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The Unfolded Protein Response Triggers Selective mRNA Release From the Endoplasmic Reticulum

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SUMMARY

The unfolded protein response (UPR) is a stress response program that reprograms cellular translation and gene expression in response to proteotoxic stress in the endoplasmic reticulum (ER). One of the primary means by which the UPR alleviates this stress is by reducing protein flux into the ER via a general suppression of protein synthesis and ER-specific mRNA degradation. We report here an additional UPR-induced mechanism for the reduction of protein flux into the ER, where mRNAs that encode signal sequences are released from the ER to the cytosol. By removing mRNAs from the site of translocation, this mechanism may serve as a potent means to transiently reduce ER protein folding load and restore proteostasis. These findings identify the dynamic subcellular localization of mRNAs and translation as a selective and rapid regulatory feature of the cellular response to protein folding stress.

INTRODUCTION

Protein synthesis is tightly coupled to protein homeostasis so that proteome function can be sustained during environmental and physiological stress. Among the most rapid and specific means to this end is the reprogramming of translational activity (Holcik and Sonenberg, 2005; Spriggs et al., 2010). One such program, the unfolded protein response (UPR), is initiated by the accumulation of unfolded proteins in the endoplasmic reticulum (ER)

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(Walter and Ron, 2011; Wang and Kaufman, 2012). By acting to both reduce the protein folding load of the ER and increase the organelle's protein folding capacity, the UPR provides a conserved mechanism for responding and adapting to proteostatic stress.

The UPR stress response program operates within the context of a translational machinery that is compartmentalized between the cytoplasm and endoplasmic reticulum (Palade, 1975; Palade, 1956; Voeltz et al., 2002). In a typical mammalian cell, approximately half of all ribosomes and a third of all mRNAs are associated with the ER membrane (Palade, 1975; Reid and Nicchitta, 2012) Both mRNAs and ribosomes can be tethered to the ER independently of one another to confer ER association (Adesnik et al., 1976; Chen et al., 2011; Pyhtila et al., 2008). One function of this compartmentalization is to enable the co-translational insertion of membrane and secretory proteins into the ER, the entry point for the secretory pathway (Palade, 1975). In addition, recent studies have revealed that a substantial fraction of all cytoplasmic protein-encoding mRNAs are translated on the ER, making the ER a primary site for the synthesis of the proteome generally (Diehn et al., 2000; Mueckler and Pitot, 1981; Reid and Nicchitta, 2012). This large-scale compartmentalization of protein synthesis opens the possibility that mRNA localization to the ER may be an important control point in translational regulation, particularly with respect to findings that the activity of cytoplasmic and ER-associated ribosomes can be regulated independently (Stephens and Nicchitta, 2008).

The UPR, a stress response program that is also highly compartmentalized, couples translation to the protein folding status of the endoplasmic reticulum (ER) (Walter and Ron, 2011; Wang and Kaufman, 2012). There are two general categories of cellular responses to the accumulation of unfolded proteins in the ER: i) a reduction in the protein folding load in the ER and ii) increased ER protein folding capacity. Mechanisms identified to date that reduce protein folding load include a general suppression of translational activity through phosphorylation of eIF2α (Prostko et al., 1993), retrotranslocation and degradation of translocated proteins (Vembar and Brodsky, 2008), and degradation of ER-associated mRNAs (Maurel et al., 2014). Protein folding capacity is enhanced by increased synthesis of ER chaperones (Lee et al., 2003) and expansion of ER volume (Schuck et al., 2009). Because the UPR is activated in response to a highly compartmentalized stress (only ER protein folding is perturbed), we hypothesized that the UPR may take advantage of the intricate compartmentalization of mRNAs and ribosomes between the cytosol and ER as a means to reduce protein flux into the ER.

Here, we report that the UPR drives a large-scale re-compartmentalization of translation between the cytosol and ER, where mRNAs that encode membrane and secreted proteins are rapidly released from the ER upon induction of stress. The specific release of this class of mRNAs serves as a significant component of the transient reduction of protein flux into the ER, providing a potent means to support the restoration of ER proteostasis. These findings demonstrate that the dynamic re-localization of mRNAs and translation between the cytosol and ER can serve as a rapid and selective means of translational regulation during cell stress and recovery.

RESULTS

Translational profiling in the UPR

We treated mouse embryonic fibroblasts (MEFs) with 1μM thapsigargin (Tg), which elicits ER protein folding stress by inhibition of the ER Ca^{2+} pump SERCA (Thastrup et al., 1990), and used cell fractionation and ribosome footprinting approaches to assess how UPR activation reprograms the dynamics and subcellular organization of mRNA translation (Ingolia et al., 2009; Jagannathan et al., 2011). A 4h time course of Tg treatment was investigated and a characterization of UPR pathway activation status over this time course is depicted in Fig. 1. Tg elicited an increase in eIF2α phosphorylation, peaking at 0.5–1h of treatment, which subsequently declined to a new steady state (Fig. 1A). The UPR-elicited increase in eIF2α phosphorylation coincided with enhanced autophosphorylation and activation of the eIF2α kinase PERK (Fig. 1A) (Su et al., 2008). Intriguingly, phospho-eIF2α levels declined even as phospho-PERK levels remained elevated, pointing towards increased eIF2α-directed phosphatase activity (Fig. 1A). CHOP and ATF4, two UPR-related transcription factors whose transcription and translation are enhanced under conditions of elevated eIF2α phosphorylation, displayed elevated levels, though at time points where phospho-eIF2α levels had declined. Total translational activity as measured by [^{35}S]Met/Cys incorporation was reduced by ~50% at 0.5h, then recovered, on a time course distinct from eIF2α phosphorylation, to a new steady state (Fig. 1B). The recovery of protein synthesis occurred despite the ongoing presence of Tg and continued PERK activation (Fig. 1A). Having determined that this treatment period encompasses the detection, response, and adaptation to unfolded protein accumulation, we treated MEFs with Tg, isolated cytosolic and ER-associated polyribosomes by sequential detergent extraction, and analyzed translational status by ribosome profiling to provide a transcriptome-wide, quantitative, nucleotide-resolution snapshot of ribosome position and density in each cellular compartment (Ingolia et al., 2009; Reid and Nicchitta, 2012). To determine the subcellular localization of mRNAs, we purified mRNA from each compartment for analysis by RNA-Seq. Combined, these data define the subcellular localization of each mRNA, of its translation, and its ribosome loading density over time.

To assess Tg-induced changes in translation, we plotted the positions of ribosomes relative to the start codon across all mRNAs. In untreated cells (t = 0h), ribosomes were distributed in coding sequences with a standard three-nucleotide periodicity with two exceptions: a large peak at the start codon and a large peak at 13 nt, each of which has been observed previously (Fig. 1C) (Liu et al., 2013). Upon induction of the UPR, the pattern of ribosome positions on mRNAs changed such that ribosomes were clustered heavily in the first 50nt of the coding sequence. A similar profile was observed in treatments with non-natural amino acids, indicating that this translational response, which is consistent with an inhibition of the elongation stage of translation, may be a general feature of proteotoxic stresses (Liu et al., 2013).

