC1* in yeast or *XBP1* in metazoans. In *S. cerevisiae*, the splicing reporter (SR-GFP) in which the first exon of the HAC1 open reading frame is replaced by GFP) (**Figure 2**). The *HAC1* intron represses translation of the mRNA, so GFP is only expressed once active Ire1 removes the intron. Therefore, the reporter can report on Ire1 activity independently of *HAC1* transcriptional activity [24]. Like the transcriptional reporters, fluorescence levels of SR-GFP can be measured by fluorescent microscopy and flow cytometry. Similar approaches have been used in other organisms including *C. elegans* [76], *D. melanogaster* [77], and mammalian cells [78, 79].

2.4 Quantitative assessment of the ER misfolded protein burden in living cells.

One of the challenges in detecting ER stress in living cells has been to visualize and quantitate changes in the ER misfolded protein burden. Methods highlighted in this review mostly rely on indirect measurements reflecting either activation of UPR sensors (splicing reporter, Ire1-GFP etc.). Few methods exist to assess global changes in misfolded protein accumulation. Biochemical techniques such as BiP/Kar2 sedimentation have been used to quantitate the chaperone binding to misfolded substrates [80]. However, until recently, no option was available for imaging intact cells. No general dyes, antibodies or other tools recognize and report on unfolded protein levels.

However, there are molecules that can recognize unfolded proteins- chaperones. Our group has developed an assay that exploits the ability of the chaperone BiP to bind to unfolded proteins using a fluorescence microscopy technique termed Fluorescence Recovery After Photobleaching (FRAP). FRAP relies on the ability of fluorescently tagged BiP (such as BiP-GFP) to freely diffuse and sample the entire volume of the ER lumen. When a BiP-GFP molecule encounters and binds a misfolded protein, diffusional mobility of BiP-GFP decreases [66, 81]. Changes in BiP-GFP mobility can be quantitated by calculating the protein diffusion coefficient (*D*) using inhomogeneous simulations [82]. *D* of the protein, which can reveal changes in protein size (R_h), binding interactions, and ER lumen viscosity. *D* is inversely proportional to the protein R_h and environment viscosity. Thus, an increase in molecular size (complex formation) or environmental crowding decreases *D*. Conversely increased *D* indicates release of a protein from a complex or decreased viscosity [49]. We have shown that this method can detect early changes in the ER misfolded protein burden that cannot be detected via classical UPR assays [81]. Mobility of the yeast BiP homologue, Kar2 can also report on the ER misfolded protein burden [62] (**Figure 4**). Interestingly, we have shown that Kar2 mobility can reveal different modes of Ire1 activation. In yeast, inositol depletion can trigger UPR activation in the absence of significant changes in the ER misfolded protein burden [62]. The ability of membrane lipid perturbations to induce UPR independently of accumulation of misfolded proteins has been described using genetic and biochemical methods in both yeast and mammalian cells [25, 83]. Therefore, changes in BiP/Kar2 mobility, when coupled to other biochemical analysis, can directly assess changes in the misfolded protein burden in living cells.

2.5 Imaging calcium levels in the ER

One of the major cellular perturbations that triggers UPR activation in mammalian cells is the depletion of ER calcium stores. For this reason, the SERCA pump inhibitor thapsigargin is often used to induce ER stress. While multiple dyes and genetically encoded reporters are available to measure calcium release in the cytoplasm (see review by McCoombs and Palmer [84]), the tools to monitor calcium levels in the ER are limited. The most commonly used reporter for ER calcium levels is a modified version of the original cameleon construct used to measure cytoplasmic calcium. This reporter consists of calmodulin and a calmodulin-binding peptide derived from skeletal muscle myosin light chain kinase (skMLCK)] that undergo a conformational change upon binding. The binding of calmodulin to the peptide is maximal at high concentrations of calcium and the interaction is rapidly inhibited when calcium levels decrease [85]. The calmodulin/peptide pair was cloned between two FPs, Venus and cerulean, to generate and efficient FRET sensor that can respond to small changes in ER calcium levels. The reporter is targeted to the ER using a calreticulin signal sequence and contains an ER retrieval motif (KDEL). The FRET ratio of D1ER rapidly decreases upon depletion of the ER calcium store induced by thapsigargin [85]. This signal change has also been successfully measured by fluorescence lifetime imaging microscopy (FLIM) [86]. As with any FRET biosensor, it is critical to calibrate signals in the cells of interest and not rely solely on *in vitro* measurements [87]. Cameleon sensor alternatives are available and have some caveats. Luminescent aequorin probes can be targeted to the ER. These are significantly less sensitive than the FRET reporter. Moreover, aequorin luminescence requires the investigator to supply a coelenterazine cofactor. The initial reaction requires that the ER be depleted of calcium, which will induce ER stress, making this reporter unattractive to measure small changes in ER calcium concentrations [88]. A more recent reporter combines bioluminescence and a photoswitchable FP to achieve a robust range of detection of calcium concentrations [89].

