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Cover Page Footnote

The authors are grateful to Mariappan members, Jacob Culver and Xia Li for timely advice, discussions, help with experiments, and comments on the manuscript. \Written for Crystal Adamchek's course MCDB 474: Independent Research.

Tail-Anchored Protein Insertion Under ER Stress Conditions: Calcium is Key

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ABSTRACT

Tail-anchored (TA) membrane proteins are critical for protein translocation, intracellular trafficking, and programmed cell death. TA proteins contain hydrophobic transmembrane domains and traverse through the cytosol to post-translationally insert into cellular membranes. It is unclear how this post-translational insertion is affected by Endoplasmic Reticulum (ER) stress. Here, we find that TA protein insertion is significantly reduced with ER stress inducer thapsigargin (Tg), a calcium pump inhibitor that blocks the import of calcium into the ER causing ER stress, but not when treated with other ER stress inducers. Interestingly, our data suggests that increased calcium in the cytosol may decrease TA protein insertion rather than ER stress. One potential mechanism for this is the calcium-dependent activation of chaperone, calmodulin, which acts like a trap for TA proteins under certain physiological conditions.

INTRODUCTION

Membrane proteins account for approximately 35% of the cellular proteome and are essential for a variety of cellular activities including cell-cell signaling, transport of nutrients and waste products across cells, capture of energy and transduction into usable form, and communication with the environment¹. A shared feature of membrane proteins is the highly hydrophobic transmembrane domain (TMD), which anchors the protein inside the lipid bilayer. Thus, a fundamental challenge in membrane protein insertion is the movement of these hydrophobic TMDs from the aqueous cytosol, where they are synthesized, to the lipid bilayer, where they are energetically most stable². The process requires selective TMD recognition, shielding of the hydrophobic TMD from the aqueous cytosol, targeting to the correct membrane surface, and integration of the TMD into the lipid bilayer in the correct orientation³. The solution couples protein synthesis and insertion at the ER, termed the co-translational protein insertion pathway⁴. The TMD of the membrane protein is co-translationally recognized and bound by the signal recognition particle (SRP) in the cytosol⁵. The SRP-bound ribosome nascent chain complex is then delivered to the ER-localized SRP receptor. The nascent chain is co-translationally integrated into the ER membrane via the Sec61 translocon pore⁶. Fidelity is maximized as protein synthesis and insertion of the hydrophobic TMD are coupled at the ER membrane, thereby minimizing exposure and aggregation of the TMD in the cytosol (Fig. 1a).

Tail-anchored (TA) membrane proteins are a special class of membrane proteins that are critical for protein translocation, intracellular trafficking, and programmed cell death. TA proteins are highlighted by a TMD on the C-terminus that dictates its membrane orientation and an N-terminal domain that provides functionality and faces the cytoplasm. Due to the topology of TA proteins—TMD on C-terminus—these membrane proteins are precluded from SRP binding⁷.

Protein synthesis occurs in the N-C direction. Since TA proteins contain a TMD on the C-terminus, SRP is unable to bind and pause translation because translation is terminated prior to the full emergence of the TMD from the ribosomal exit tunnel.

Targeting and insertion occur after complete synthesis of the membrane protein substrate. Additional factors shield the hydrophobic TMD of the TA protein and deliver it to the correct membrane. The basic paradigm of post-translational membrane insertion consists of cytosolic chaperones which mediate recognition, shielding and transport. These chaperones interact with a specific ER-localized receptor that dictates targeting and insertion. Genetic and biochemical research have identified cytosolic and membrane factors involved in this post-translational (GET/TRC) pathway¹¹. The GET/TRC pathway in yeast/mammals routes TA proteins, especially those with higher hydrophobicity, to the ER for insertion¹² (Fig. 1b).

Despite significant progress in elucidating the mechanism of TA protein trafficking, delivery, and insertion, it remains unclear how TA proteins are regulated under stress conditions¹. We believe that stress conditions affects TA protein synthesis since ER stress elicits a set of cellular responses which increases the ER machinery's capacity to fold and degrade proteins, while upregulating the production of chaperones that prevent protein misfolding in the cytosol¹⁷. Here, we find that TA protein insertion is significantly reduced with ER stress inducer thapsigargin (Tg) but not with other ER stress inducers.