We quantified the translational status of mRNAs in control and UPR-activated cells by counting the length-normalized number of ribosome footprinting reads in each transcript's coding sequence. This metric was highly reproducible across experimental replicates and

serves as a strong proxy for translation status (Fig. S1A, S1B). Each time point of Tg treatment induced a translational response that diverged widely from experimental error (Fig. S1C, Table S1). The translational response to Tg changed significantly over time; following a large change in translation after 0.5h treatment, the response continued to evolve such that at 4 h it had little resemblance to that at 0. h (Fig. 1D, Fig. S1D). Each time point resembled those close to it, while diverging substantially from more distant time points, indicating that the UPR comprises a multiphasic program with distinct stages. These changes in total translation can be explained by two variables: the concentration of a particular mRNA and the efficiency of translation (ribosome loading) of that mRNA. To determine which of these variables is the primary driver of UPR-induced translational remodeling, we calculated the degree to which ribosome loading density and mRNA levels are modified as the UPR progresses. At early time points, changes in translation were determined mostly by changes in translational efficiency, while mRNA levels were only modestly altered (Fig. 1E). At later time points however, the UPR had shifted significantly in character, becoming equally reliant on mRNA levels and translational changes. We therefore divided the UPR-induced translational remodeling into three categories: down-regulated genes (reduced translation throughout Tg treatment), primarily translation-dependent early response (enhanced translation at the 0.5h time point), and relatively mRNA-dependent late response (translation induced at 4h relative to untreated and 0.5h).

In each of these three categories, a number of genes were highly responsive to UPR activation, providing clues to how the UPR modifies cell functionality to adapt to unfolded proteins (Fig. 2). To determine which variables were most important for the regulation of each gene, we separately analyzed total translation, mRNA levels, and ribosome loading density over time. Intriguingly, many mRNAs that were translationally down-regulated encode membrane or secretory proteins, with 13% of mRNAs encoding ER-targeted proteins experiencing at least 50% reduction in translation after 30 min Tg treatment, compared to 4% of mRNAs encoding cytosolic proteins (median change of −8% for mRNAs encoding ER-targeted proteins vs. +4% for mRNAs encoding cytosolic proteins; $p<10^{-24}$ by Student's t-test). This, along with reduction in the translation of poly-A binding protein, likely contributes to decreasing the protein folding load in the ER. The translation of these mRNAs remained low even as overall translation recovered substantially, perhaps representing a portion of a new steady state established with ongoing Tg exposure. For the majority of down-regulated mRNAs, translational efficiency was substantially reduced, while mRNA levels were relatively unchanged, indicating that for this category of genes, the UPR-elicited reduction in expression relied primarily on translational regulation (Fig. 2, Fig. S2). Many mRNAs displayed enhanced translation following UPR induction, with 12% of mRNAs at least doubling their translation after 30 min Tg treatment. Of these transcripts, several encode previously identified UPR proteins such as GADD34 and ATF4. Several such mRNAs encode proteins that point towards previously unanticipated responses of cells to unfolded protein accumulation. Prominent among the mRNAs undergoing enhanced translation were those encoding histones; on average, their translation increased by ~300%. The role of their up-regulation in the UPR is currently unclear, although it may be relevant to the transcriptional component of the response. For those proteins whose synthesis was induced at early time points, their ongoing induction displays a biphasic character: at early

time points, enhanced translation drives the up-regulation, but by later time points, translational efficiency drops as mRNA levels begin to increase. This interplay between translation and transcription points towards an intricately timed system where translation can act rapidly at early time points, then give way as transcription has time to progress. By the 4h time point, a number of other mRNAs displayed enhanced translation that point towards distinct strategies to restore proteostasis. In contrast to early time points, mRNAs encoding proteins whose activities are associated with increasing protein folding capacity displayed enhanced translation. BiP translation increased more than four-fold, while GRP94 translation doubled. Simultaneously, Adamts6, which encodes a secreted peptidase, displays enhanced translation at late time points. This demonstrates that cells use distinct strategies in late stress periods relative to early periods: while protein chaperones did not display enhanced translation in early time points, their translation is substantially enhanced in later periods. For proteins whose synthesis was induced late in the time course, mRNA levels were generally increased, at times in tandem with ribosome density. In contrast to those early-induced proteins, however, mRNA levels were generally quite predictive of total protein synthesis. For each category of genes, these patterns were generalizable outside of the examples discussed above (Fig. S2). To provide an accessible framework for further analysis of the translational response to UPR activation, gene ontologies (GO) associated with each of the three response categories are provided in Table S2. Together, these changes in translation demonstrate the diverse strategies to restore proteostasis in the face of continued protein folding stress.

Release of mRNAs encoding ER-targeted proteins from the ER upon UPR induction

Seeing as the regulation of ER-targeted protein synthesis is of particular relevance in the UPR, we next focused on the regulation of the mRNAs encoding membrane and secreted proteins. To assess the relative contributions of mRNA concentration and ribosome loading to the regulation of ER-targeted protein synthesis, we plotted the changes in total mRNA levels, ribosome loading density, and total mRNA-associated ribosome levels over the Tg treatment time course (Fig. 3A). Total ribosome engagement with mRNAs encoding ER-targeted proteins decreased modestly after 0.5 hTg exposure. This decrease was driven primarily by changes in ribosome loading rather than mRNA levels. At longer periods of Tg treatment, mRNA levels and translation efficiency began to diverge, with mRNA levels increasing and ribosome loading density decreasing.

We next asked whether the distribution of translation between ER-bound and cytosolic ribosomes was modulated during stress. The translation of mRNAs encoding ER-targeted proteins was highly compartmentalized to ER-bound ribosomes in resting cells (Fig. 3B). This compartmentalization, however, was dramatically disrupted after 0.5h Tg treatment, dropping by around half, then recovering over the remainder of the time course. In contrast, the translation of mRNAs encoding cytosolic proteins was generally unchanged. These data indicate that UPR activation elicits a substantial release of signal sequence-encoding mRNAs from the ER. On average, translation of mRNAs encoding ER-targeted proteins decreased in ER localization by 34%. In contrast, there was only a 2% decrease in ER localization for translation of mRNAs encoding cytosolic proteins ($p < 10^{-25}$, Student's t-test). This divergence indicates that mRNAs encoding ER-targeted proteins are specifically

released from the ER. In parallel, there was a small release of ribosomes from the ER that mirrored the time course of mRNA release, likely representing those ribosomes that remained bound to released mRNAs (Fig. S3). When analyzing the subcellular localization trajectories of individual transcripts over time, we observed that for ribosomes bound to mRNAs encoding ER-targeted proteins, virtually all transcripts were re-compartmentalized to the cytosol at 0.5h, and mostly returned to the ER by 1h as the translational phase of the UPR transitioned into the transcription-driven phase (Fig. 3C). A similar shift was apparent was apparent for the mRNAs themselves, further demonstrating that mRNAs re-compartmentalize along with their translation in response to UPR activation (Fig. 3D). The compartmental shift of mRNAs and of their translation was well correlated during the initial re-compartmentalization (Fig. 3E). Because both the mRNA distribution and ribosome footprint reads transitioned in tandem, it appears that UPR activation elicits the release of mRNAs from the ER largely as polyribosomes. The slope of the best fit line, however, was less than 1 (0.67), indicating that on average, when a mRNA encoding an ER-targeted protein was released from the ER, 33% of its ribosomes lost mRNA association remained bound to the ER. This correlation broke down as the UPR shifted to its transcriptional phase. Furthermore, changes in the subcellular localization of mRNAs encoding ER-targeted proteins are not correlated with ribosome density after release, demonstrating that the effect we observe here is a specific, targeted stress response rather than a byproduct of translational down-regulation (Fig. 3F). Ultimately, the selective release of mRNAs encoding ER-targeted proteins represents an unanticipated yet primary stress response mechanism for decreasing ER protein folding load.