2.6 Reporters for ER-associated degradation

A major consequence of ER stress is the accumulation of misfolded proteins in the ER. Misfolded proteins then need to be exported out of the ER to be accessible for degradation by the ubiquitin-proteasome machinery via the retrotranslocation process termed ERAD [44]. Therefore, monitoring accumulation of ERAD substrates can provide insight into the functionality of the ER folding environment. This can be done by expression known ERAD-substrate tagged with FPs. CD3δ has been used extensively in mammalian cells as an ERAD reporter. CD3δ with a yellow FP tag (YFP) accumulates in cells treated with ER stressors [90]. Using fluorescence microscopy with a similar CD3δ-sfGFP tool, we observed UPR activation and accumulation of CD3δ-sfGFP in cells expressing the mutant huntingtin protein, which is associated with Huntington's disease, [66].

Other methods exploiting the ability of GFP moieties to be recombined to form a FP in living cells (bimolecular complementation or split-GFP method) [91] can also be used to monitor ERAD. In this technique, the C-terminal β-strand of GFP (S11) is fused to an ERAD substrate such as CD3δ. The remaining portion of the GFP (β-strands 1-10) is expressed in the cytoplasm. Therefore, only after the ER protein is retrotranslocated into the cytoplasm can the two GFP fragments can come together to generate a fluorescent signal that can be monitored by fluorescence microscopy. This reporter is termed drGFP (dislocation reporter GFP) [92]. To prevent aberrant expression of the ER targeted protein in the cytoplasm due to transient transfection, it is recommended to use stable cell lines expressing both reporters.

For transient transfections and improved signal to noise, another reporter was developed. Grotzke et al. identified mutant variants of the FP Venus that can only become fluorescent when first glycosylated in the ER and then deglycosylated in the cytoplasm following ERAD retrotranslocation [93]. The reporter, termed ddVenus (for deglycosylation dependent) [93], exploits the conversion of glycosylated asparagines to aspartates by PNGaseF in the cytoplasm. By expressing half of ddVenus in the ER and the other half in the cytoplasm, a highly specific ERAD-dependent fluorescent signal can be achieved and quantitated by both fluorescence microscopy and flow cytometry [93].

2.7 Measuring changes in the ER lumen redox potential

Many secretory proteins in the ER require the formation of intra- and intermolecular disulfide bonds. This posttranslational modification is possible due to the high oxidizing ER lumen relative to the cytoplasm. The oxidizing potential of the ER lumen is controlled primarily by Ero1 in yeast [94, 95] and ERO1-β and peroxiredoxin IV in metazoans [96, 97]. In turn, members of the PDI family regulate formation of the correct disulfide bonds in a protein [98]. To monitor changes in the redox poise during ER stress in live cells tools have been developed that quantitate changes in fluorescence that occurs upon aberrant disulfide bound formation at the surface of the GFP β-barrel. Introduction of additional cysteine residues within the GFP β-barrel changes the excitation properties of the protein between the dithiol and the disulfide state. The ratio of emitted fluorescence when excited at 390 nm versus 475 nm reports on the ratio of oxidized vs. reduced GFP molecules in cells [99]. These probes are termed redox-sensitive GFP (roGFP) [100]. Unfortunately, the reducing properties of roGFPs make them generally insensitive to the strongly oxidizing environment of the ER. An ER-targeted modified redox-sensitive GFP (roGFP1-iL) has been used to measure changes in the ER redox potential in mammalian cells. This reporter contains an amino acid insertion to destabilize the disulfide bond between cysteines 147-204, creating a less reducing protein. However, this insertion impaired reporter sensitivity due to the dimness of the FP [101]. Recently, it was reported that FLIM could circumvent this problem to quantitatively report the ER redox and monitor activity of PDI in mammalian cells [86]. Interestingly, FLIM successfully reported changes in the ER redox induced by DTT and thapsigargin, but not by other drugs that induce gross secretory protein misfolding, such as Tm [86]. The latter result contrasts with a previous study in yeast showing that both DTT and Tm induced changes in ER redox poise [61]. Changes in eroGFP signal in yeast appear to reflect accumulation of the ER-targeted reporter to the cytoplasm during prolonged stress as a result of poor secretory protein translocation [12]. Future studies and development of new tools and assays should help to better understand the functional differences between the yeast and mammalian ER regarding changes in the redox poise during ER stress.