¹ ER stress occurs when the capacity of the ER to fold becomes saturated, or when demand for protein folding cannot be met by the capacity of ER to fold proteins. A number of physiological and pathological conditions are able to perturb proper ER function and induce ER stress. Triggers for ER stress include intracellular alterations (calcium or redox imbalances), certain microenvironmental conditions (hypoxia, acidosis, hypoglycemia), natural compounds (thapsigargin, tunicamycin, dithiothreitol), or overexpression of or mutations in proteins that prevent proper folding.⁹

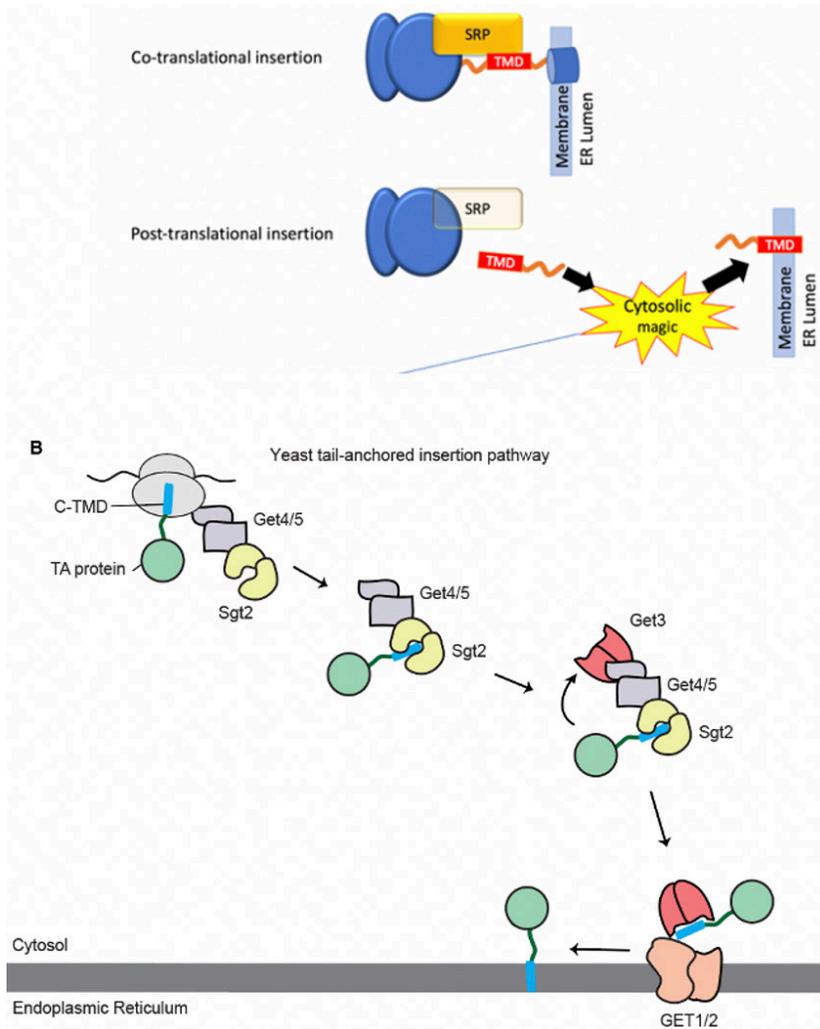


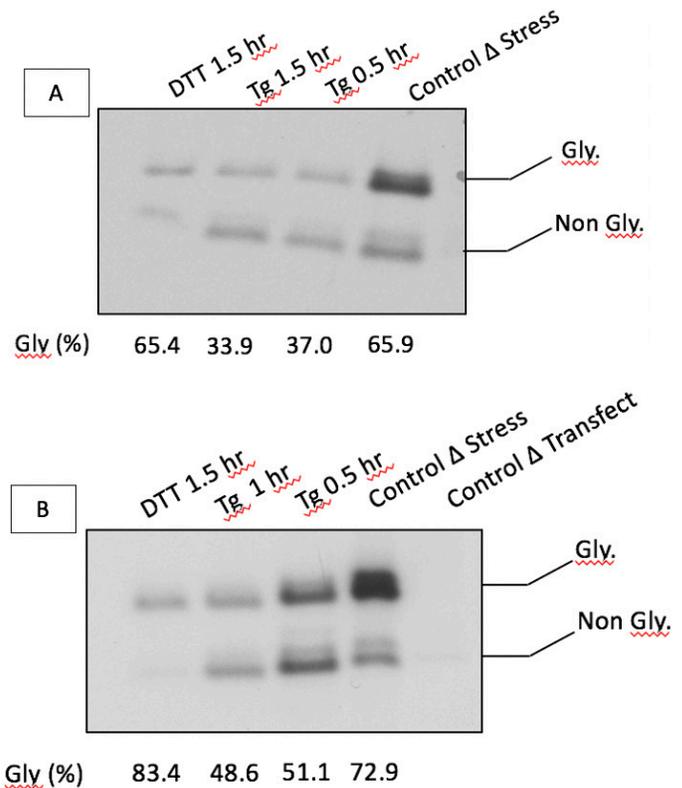
Figure 1a. Schematic diagrams of co- and post-translational protein insertion mechanisms.