Despite the large-scale re-compartmentalization of mRNAs to the cytosol, a small subset of mRNAs encoding ER-targeted proteins gained or retained ER localization. Those mRNAs that remained on the ER during this early restructuring of the transcriptome encode proteins that likely contribute either to the decrease in protein folding load or to an increase in protein folding capacity of the ER – generally, functions essential to the recovery of homeostasis in the ER. Several ER-retained mRNAs encode proteins that degrade or remove proteins from the ER; genes involved in proteolysis, exocytosis, and ER-associated degradation were all represented (Fig. 3G). Notable was the substantial retention of the syntaxin mRNAs and other mRNAs whose protein products are involved in protein export from the ER (Teng et al., 2001). In principle, the sequestration of unfolded proteins into transport vesicles could provide an important means to decrease protein folding load, and there is precedent for such activity in bacterial unfolded protein stress (Schwechheimer and Kuehn, 2013). mRNAs encoding proteins associated with ER proteolysis, including Rhbdf1 and Edem2, were also mostly retained on the ER. Conversely, mRNAs encoding ER lumenal chaperones were released from the ER similarly to most mRNAs encoding ER-targeted proteins; mRNAs encoding GRP94 (−43%), BiP (−38%), and calreticulin (−44%) were all efficiently released. These data indicate that the early portion of the UPR focuses primarily on reducing protein folding load rather than increasing in protein folding capacity. Also retained on the ER was the mRNA encoding SCAP, which promotes sterol synthesis, perhaps to allow for expansion of ER volume. Gene ontology analysis revealed that mRNAs encoding components of the SNARE complex were the most effectively retained, pointing towards their central role in unfolded protein recovery (Fig. 3H). Several GOs were

associated with ion homeostasis, specifically calcium (Table S3). Seeing as Tg specifically disrupts calcium influx into the ER, this observation solidifies the concept that mRNAs that are retained on the ER are selected by virtue of their importance for recovery of homeostasis. Together, these features define new regulatory approaches used by cells to rapidly adapt to unfolded proteins in the ER and demonstrate the specificity of this re-compartmentalization of translation.

Kinetics of translational reorganization and recovery

To assess the short-term kinetics of stress-induced mRNA re-compartmentalization, we used an alternative stressor, DTT, which prevents disulfide bond formation in the ER (Braakman et al., 1992). DTT is a rapidly reversible stressor, allowing for washout studies that can provide insight into the recovery from the UPR (Rubio et al., 2011). In these experiments, we first treated MEFs with DTT for time periods of 2–30min. Following DTT addition, total translational activity initially dropped sharply and continued to decline throughout the time course of the experiment (Fig. 4A). Elevated levels of phospho-eIF2α could be detected by 10 min of DTT treatment (Fig. 4B). Over this time course, we determined the localization of mRNA encoding the ER-targeted protein GRP94 (*Hsp90b1*) and of the mRNA encoding the cytosolic protein GAPDH by qPCR (Fig. 4C). Following treatment with DTT, *Hsp90b1* mRNA re-compartmentalization to the cytosol was discernible by 10min and continued at a rate of ~1% per min. In contrast, the steady state levels of ER-associated *Gapdh* mRNA remained unchanged. These results demonstrate that re-localization is a selective and rapid means of post-transcriptional gene regulation.

Having established that UPR-induced mRNA re-localization is rapid, we next assessed the kinetics of recovery following release of the stress. Here, we treated cells for 30min with DTT, switched to fresh media lacking DTT, incubated the cells for 10min or 20min, and then analyzed mRNA translational status in each compartment by ribosome profiling. Ribosomes were modestly released from the ER upon treatment, then began to return within 20min (Fig. 4D). The release of translation of ER-targeted mRNAs was recapitulated after 30min of DTT treatment at a transcriptome scale, with the average mRNA encoding an ER-targeted protein dropping from ~90% translation on the ER to ~40% (Fig. 4E). Following DTT washout, mRNA localization recovered rapidly, with recovery beginning by 10min and accelerating at 20min. Together, these results demonstrate that both the release and retrieval of mRNAs encoding ER-targeted proteins occurs within minutes of UPR induction.

Also notable was the rapid recovery of gene-level translational efficiency upon the release of stress. Canonical targets for UPR-induced translational up-regulation had significantly enhanced translation after DTT treatment, and this enhancement dropped off rapidly after washout of DTT (Fig. 4F). This pattern was also apparent at a transcriptome scale. Upon induction of stress with DTT, both ER and cytosolic translational efficiency deviated prominently from experimental noise (Fig. 4G). Following DTT washout, each compartment rapidly began to return to the translational state of untreated cells, with about half of the deviation absent after 10min and continuing to decrease after 20min. This deviation had similar kinetics in each compartment. Additionally, these short time points allowed for the identification of targets of translational regulation in the UPR – in this time scale, translation

would be the dominant factor in gene regulation. We scored genes based on how closely they follow a pattern of translational up-regulation in the UPR and rapid recovery following release of stress (Fig. S4A). Rapidly responsive genes clustered into several functional categories, with ER-targeted proteins largely down-regulated and several regulatory functions up-regulated (Fig. S4B). Curiously, although the presence of upstream open reading frames have been characterized as leading to enhanced translation in the UPR for several genes (Lu et al., 2004), we found only a weak correlation between these gene elements and translational changes in the UPR (Fig. S4C, S4D), suggesting that factors in addition to uORFs participate in the translation regulation of these mRNAs. A complete listing of gene-level correlations and gene ontology enrichments are provided in Table S4. Together, these results reveal a rapid, stress-responsive translational regulatory system that operates by a mechanism largely independent of upstream open reading frames.

Translational activity of released mRNAs

Upon release into the cytosol, mRNAs encoding ER-targeted proteins could be translationally repressed or they could continue to be translated. This distinction carries important ramifications for the nature of the UPR – the synthesis of membrane proteins in the cytosol, for example, could result in the accumulation of protein aggregates. To address the translational state of cytosol-relocalized mRNAs, we first compared the distribution of ribosomes along mRNAs encoding ER-targeted proteins to ribosome position along mRNAs encoding cytosolic proteins. In untreated cells, both classes of mRNAs displayed ribosome positioning patterns indicative of robust translation, with high and consistent density in the coding sequence (Fig. 5A, top panels). The total density of ribosomes, however, was substantially lower for mRNAs encoding ER-targeted proteins in the cytosol than on the ER (Fig. 5B), demonstrating that while ribosomes can associate with these mRNAs in the cytosol, they do so at low levels. Upon treatment with Tg, ribosome positioning changed substantially for mRNAs encoding both cytosolic and ER-targeted proteins, each displaying a large buildup of ribosomes in the first 50nt (Fig. 5A, bottom panels). However, the lack of any clear differences between mRNAs encoding ER-targeted and cytosolic proteins argues against a specific translational suppression of mRNAs encoding ER-targeted proteins in the cytosol. Furthermore, the difference in density of ribosomes on mRNAs encoding ER-targeted proteins between the cytosol and ER disappeared upon Tg treatment, likely representing mRNAs released from the ER along with their associated ribosomes (Fig. 5B). Together, these data indicate that mRNAs encoding ER-targeted proteins are suitable substrates for translation in the cytosol and that these mRNAs are not specifically translationally repressed after their release from the ER.