2.8 Mouse models to study UPR signaling *in vivo*

While biochemical and *in vitro* tools have proven effective for investigating ER stress mechanisms, the development of *in vivo* systems will be essential for a full understanding the co-ordination of UPR activation within and between tissues, as well as for understanding disease-related mechanisms. To date, two transgenic mouse lines have been developed and studied.

The first transgenic mouse line generated to assess UPR activity *in vivo* was the ER stress Activated Indicator (ERAI) [79], that exploits the ER-stress dependent splicing of *XBP1* mRNA. In this model, the yellow GFP variant, Venus, is fused downstream of the promoter and partial sequence of the human XBP1 gene, which includes the 26 base pair fragment that is excised during ER stress and regulates translation of the functional XBP1 protein. Venus expression occurs only during ER-stress-induced splicing of *XBP1* [79]. This model reports on both physiological and exogenous ER stress *in vivo* and provides a robust read-out of IRE1-dependent UPR activation. The specificity of this model for IRE1 activity does not consider the contributions of the ATF6 and PERK pathways. Furthermore, this model is relatively insensitive to low levels of ER stress, which could make assessment of the UPR in chronic disease or due to low levels of physiologic stress difficult.

The second transgenic mouse model generated to assess UPR activation *in vivo* exploits the UPR-dependent upregulation of the protein folding chaperone, Grp78. This mouse strain expresses the LacZ reporter fused to 3 kilobases of the Grp78 promoter, which contains a cAMP response element (CRE) and three ER stress response element (3xERSE) repeats (ERSE-LacZ) [102]. In this model, LacZ expression correlates with the endogenous Grp78 expression profile, a robust indicator of UPR activation. Accompanying the wild type ERSE-LacZ are two complementary transgenic lines that carry a deletion of either the Cre and the 3xERSE (D300), or the 3xERSE alone (D170) [102]. The use of these complementary lines, in conjunction with the WT ERSE-LacZ, allows for identification of distinct UPR activation mechanisms for different types of stresses based on their requirement for the Cre, the 3xERSE, or both, for UPR induction. The use of this model comes with the caveat that Grp78 activation represents overall UPR activation and does not allow for assessment of individual UPR pathways activity.

Both the ERAI and ERSE-LacZ models are powerful tools for the *in vivo* detection of UPR activation. As both models provide distinct advantages, they can be used in a complementary way to answer both global and specific questions relating to UPR activation *in vivo*.

3 Perspective

The toolbox for studying ER stress and the UPR in cells has become diverse and powerful for investigators. Modern microscopes and FACS setups can easily monitor three to four fluorescence reporters. To fully realize the live cell toolbox, implementation of current and future technologies will be necessary.

First, it would be ideal to avoid overexpression artifacts for fluorescently tagged proteins, such as BiP. In yeast, chromosomal tagging is a standard technology. In metazoans, CRISPR [103, 104] technology is coming online and appears to be capable of chromosomal tagging of endogenous genes with FPs to generate cell lines and even whole animals [105]. Even with homologous recombination and tagging, a significant impediment to imaging a number of proteins is the low expression levels of most cellular proteins. FPs are generally not bright enough to image at low expression levels in live cells [106]. Some newer brighter FPs, such as mNeonGreen [59] have been reported and suggest that it may be possible to image low expressed proteins at physiologic levels in cells.

Second, transcriptional and translational reporters would be even more useful if they could be rapidly cleared from the cell. Such a tool would enable continuous interrogation of stress reporters. Addition of a ubiquitin [107] or proteasome degron [108] to the reporter can achieve this goal. However, the available systems tend to either degrade reporters too quickly for low abundance signals or too slowly for rapidly changing processes. In addition, proteasome-dependent degradation is compromised in situations where proteasome inhibitors are used or when the proteasome can be overwhelmed during extreme stress. An ideal system would be independent of other cellular processes. One possibility may be to exploit properties of fluorescent proteins. Proteins that change color with age [109] potentially could be evolved to become dark with age and thus provide information on levels of recently synthesized FP reporter without forfeiting a color channel for multicolor reporter imaging.

Finally, FPs are powerful, but suboptimal tagging reporters. A small peptide with a bright associated dye would be an ideal tag and may be possible. The FlAsH/ReAsH peptide tagging system [110, 111] is currently the smallest commercially available tag. One of the major shortcomings is the lack of suitability for the secretory pathway. The cysteines in the peptide make contact with a biarsenical-linked dye and these cysteines are prone to inactivation by inappropriate disulfide bond formation in the oxidizing environment of the secretory pathway lumen [112].