Figure 1b. The additional factors needed to shield TA proteins shown in second image (GET/TRC pathway)¹²:

1. After the nascent TA peptide chain is synthesized and released from ribosome, it is captured by Sgt2 with the aid of Hsp70.
2. Sgt2 loaded with TA-protein binds to pre-targeting complex and transfers client to Get3, which is in its closed, ATP-bound form (tense state)
3. Cargo-loaded Get3 dissociates from pre-targeting complex
4. Cargo-loaded Get3 is captured—most likely after ATP hydrolysis—by Get2 at the ER membrane
5. Interaction of carbo-loaded Get3 with Get1 drives transition of Get3 to open state with the release of ADP (relaxed state) and insertion of TA TMD into bilayer
6. ATP binding and interaction with Get4/Get5 drive dissociation of Get3 from ER receptor and its recruitment to another cycle of substrate engagement¹²

Figure 1 drawn by Jacob Culver

Figure 2. (A + B) FLAG-tagged β Vamp2 was transfected into Hek293 cells and subject to ER-stress inducers: Tg (1 μ g/mL) and DTT (4 mM). The percentage of glycosylation (Gly) is a proxy for successful TA protein insertion into the ER, which is calculated by glycosylated protein/(glycosylated protein + non-glycosylated protein)*100. Intensity of bands were quantified in ImageJ



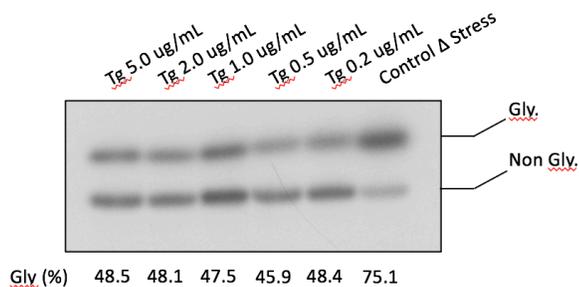


Figure 3. FLAG-tagged β Vamp2 was subject to ER-stress inducer, Tg (1 ug/mL), for 1 hour at different concentrations (0 ug/mL- \rightarrow 5 ug/mL).

RESULTS

The first question we wanted to address was how would TA protein insertion be affected under stress conditions? We hypothesized that it was not stress conditions, but rather changes in intracellular calcium that affected the TA protein insertion rate. To test this hypothesis, we compared ER stress inducer, Thapsigargin—a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase pump that increases cytosolic calcium concentration—to the ER stress inducer dithioltrithol (DTT) that do not affect calcium levels but interferes with the disulfide bond formation of proteins in the ER. First, we co-transfected HEK293 cells with FLAG-tagged Vamp2, an abundant TA protein involved in the targeting and fusion of transport vesicles to their target membranes. We then treated cells with ER stress inducers, Tg and DTT, across different time points (Figure 2). Following this treatment, we starved the cells with medium lacking methionine/cysteine (M/C), radiolabeled with 35S methionine, and harvested in RIPA buffer. The cell lysates were immunoprecipitated for β Vamp2 using anti-FLAG beads. The immunoprecipitated samples were separated on a SDS-PAGE gel and process for autoradiography. Since we appended a C-terminal glycosylation tag to the C-terminus of β Vamp2, the insertion into the ER can be monitored by its glycosylation from ER-localized N-glycosylation machinery (Gly %). The result showed that β Vamp2 glycosylation for Tg treated samples at all timepoints decreased in comparison to the control (no stress treatment), whereas the glycosylation for DTT samples were similar to controls (Figure 2A, B). Taken together, these results indicate that TA protein insertion, represented by the glycosylation band, was reduced following Tg treatment, but not DTT treatment.