To directly test whether mRNAs encoding ER-targeted proteins are translated, we treated cells with Tg, then added the translation initiation inhibitor pactamycin (150nM) for the final 10 min. The use of a translation initiation inhibitor allowed us to distinguish between mRNAs undergoing continued translation from mRNAs that were present in translationally-stalled polysomes. Cells were fractionated to obtain cytosolic and ER polysomes and the fractions analyzed by sucrose gradient density sedimentation. Assessing the location of an mRNA in the gradient allows for identification of translational runoff, where movement of mRNA from polyribosomes to monosomes or non-ribosome associated is indicative of

translational activity. Gradient profiles from the cytosolic fractions are depicted in Fig. 6A–D, with the paired membrane fractions in Fig. S5A. In cells not treated with Tg, *Bactin* mRNA, which encodes the cytosolic β-actin protein, was primarily associated with heavy polysomes (Fig. 6A) and collapsed to associate with the 80S ribosome peak following pactamycin treatment (Fig. 6B), indicative of robust translation. For *Hspa5* and *Hsp90b1*, both of which encode ER luminal proteins (BiP and GRP94, respectively), the small fraction of mRNA that was present in the cytosol was mostly associated with the 80S ribosome peak even before pactamycin treatment (Fig. 6A). Upon treatment with Tg, there was a large increase in *Hspa5* and *Hsp90b1* associated with polysomes in the cytosol, likely representing those mRNAs that were released from the ER membrane (Fig. 6C). When pactamycin was included for the final 10min of Tg treatment, both of these mRNAs lost polysome association to a large degree, although a substantial amount of each remained associated with small polyribosomes (Fig. 6D). These data indicate that released mRNAs display divergent fates: some are translationally stalled, while others actively engage in translation.

While some released mRNAs encoding ER-targeted proteins had translational activity in the cytosol, there was a substantial subset not sensitive to pactamycin. To assess the translational status of these mRNAs, we next asked whether their associated ribosomes retain nascent proteins that were in the process of translocation into the ER at the time of release. To test this, we pulse labeled cells with [^{35}S]Met/Cys, then chased with cold amino acids for 30 min in the presence or absence of 1μM Tg. At the end of the chase, labeling was halted with cycloheximide and cells fractionated. Nascent protein chains that were in the ER during the pulse labeling period were detected by purifying ribosomes by ultracentrifugation, then using ConA beads to purify the nascent glycoprotein fraction, noting that the high mannose oligosaccharide core is added co-translationally and uniquely in the ER (Ruiz-Canada et al., 2009). Glycosylated nascent chains from each cell fraction were eluted with 500mM α-D-mannose and quantified by liquid scintillation counting. In experiments with a pulse but no subsequent chase, glycosylated nascent chains were detected almost exclusively in the ER fraction (Fig. 6E). As a control, treatment with EndoH prior to ConA addition abolished the nascent chain signal (Fig. S5B). Following a 30min chase with non-radioactive amino acids, most of the nascent chains present during the pulse had run off, indicating that their synthesis had been completed and ribosome association lost. When 1μM Tg was included in the chase, there was a ~125% increase in glycosylated nascent chains detected in the cytosol (p<0.004; Student's t-test). This increase corresponds to nascent chains that were present in the ER lumen and glycosylated at the beginning of Tg treatment and were subsequently released into the cytosol. If we assume that the ER signal at the beginning of the chase represents the entirety of nascent ER proteins, the signal in the cytosol indicates that ~20% of all ER nascent chains were present in the cytosol after 30 min Tg treatment. These ~20% of nascent chains were present on the roughly half of all ribosomes associated with ER-targeted proteins that were release, indicating that ~40% of ribosomes released from the ER retain nascent protein chains. These data demonstrate that while some released mRNAs are translated, other released mRNAs retain nascent chains in a translationally stalled state. The release of these nascent chains from the ER likely involves the retrotranslocation machinery (Tsai et al., 2002).

DISCUSSION

The UPR develops in response to a highly compartmentalized stress and couples proteostasis in the ER lumen to translational regulation of the transcriptome. Here, we have demonstrated that the subcellular architecture of translation is remodeled in a manner that is well suited for this stress response process. In order to stem the influx of unfolded proteins into the ER, the mRNAs that encode ER-targeted proteins are selectively released from the ER membrane. Simultaneously, the translation of mRNAs encoding cytosolic proteins is largely unchanged, demonstrating that the UPR-induced release of ER-associated mRNAs is specific to those encoding particular proteins, rather than a general effect. By releasing mRNAs encoding ER-targeted proteins into the cytosol, cells can specifically reduce the flux of proteins into the ER while retaining general translational activity on the ER. These findings define mRNA and ribosome localization to the ER as a new variable in translational regulation that is relevant to virtually all transcripts and is dynamic in response to stimuli.

It is useful to consider the impact of this re-localization of translation on protein flux into the ER relative to previously identified mechanisms. At the peak of translational suppression after 0.5h Tg treatment, these mechanisms include general suppression of translational activity (~50%, Fig. 1B), reduction in levels of mRNAs encoding ER-targeted proteins (~5%, Fig. 3A), and reorganization of ribosome from mRNAs encoding ER-targeted proteins to those encoding cytosolic proteins (~15%, Fig. 3A). When including effects of translation re-localization which we have identified here (~50%, Fig. 3B), these factors combine to result in an approximately 80% reduction in protein flux into the ER – a reduction that affords the ER an opportunity to restore proteostasis. In addition to contributing substantially to the reduction in protein flux into the ER, UPR-induced translational re-localization functions on a time scale that is more rapid than other mRNA-specific mechanisms analyzed. While re-localization is effective within ~20 min (Fig. 4C), the impact of ribosome loading comprises a small component of the translational response at early timepoints and grows over time (Fig. 3A). mRNA levels do not change markedly until the late 4h time point (Fig. 3A). These observations distinguish mRNA relocalization as a uniquely fast form of gene regulation, pointing towards its potential utility in other sorts of cell stimuli that require a rapid response.

Dynamic association of polyribosomes with the ER

The observations discussed above raise a number of questions regarding the mechanisms by which polyribosomes associate with the ER and how these might change in order to confer stress-elicited polyribosome release. There are three general classes of interactions between the ER and polyribosomes, each of which is likely conferred by several molecular mechanisms (Fig. 7A). First, nascent protein chains can provide affinity for the ER (Kalies et al., 1994). Second, ribosomes can associate with the ER independently of translation and can initiate translation while maintaining ER association (Borgese et al., 1973; Gorlich et al., 1992; Hortsch et al., 1986; Potter and Nicchitta, 2000; Savitz and Meyer, 1990). Finally, mRNAs themselves can retain ER association independently of ribosomes (Adesnik et al., 1976; Chen et al., 2011; Pyhtila et al., 2008). Each of these interactions must be absent or disrupted to allow for the release of a polyribosome. Alternatively, a ribosome may

dissociate from an mRNA, allowing for release of the mRNA while retaining its ER association. Within this framework, it is useful to consider how changes in each of these variables could give rise to the observed selective release of mRNAs encoding ER-targeted proteins.