The current toolbox provides investigators with many opportunities to study dynamic changes in ER function and cell coping with ER stress. Combined with powerful commercial microscopes, high content and high throughput imaging systems (such as the Perkin Elmer Operetta), and systems biology computational analyses, the ER stress imaging toolbox will help investigators gain new insights into the cell biology of secretory protein quality control and stress resolution.

Acknowledgments

The authors would like to thank Peter Walter (University of California at San Francisco) for the IRE1-GFP cells and the SR-GFP plasmid and Yukio Kimata (Nara Institute of Science and Technology) for the Ire1-HA plasmid. We thank Feng Guo (Albert Einstein College of Medicine) for immunofluorescence images in Figure 3A.

Work leading to this review was supported by a grant from the National Institute of General Medical Sciences (NIGMS) 1R01GM105997-01 to ELS. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIGMS or the NIH. PL was supported by a postdoctoral fellowship from the American Heart Association and is the recipient of a Schulich Strategic Support Award from the University of Western Ontario. ENF is supported by the CIHR Strategic Training Program in Vascular Research.

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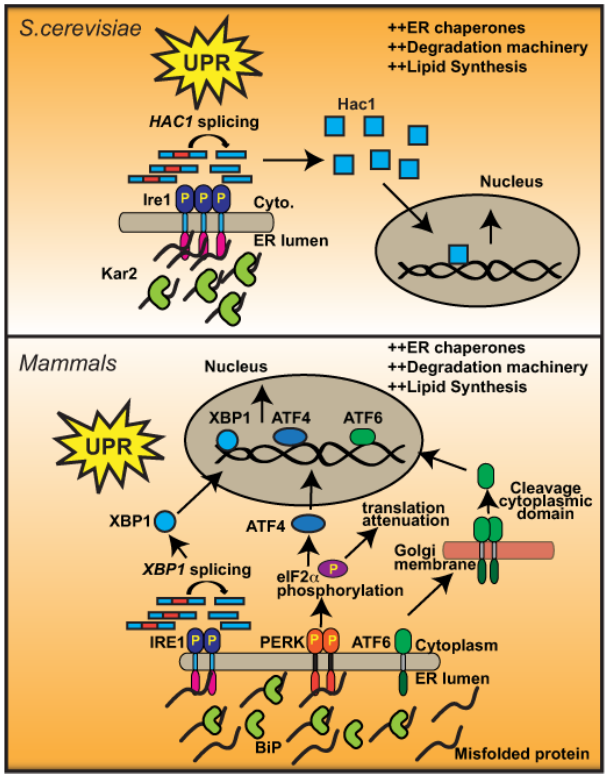

Figure 1.

Features of the UPR sensors and their effectors in *S. cerevisiae* and mammals.

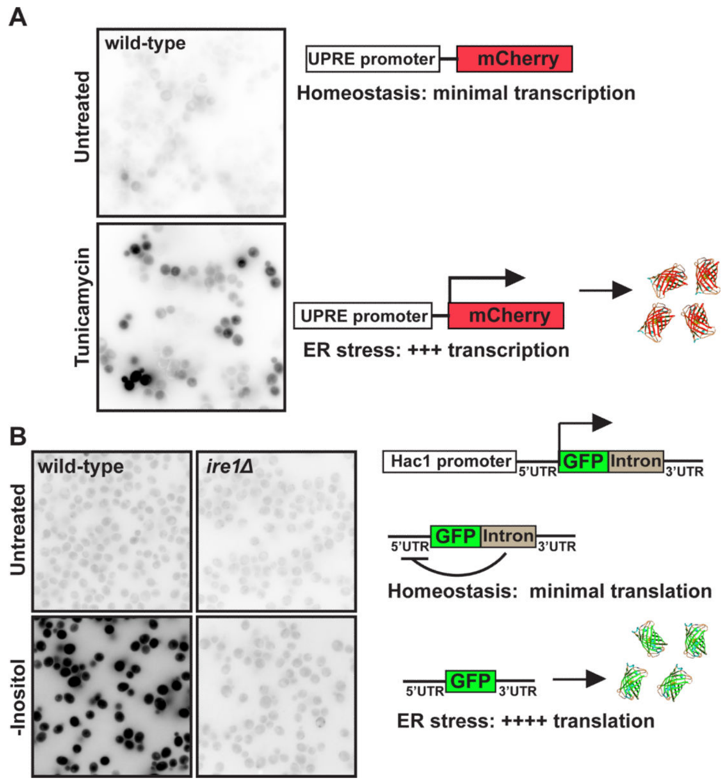

Figure 2.