We next asked whether the reduced TA protein insertion was dependent on Tg concentration. We hypothesized that TA protein insertion would decrease proportionally as Tg concentration increased. Our results show that the reduced TA protein insertion—decreased glycosylation-to-total protein ratio compared to the control—occurred across all Tg concentrations (Figure 3). However, we do not observe a proportional decrease in TA protein insertion with increasing Tg concentration. We will need to rerun this experiment

ⁱⁱ When referencing the “hydrophobicity of TA proteins” I am referring to the hydrophobicity of the TA proteins’ TMD which can be calculated through online protein sequencing databases¹⁶. Hydrophobicity was chosen as a variable to test different TA protein constructs since TMD hydrophobicity is an important factor that determines targeting and function.¹

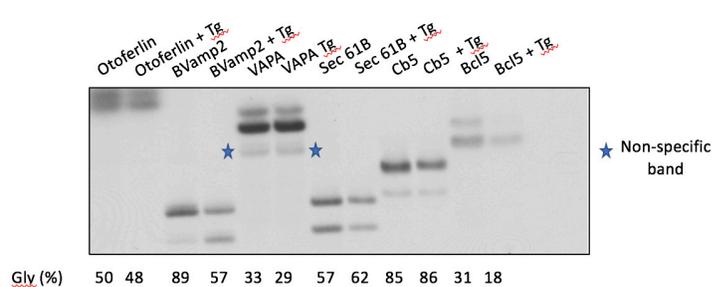


Figure 4. TA protein constructs (Otoferlin, β Vamp2, VAPA, Sec61B, Cb5, Bcl2) were transfected into HEK293 cells and treated with Tg (1 ug/mL) for 1 hour. Constructs arranged left-to-right in the order of decreasing TMD hydrophobicity.

to confirm the relationship of Tg concentration to TA protein insertion using a larger range of Tg concentrations.

We next hypothesized that the decreased protein insertion observed in β Vamp2 upon introduction of Tg should hold across a range of TA protein constructs. We performed the same experiment using ER-targeted TA protein constructs of varying hydrophobicityⁱⁱ (Otoferlin, β Vamp2, VAPA, Sec61B, Cb5, Bcl2) treated with Tg (1 ug/mL) for 1 hour. Control samples were not treated with Tg. We observed clear reduction in TA protein insertion in β Vamp2 and Bcl2 along with minor reduction in Otoferlin and VAPA (Figure 4). This evidence supports our hypothesis that Tg-induced reduction in TA protein insertion occurs in a variety of TA protein constructs and is indicative of an underlying mechanism. However, it remains unclear why protein insertion is not reduced in all TA protein constructs.

In a final set of experiments, we investigated the underlying mechanisms behind Tg-induced reduction in TA protein insertion. We hypothesized that the key to decreased TA protein insertion was calcium. Thapsigargin is a noncompetitive inhibitor of sarco/endoplasmic reticulum Ca^{2+} ATPase that blocks calcium flow into the ER and raises the cytosolic calcium. It has been shown that increases in Ca^{2+} concentration occur during cell death conditions and ER stress, with calcium acting as a key signaling molecule to trigger apoptosis¹⁴. We hypothesized that one of these calcium-induced mechanisms to stimulate apoptosis or reduce ER stress is to reduce TA protein insertion—TA proteins such as Bcl2 are key anti-apoptotic factors and decreasing the rate of protein insertion helps relieve ER stress. One potential mechanism for this to occur is for calcium to activate abundant, Ca^{2+} -dependent chaperone, calmodulin, which acts like a trap for TA proteins under certain physiological conditions¹³. To test this hypothesis, we performed chemical crosslinking experiment between β Vamp2 and calmodulin.

We co-transfected HEK293 cells with FLAG-tagged β Vamp2 and induced protein expression with 100 ng/mL of doxycycline. We then treated cells with 1 ug/mL Tg for 1 hour. Following treatment, we starved the cells with medium lacking M/C, radiolabeled with 35S methionine, and added 0uM, 200 uM, and 500 uM of DSS cross linker into 1 mL KHM buffer. After 30 min incubation at room temperature, we quenched the reaction by adding 0.1 M Tris pH 8.0. The cell lysates were immunoprecipitated for β Vamp2 using