During homeostasis, polyribosomes synthesizing ER-targeted proteins are anchored to the ER by at least two of the three mechanisms: by their nascent protein chain, by their ribosomes and/or by their mRNAs (Chen et al., 2011). Polyribosomes synthesizing cytosolic proteins, in contrast, do not have affinity conferred by their nascent proteins and their mRNAs are generally not tethered to the ER. These polyribosomes, therefore, must be anchored to the ER by ribosomes, which have their own independent ER affinity (Borgese et al., 1973). It follows that selective release of mRNAs encoding ER-targeted proteins could be achieved by disrupting ER:nascent chain and ER:mRNA interactions and necessarily a subset of the ER:ribosome interactions (Fig. 7B). mRNAs that have at least one ribosome stably associated with the ER would retain their ER association, allowing for the retention of mRNAs encoding cytosolic proteins and a fraction of mRNAs encoding ER-targeted proteins (Fig. 7C). Specific mRNAs, such as those whose translation products are important for homeostasis recovery in the ER lumen, could also be retained on the ER by association with ER resident RNA-binding proteins. Together, these variables provide a framework for understanding the dynamics of polyribosome association with the ER. Indeed, some aspects of this system have already been described. ER:nascent chain interactions have been shown to be disrupted in the UPR in a system where the proteasome degrades nascent ER proteins cotranslationally (Oyadomari et al., 2006). A diversity of ribosome receptors has been identified and the data provided here suggest that multiple and regulated mechanisms contribute to ribosome-ER interactions (Kalies et al., 1994; Kreibich et al., 1978; Savitz and Meyer, 1990). This finding also highlight the need for a better understanding of the entirety of the mechanisms that regulate ribosome association with the ER and how these interactions might be dynamically regulated. In the same vein, our understanding of mRNA tethering to the ER is quite limited, and the identification and functional characterization of mRNA receptors and their dynamics is in need of further study (Blower, 2013; Cui and Palazzo, 2014)

Points of regulation for mRNA compartmentalization

We have shown here that the UPR-regulated re-compartmentalization of mRNA and of translational activity can serve as a rapid means by which specific cohorts of mRNAs can be targeted for substantial re-programming of protein synthesis. These findings raise the possibility that the response we observe here, with specific mRNAs being re-compartmentalized, may in fact be generalizable to a number of cellular responses. In this context, the ER membrane can serve as a fundamentally distinct compartment for protein synthesis, with ribosomes, mRNAs, and tRNA synthetases all tethered by their own independent means (Chen et al., 2011; Dang et al., 1983). This subcellular localization may serve as a precise means of translational control – another instance of an emerging understanding that biochemical processes within the cell are carefully organized even when not separated by membranes (Campanella et al., 2005). We envision a system where the localization of each component of the translational apparatus can be recruited or removed

from the ER as a cell grows, divides, differentiates, and responds to external stimuli. Indeed, cells differ widely in their ER enrichment of ribosomes, from professional secretory cells where virtually all ribosomes are ER-bound to leukocytes where the ER is barely detectable (Palade, 1956). We propose that the localization of mRNAs, ribosomes, and regulatory factors to the ER comprises a dynamic system that can be a critical aspect of translational regulation.

EXPERIMENTAL PROCEDURES

Full descriptions of experimental procedures and gene-level sequencing data are included in the Extended Experimental Procedures and in Tables S1, S5, and S6.

Cell culture, treatment, and fractionation

Cells were cultured in DMEM + 10% FBS at 37° in 5% CO_2 and were harvested at ~80% confluence. Cells used for all experiments were wild type SV40-immortalized mouse embryonic fibroblasts. Cell fractionation was carried out according to (Jagannathan et al., 2011). Cell fractionation was carried out according to (Jagannathan et al., 2011). Cells were treated with 180μM cycloheximide, washed with cold PBS, and the plasma membrane permeablized by addition of a cytosol buffer consisting of 110mM KOAc, 25mM K-HEPES pH 7.5, 15mM $MgCl_2$, 4mM $CaCl_2$, 0.03% digitonin. Digitonin-permeabilized cells were then washed in the cytosol buffer with 0.015% digitonin. The ER was solubilized in 200mM KOAc, 25mM K-HEPES pH 7.5, 15mM $MgCl_2$, 4mM $CaCl_2$, 2% dodecylmaltoside. Where indicated, cells were treated with 1μM thapsigargin (Calbiochem) or 1mM DTT (Sigma-Aldrich).

Radioactive labeling and ribosome counting

Cells were starved of Met/Cys for 30min by incubation in Met/Cys-free DMEM. Cells then were pulsed with 50μCi/mL [^{35}S]Met/Cys for 5min, followed by addition of 180μM cycloheximide to halt labeling. Cells were lysed in 1% CHAPSO, 200mM KOAC, 15mM K-HEPES pH 7.2. Trichloroacetic acid was added to 10%, incubated for 20 min on ice, and precipitated proteins collected on glass fiber filters by vacuum filtration. Filters were rinsed thoroughly with 10% TCA + 10mM Met, EtOH, and air dryed. Filter-associated radioactivity was determined by liquid scintillation counting.

Ribosome quantification

Cells were fractionated and ribosomes pelleted through a 500mM sucrose cushion (90,000 rpm, 30min, TLA 100.2 rotor). The ribosome pellet was resuspended and RNA purified by GT/phenol extraction. The concentration of ribosomes was then determined by UV absorbance at 260nm, using a U-2000 spectrophotometer (Hitachi).

Immunoblotting

Treated cells were lysed in 1% CHAPSO, 200mM KOAC, 15mM KHEPES pH 7.2, precipitated with trichloroacetic acid, separated by SDS-PAGE, transferred to nitrocellulose, and processed by standard procedures. Antisera used are in Table S5. All lanes were loaded as cell equivalents.

Ribosome profiling and mRNA-seq

Ribosome profiling was performed by digesting subcellular fractions with micrococcal nuclease, pelleting ribosomes, and purifying the ribosome-protected RNA footprint by gel electrophoresis. Deep sequencing libraries were prepared using the NEBNext Small RNA kit (New England Biolabs). For mRNA-seq, mRNAs were enriched by rRNA depletion, then fragmented for library preparation. All sequencing was performed on the Illumina HiSeq 2500. Experimental duplicates were performed for each time point.

Data analysis

Reads were mapped to a Refseq transcriptome using Bowtie (Langmead et al., 2009) and normalized by coding sequence length, library size, and experimentally determined ribosome quantities to give total translation and mRNA counts. Unless otherwise noted, error bars represent the SD and the medians of experimental replicates. For purposes of analyzing mRNAs encoding ER-target proteins, we defined ER-targeted proteins as those that contain either a signal sequence or transmembrane domains as determined by TMHMM (Krogh et al., 2001). Cytosolic proteins are defined as those proteins that do not contain topogenic signals.