UPR transcriptional reporters. A. Yeast expressing an mCherry reporter under the regulation of a UPRE exhibit increased mCherry expression following Tm treatment. B. Yeast expressing a splicing reporter under the control of the native *HAC1* promoter. The intron suppresses translation, but splicing following cleavage by Ire1 enables translation. Resulting levels of GFP reflect relative levels of *HAC1* splicing. The inverted fluorescence micrographs establish that *HAC1* splicing (darker more intense fluorescent signal in yeast) reported following inositol withdrawal depends on a functional copy of Ire1, as mutant yeast cannot splice *HAC1*.

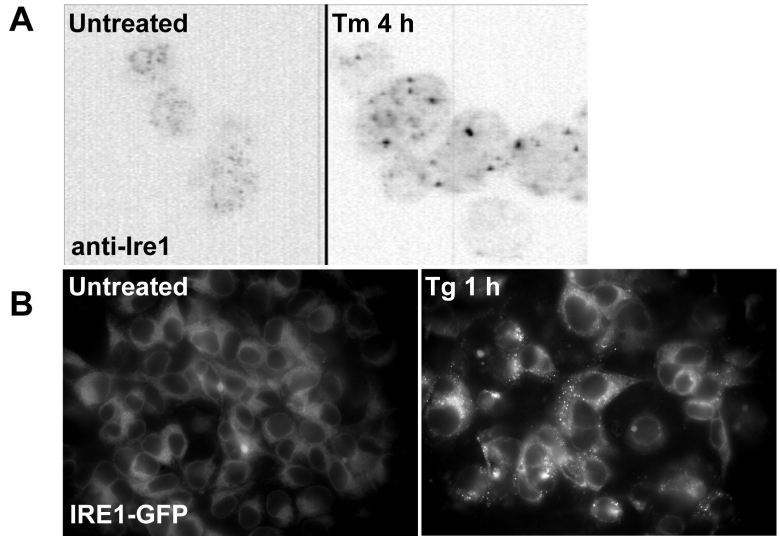

Figure 3.

Assessing Ire1 and IRE1 clustering in cells. A. Immunofluorescence of HA-tagged Ire1 in homeostatic (left) and Tm stressed yeast cells (right). Robust clustering is observed in the stressed cells. Images were provided by Feng Guo. B. IRE1-GFP expressed in tet-inducible HEK293 cells. Clustering is apparent following a 1 h stress with 10 nM thapsigargin (Tg) treatment.

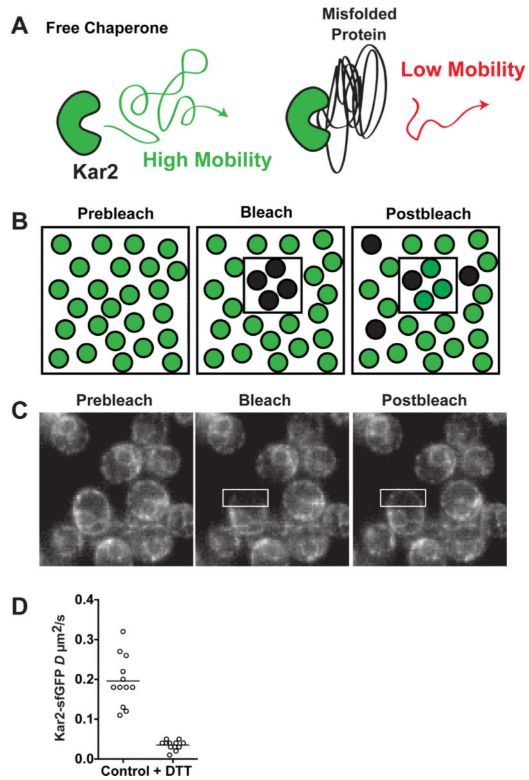

Figure 4.

Kar2-sfGFP ER stress reporter and FRAP analysis. A. Unbound Kar2 freely diffuses with high mobility while Kar2 bound to unfolded proteins will form a large complex that will diffuse much more slowly. B. In FRAP, a population of FPs can be selectively photobleached with a laser scanning confocal microscope (box in middle panel). The FPs are still present, but now dark. Movement of unbleached molecules into the bleach region can be measured and used to calculate *D*. C. Example of a FRAP experiment for Kar2-sfGFP-HDEL expressing yeast. A region of interest (white box) is photobleached and fluorescence recovery can be observed over time. D. Example of diffusion coefficients (*D*) for cells expressing Kar2-sfGFP-HDEL. Unstressed cells exhibit a much higher *D* value, while DTT treatment dramatically decreases Kar2 mobility and *D*.