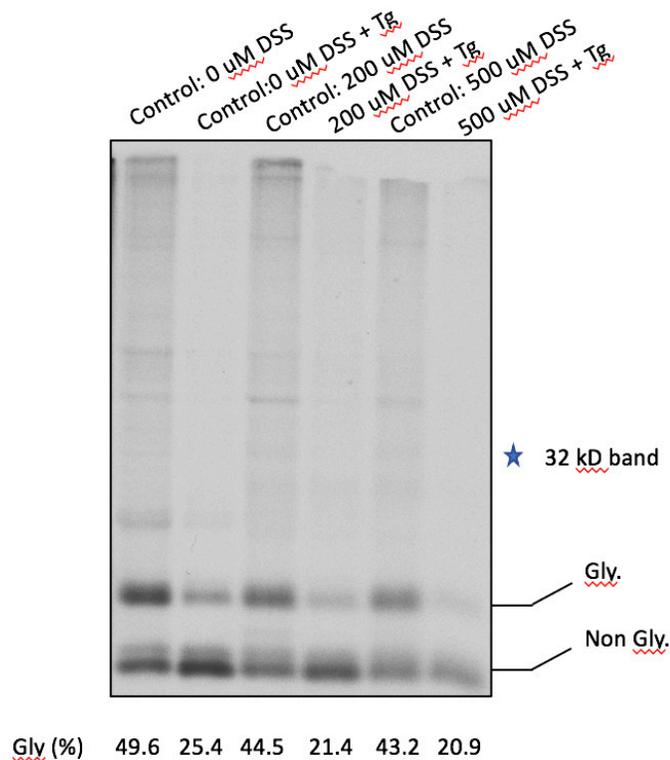


Figure 5. HEK293 cells were transfected with FLAG-tagged β Vamp2 and treated with Tg (1 μ g/mL) for 1 hour. Cells were treated with 0 μ M, 200 μ M, and 500 μ M of DSS crosslinker before harvesting and immunoprecipitating for β Vamp2 samples.

anti-FLAG beads. Each DSS cross linker concentration had a non-TG treated control. If our cross-linking experiment was successful, and our hypothesis is correct—calmodulin interacts with and inhibits TA proteins from being inserted into the ER—we expect to see a 32 kD band from immunoblotting analysis (both calmodulin and β Vamp2 are roughly 16 kD) for the 200 and 500 μ M DSS samples. Our result showed minimal cross-linking around the 32 kD band in the DSS treated samples (Figure 5). From our data, we cannot definitely determine the mechanism behind the TG-induced reduction in TA protein insertion. We will perform more experiments to optimize the DSS cross linker or via an alternative crosslinker to determine whether the Ca^{2+} activated calmodulin-trap is the mechanism by which TA protein insertion is reduced under TG-induced conditions.

DISCUSSION

Despite a better understanding of the mechanisms undergirding TA protein trafficking, delivery, and insertion, our understanding of how TA proteins—such as Bcl2 that provide crucial anti-apoptotic activity⁹—are regulated under stress physiological conditions remains limited. This paper shows that TA protein insertion is reduced with ER stress inducer thapsigargin (TG) but not with other ER stress inducers.

Our data suggests increased calcium in the cytosol is the key. Increased intracellular calcium may act as a signal to inhibit TA protein insertion during stress or cell death conditions. One potential

mechanism, for this is by activating targeting inhibitory calmodulin, which acts like a trap by binding to TA proteins under certain physiological conditions. Although our experiments did not definitively demonstrate that Ca^{2+} -activated calmodulin serves as the mechanism to trap TA proteins in the cytosol and prevent their insertion into the ER, we are hopeful that future experiments will elucidate the mechanism. Our next step will be to determine if a shift in calcium disrupts TA protein insertion. To do this, we can increase intracellular calcium by treating cells with ionomycin, which binds to calcium, and monitoring TA protein insertion. This could also be done using forward chemical genetics, to screen for and determine a small molecule inhibitor of calmodulin. Additionally, we can measure cytosolic calcium concentration changes in the presence of TG through calcium sensors like GCamp to determine whether the change in intracellular Ca^{2+} is significant. Our other goal is to determine whether calmodulin is the factor that slows TA protein insertion after TG treatment. Since our results were inconclusive we plan to modify the TA protein-calmodulin chemical cross-linking experiment by optimizing the DSS cross linker or using other crosslinkers (DSS is a lysine-mediated cross linker, some proteins may not have a lysine properly positioned). Another option to test our hypothesis that calmodulin under increased intracellular calcium conditions acts like a TA protein trap, is to perform siRNA mediated knock down of calmodulin under TG stress conditions and observe the effects of TA protein insertion. Finally, we wish to delve deeper into the characterization of TG's effect on TA protein insertion. To do this, we will perform more experiments analyzing the concentration (minimum threshold), time, and substrate specificity of TG's effect on TA protein insertion.