Translational runoff

Cells were treated for 10min with 150nM pactamycin and elongation then halted by addition of 180μM cycloheximide. Cell fractions were prepared and analyzed on 20–55% sucrose gradients by centrifugation for 4h at 35,000 rpm in the SW41 rotor. Gradient fractions were collected as described above, total RNA extracted, and mRNAs quantified by qRT PCR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- The UPR encompasses a multistage translational response.
- mRNAs encoding ER-targeted proteins are released from the ER upon UPR induction.
- UPR-elicited mRNA release is rapid and reversible.
- Selective mRNA release is a mechanism for reducing protein flux into the ER

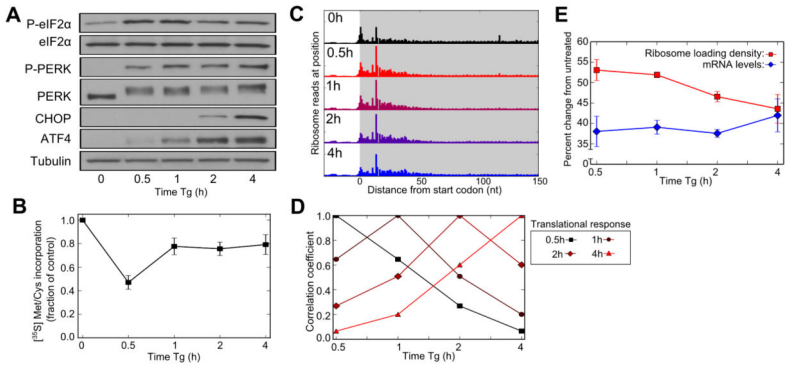


Figure 1. Disruption and recovery of translation in the UPR

(A) Immunoblots against UPR-associated proteins over a time course of Tg treatment **(B)** Time course of translational activity during Tg treatment as measured by [^{35}S]Met/Cys incorporation. Error bars represent SD (n=3) **(C)** Distribution of ribosome positions relative to the start codon at each experimental time point. **(D)** Translational response at each time point compared to translational responses at other time points. For each time point of Tg treatment, the $\log_2$ change in change translation was calculated for each mRNA. A correlation coefficient was then calculated between that time point and all other time points. **(E)** The median divergence (absolute value of change) from untreated cells in mRNA levels and in ribosome loading density (ribosomes per mRNA) was calculated for all genes and depicted over the treatment time course. See also Figure S1, Table S1.

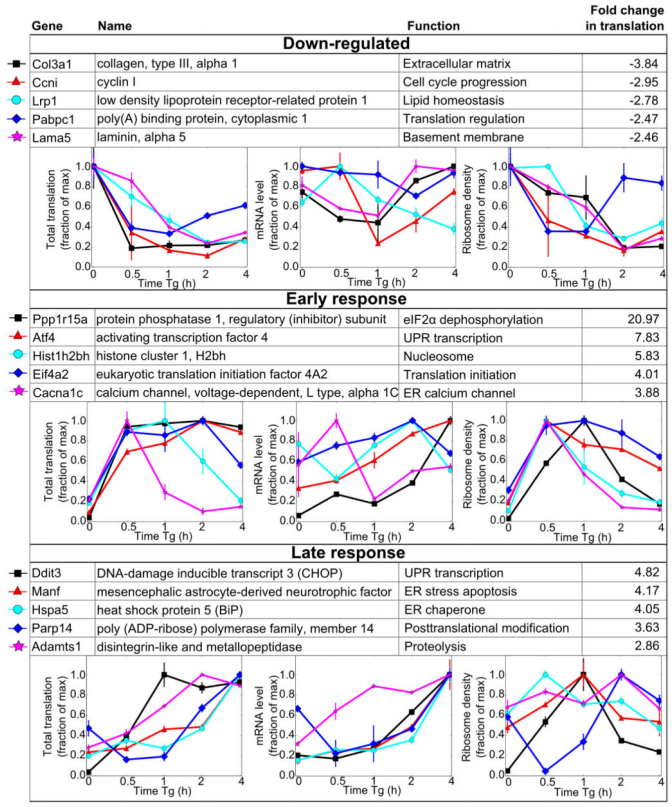
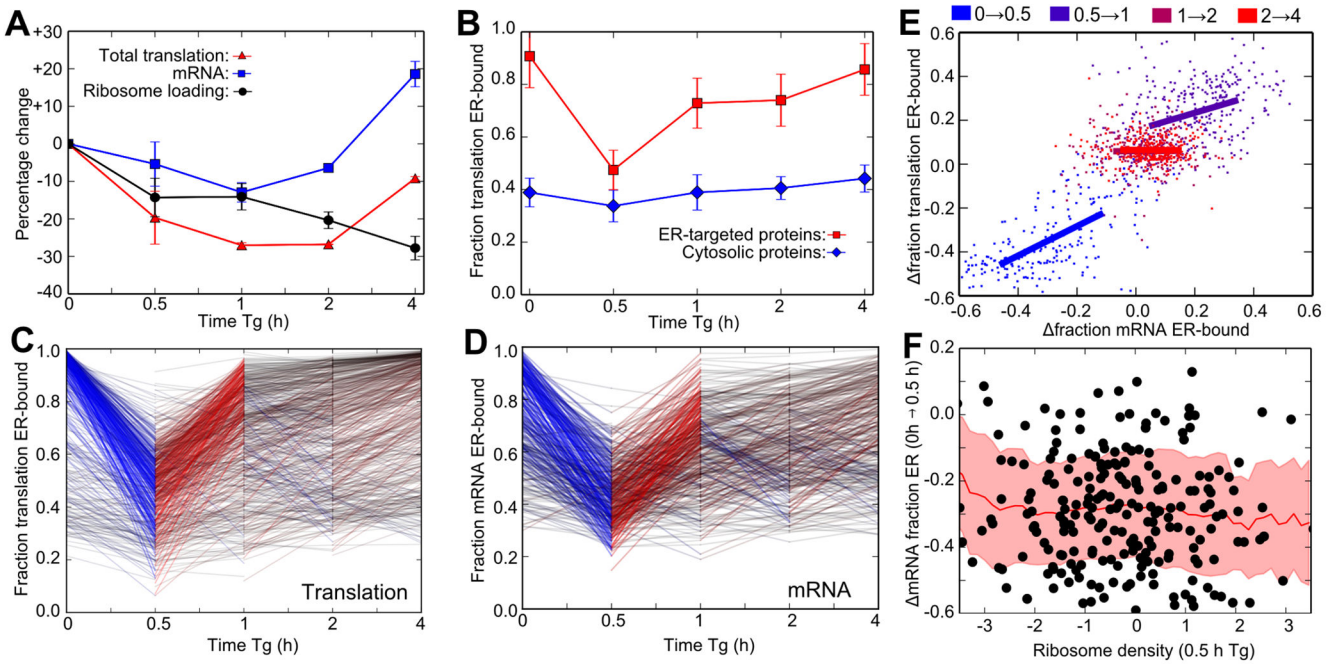


Figure 2. Divergent translation trajectories of UPR-responsive mRNAs

The total translation, mRNA levels, and ribosome density are plotted for mRNAs whose translation is altered in response to Tg treatment. Three categories are used to select mRNAs: down-regulated (average of translation at all Tg treatment times/0 h Tg), early response (translation at 0.5h Tg/0h Tg), and late response (translational at 4h Tg/average (0h Tg, 0.5h Tg)). For each mRNA, the fold change in translation during the given time is indicated. See also Figure S2, Table S2.



G

Gene	Name	Function	%ER(t=0)	%ER(t=0.5)	Change
Stx3	syntaxin 3	Exocytosis	44.7	79.5	+34.9
Rhbdf1	rhomboid family 1	Serine protease	92.2	87.1	-5.1
Scap	SREBF chaperone	Cholesterol homeostasis	93.6	87.4	-6.1
Edem2	ER degradation enhancer	Degradation of ER proteins	84.0	74.4	-9.6
Smo	smoothened	Cell signaling pathways	82.8	72.1	-10.6
Ppt2	palmitoyl-protein thioesterase	Removal of protein acyl chains	95.2	82.7	-12.6
Median	**(all ER-targeted proteins)**		92.0	47.1	-44.9

H

Ontology	Δtranslation %ER
SNARE complex	+1.3
regulation of membrane potential	-4.6
potassium channel activity	-9.3
calmodulin binding	-10.3
mitochondrial inner membrane	-12.3
intracellular protein transport	-14.1
Median (all gene ontologies)	-27.7

Figure 3. UPR activation elicits the release of mRNAs encoding ER-targeted proteins from the ER

(A) Changes in the mRNA levels, ribosome loading density, and total translation for mRNAs encoding ER-targeted proteins over time. **(B)** Subcellular localization of the translation of mRNAs encoding ER-targeted (red) proteins and cytosolic (blue) proteins over the treatment time course **(C)** Trajectories of the subcellular localization of the translation of all mRNAs encoding ER-targeted proteins over the treatment time course, where red indicates an increased ER localization, blue decreased. Each line represents the translation of an individual mRNA. **(D)** As in (C), depicting the subcellular localization of all mRNAs encoding ER-targeted proteins. **(E)** Relationships between the change in localization of translation and the change in localization of mRNAs encoding ER-targeted proteins for each time point transition. Best fit lines are depicted. **(F)** Relationship between the ribosome density (ribosomes per mRNA) after 0.5h Tg treatment and the change in mRNA localization at the same time point for mRNAs encoding ER-targeted proteins. Red line represents a moving average; shaded area is ± SD. **(G)** Selected mRNAs encoding ER-targeted proteins whose translation is retained or recruited to the ER following UPR induction. **(H)** Gene ontologies among mRNAs encoding ER-targeted proteins that are enriched for ER retention. See also Figure S3, Table S3.

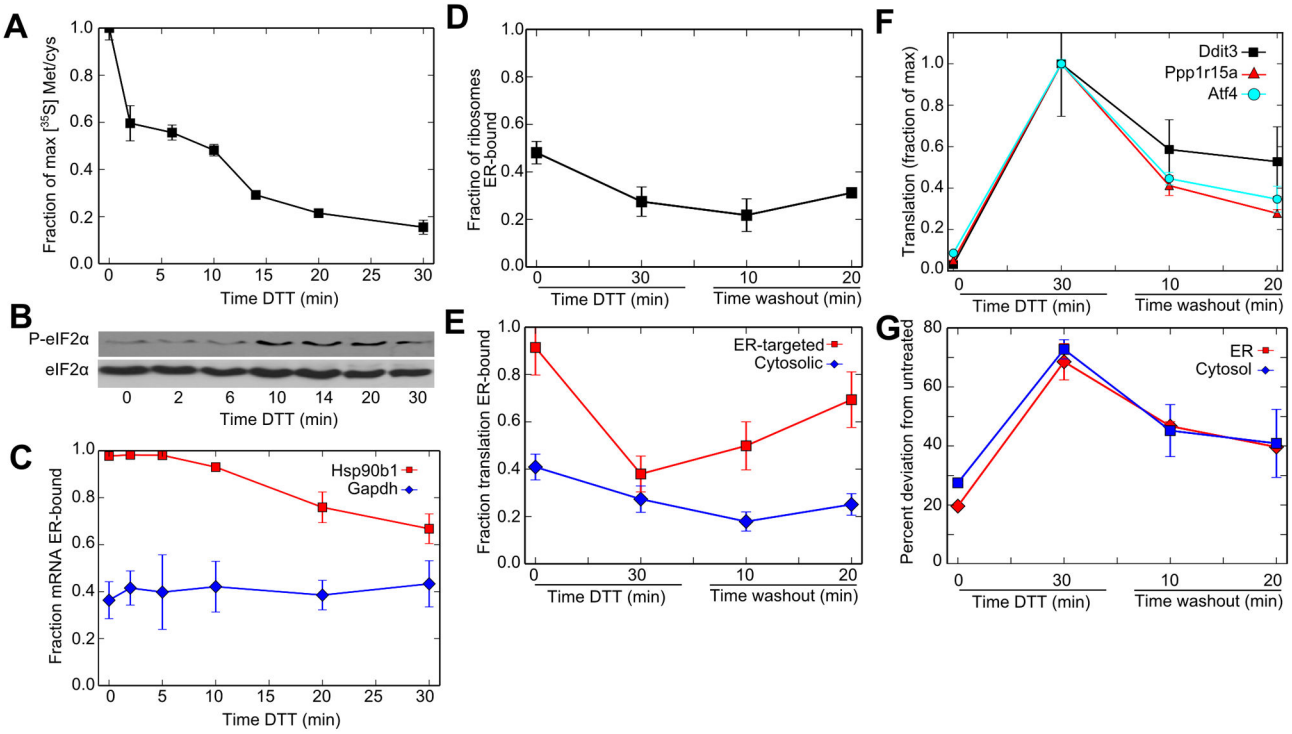


Figure 4. Kinetics of UPR-induced mRNA re-localization

(A) Translational activity as measured by [^{35}S]Met/Cys incorporation over a time course of treatment with 1mM DTT. Error bars represent SD (n=3). **(B)** Kinetics of eIF2α phosphorylation as assessed by immunoblot during DTT treatment. **(C)** Localization of an mRNA encoding a cytosolic protein (GAPDH) and an ER-targeted protein (Hsp90b1/GRP94) (n=3). **(D)** Fraction of ribosomes associated with the ER during DTT treatment and washout. Error bars represent SD (n=3). **(E)** Localization of the translation of ER-targeted proteins and cytosolic proteins as assessed by ribosome profiling during the induction and recovery from DTT treatment. Error bars represent SD (n=3). **(F)** Changes in gene-level translation for cytosolic and ER-associated ribosomes in UPR induction and recovery. The median value of the absolute $\log_2$ deviation in translation is calculated in each compartment, with error bars representing standard error between replicates. At the 0 time point, points represent the deviation in experimental replicates for Control samples. **(G)** Patterns of translational remodeling for canonical targets of UPR translational regulation. See also Figure S4, Table S2.

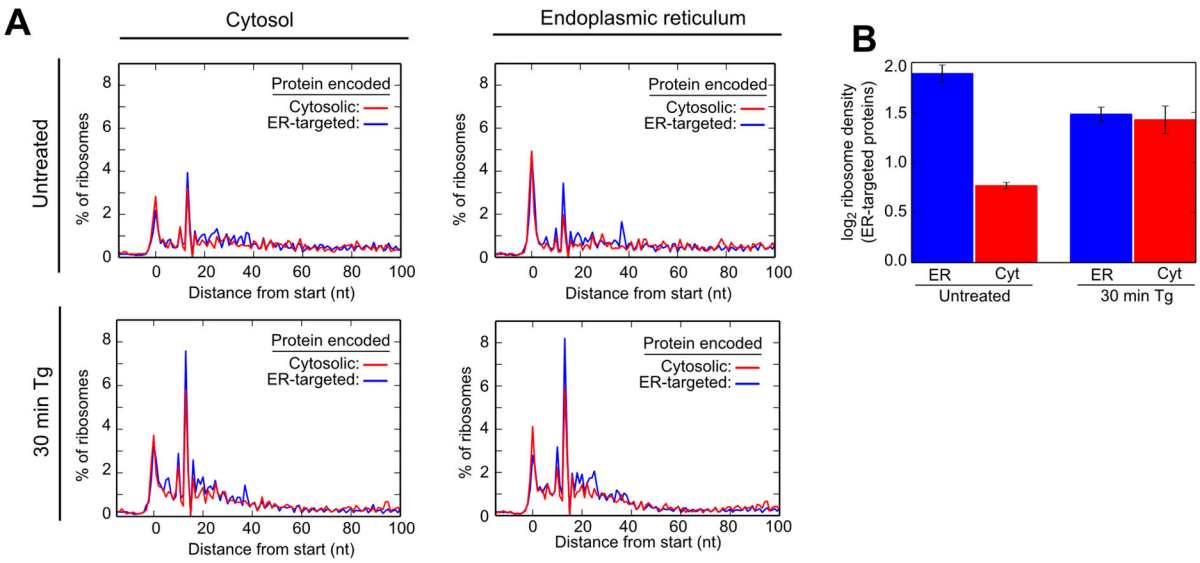


Figure 5. UPR induction effects on ribosome loading status for mRNAs encoding ER-targeted proteins

(A) The distribution of ribosomes along mRNAs encoding cytosolic (red) or ER-targeted (blue) in the cytosol and ER with or without 30min Tg treatment. **(B)** Ribosome loading of mRNAs encoding ER-targeted protein in the ER and cytosol with or without Tg treatment.