Nonetheless, our results demonstrate that thapsigargin, an important and commonly used ER-stress inducer that has also attracted cancer research—mipsagargin, a prodrug of thapsigargin is currently undergoing clinical trials for treatment of glioblastoma¹⁵—has a significant off-target effect, namely, reducing TA protein insertion into the ER.

EXPERIMENTAL PROCEDURES (MATERIALS AND METHODS)

DNA constructs + Antibodies

To construct TA model substrates, PCR amplification of the gene in question was performed with Phusion High-Fidelity DNA polymerase (Thermo Fisher). A FLAG tag (MDYKDDDDK) was added to the N-terminal primers.

Antibodies used for immunoblotting analysis are: Mouse α -HA-HRP (Cell Signaling, # 400 2999S), Rat α -FLAG L5 (BioLegend, # 12775), Rabbit α -FLAG.

Cell culture:

HEK293-Flp-In™ T-REx™ 293 from Invitrogen and HEK293T cell lines were cultured with high glucose DMEM (Corning, Corning, NY), 10% FBS (Gibco, Gaithersburg, MD), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco) at 37° 442 C and 5% CO₂. HEK 293-Flp-In T-Rex.

Transfection and Immunoprecipitation of TA proteins from cells

HEK293 cells (0.85×10^6 /well) were plated on polylysine-coated (0.1mg/mL) 6-well plates and transiently transfected with 2 μ g of the indicated TA plasmid constructs, and 5 μ L of Lipofectamine 2000 (Invitrogen) mixed in Opti-MEM (Gibco). 24 hours after transfection, cells (non-control) were exposed to 0-1 hr of stress treatment with 1 μ g/mL of Tg or 4 μ g/mL DTT followed by incubation in cysteine- and methionine-free media with 10% dialyzed FBS for 30 minutes, ER-stress added as well. Cells were then labeled with 8.5 μ L of 35S protein labelling mix for 30 mins. Cells were rinsed with 1xPBS and chased with complete DMEM medium. The cells were harvested in RIPA buffer containing 50mM Tris-HCl pH 8.0, 150mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% Sodium Deoxycholate (Millipore Sigma), 1x complete protease inhibitor cocktail. After centrifugation for 10 minutes at 20,000 g, the lysates were incubated with rat anti-FLAG beads for 1.5 hours with rotation. The beads were then washed with 1 mL of RIPA buffer x3. The washed beads were boiled with 50 μ L of 2x SDS sample buffer for 5 minutes followed by SDS-PAGE and autoradiography.

Cross linking between TA protein and cytosolic interacting partner

HEK293 cells (0.85×10^6 /well) were plated on polylysine-coated (0.1mg/mL) 6-well plates and transiently transfected with 2 μ g of the indicated β Vamp2 plasmid, and 5 μ L of Lipofectamine 2000 (Invitrogen) mixed in Opti-MEM (Gibco) and induced with 100 ng/mL of doxycycline. 24 hours after transfection, cells (non-control) were exposed to 30 minutes of stress treatment with 1 μ g/mL of Tg followed by incubation in cysteine- and methionine-free media with 10% dialyzed FBS for 30 minutes. Cells were then labeled with 8.5 μ L of 35S protein labelling mix for 30 mins, ER-stress added as well. Cells were rinsed with 2 mL of KHM buffer (20 mM Hepes pH 7.4, 110 mM KAc, 2mM MgAc) and incubated with 1 mL of KHM buffer containing 0 μ M, 200 μ M, and 500 μ M of DSS crosslinker (kept at room temperature) for 30 mins. The crosslinker solution was discarded and reaction was quenched by addition 1 mL of 0.1M Tris pH8 for 5 min on ice. The cells were harvested in RIPA buffer with 1x complete protease inhibitor cocktail. After centrifugation for 10 minutes at 20,000 g, the lysates were incubated with 12 μ L of mouse anti-FLAG beads and incubated for 1.5 hours with rotation. The beads were then washed with 1 mL of RIPA buffer x3. The washed beads were boiled with 50 μ L of 2x SDS sample buffer for 5 minutes followed by immunoblotting with anti-FLAG antibodies for TA proteins. The immunoprecipitants were analyzed by SDS-PAGE autoradiography.

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