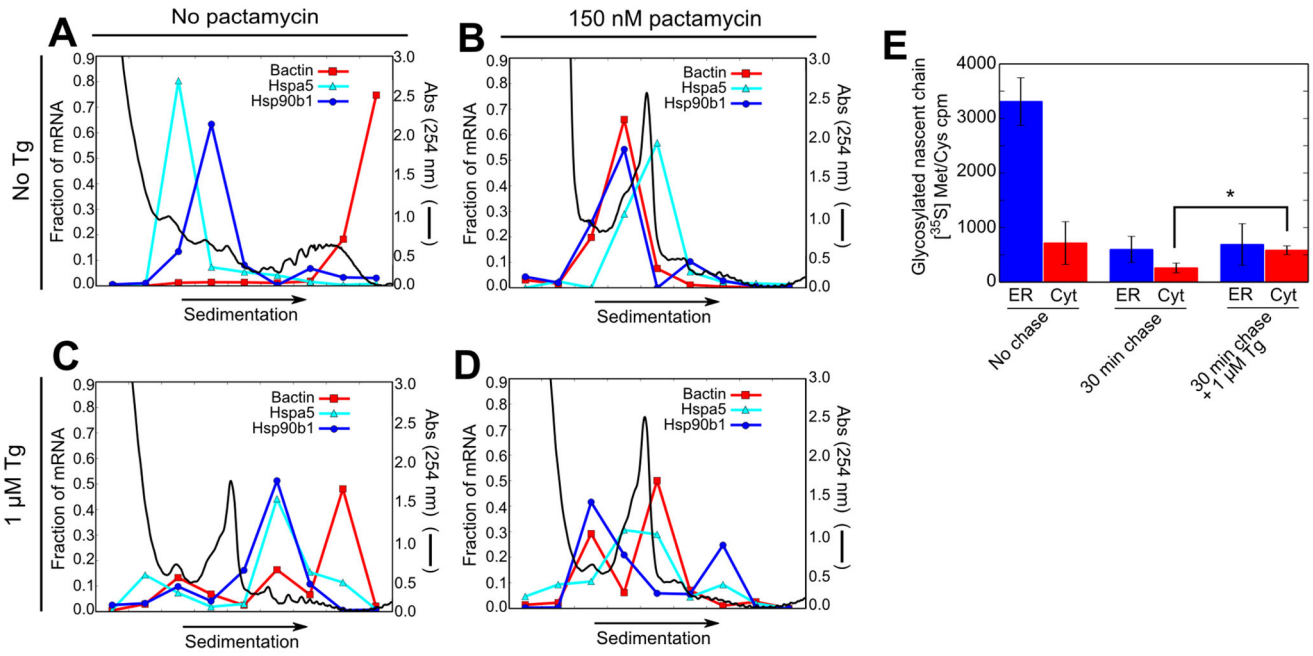


Figure 6. Divergent fates of mRNAs encoding ER-targeted proteins in the cytosol upon UPR induction

Cytosolic polyribosomes were analyzed by sucrose gradient centrifugation following **(A)** no treatment, **(B)** 10 min treatment with 150nM pactamycin, **(C)** 30 min treatment with 1μM Tg **(D)** 30 min Tg treatment with addition of 150nM pactamycin at 20 min. Gradient fractions were collected and analyzed for mRNA content by qPCR. **(E)** Radiolabelled, glycoslyated nascent polypeptide chains were quantified after a 10min pulse with [^{35}S]Met/Cys or after a 30min chase with DMSO or 1μM Tg (n=3). See also Figure S5.

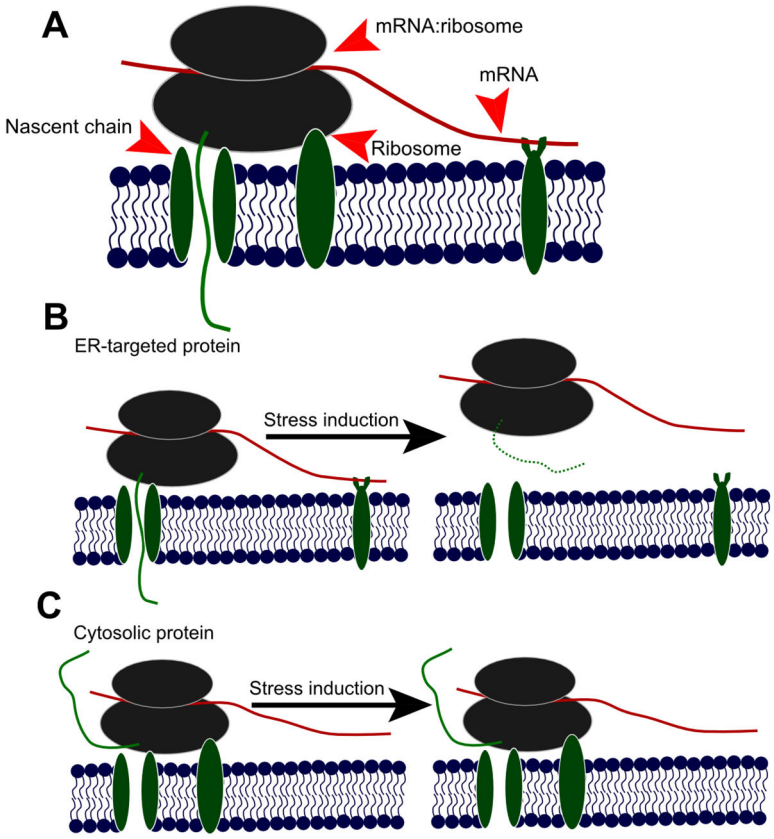


Figure 7. Model for dynamic mRNA localization to the ER

(A) Binding interactions that can confer polyribosome association with the ER are indicated by red arrowheads; red line is mRNA, green is nascent protein, black ellipses are ribosomal subunits. In addition to disruptions in mRNA, ribosome, and nascent chain interactions with the ER, an mRNA may also be released from the ER in the case where the mRNA dissociates from the ribosomes while the ribosomes itself retains ER affinity. **(B)** Model of the changes in binding interactions that occur for a ribosome engaged in the synthesis of an ER-targeted protein upon induction of ER stress. Here, ER:nascent chain and ER:mRNA interactions are disrupted, allowing for the release of the polyribosome. The dashed green line indicates a nascent polypeptide chain. **(C)** Same as (B) for a polyribosome synthesizing a cytosolic protein where the ribosome is independently associated with the ER. Having no nascent chain or mRNA interaction with the ER, the ribosome is retained on the ER due to the ongoing interaction with its ER binding partner(s).