UNIVERSITY OF CALIFORNIA SAN DIEGO

Assessing the role of TRB3 pseudokinase in ER to mitochondrial crosstalk

A Thesis submitted in partial satisfaction of the requirements for
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in

Biology

by

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The Thesis of Lisa Lien Sen is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

Assessing the role of TRB3 pseudokinase in ER to mitochondrial crosstalk

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Professor Ulupi Jhala, Chair

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Endoplasmic reticulum (ER) stress strongly induces pseudokinase TRB3 in pancreatic beta cells and is associated with mitochondrial dysfunction and death. The mechanism by which ER stress is communicated to mitochondria is not well understood. We examined the molecular mechanism by which TRB3 communicates ER stress presence to mitochondria and found TRB3 localized to mitochondria-associated ER membranes, or MAMs, a major hub of communication between the ER and mitochondria. TRB3 interacts with GRP75, an important link between IP3R on the ER to VDAC on the mitochondria to form the IP3R-GRP75-VDAC complex, an apparatus of calcium transfer from ER to mitochondria. The central finding of this study is that TRB3 plays an integral role in modulating calcium transfer proteins associated with ER-mitochondria calcium transfer – increasing, and thereby setting the stage for mitochondria dysfunction.

INTRODUCTION

While the onset of both type 1 and type 2 diabetes is pathologically characterized as having a rapid decline in pancreatic beta cell mass and decreased insulin sensitivity, the molecular mechanisms that trigger beta cell apoptosis in each vastly differ. Increased cell apoptosis in type 1 diabetes is caused by the induction of damaging cytokines in the beta cell as an autoimmune response [8], while apoptosis in type 2 diabetes is more closely associated with cellular decline due to chronic endoplasmic reticulum (ER) stress [9,14]. ER stress triggers the early stage of the unfolded protein response (UPR) which is composed of a temporary pause on translation, increased transcription of protein folding chaperones to facilitate folding and increased degradation of misfolded proteins as a remedial response [2,10]. Upon increase in severity, the early UPR pathway leads to production of pro-apoptotic C/EBP Homologous Protein, or CHOP to initiate cellular dysfunction [5]. CHOP is a transcription factor that activates TRB3 gene expression and is thought to mediate multiple effects of ER stress via TRB3 [15].

Pancreatic beta cells are particularly vulnerable to ER stress due to the high demand of insulin production. Low levels of ER stress in the beta cell is not uncommon due to the insulin molecule's high propensity to misfold being in combination with the beta cell's need to produce and rapidly secrete a million molecules of insulin per minute [16]. The insulin molecule requires two disulfide bridges, making folding critical. In fact, human mutations that disrupt this disulfide bridge formation have been associated with severe diabetes [25,26]. Obesity also creates a high demand for insulin production and thereby exacerbates pancreatic beta cell ER stress [27]. Understanding TRB3's role in communicating ER stress caused by misfolded protein accumulation within the ER gives insight to alleviating and perhaps combating the damaging symptoms of diabetes caused by chronic ER stress.

Research previously conducted in our lab highlights the importance of the pseudokinase tribbles3, or TRB3, in augmenting or disrupting protein interaction. A limitation of studying TRB3

is that as a pseudokinase, it lacks the catalytic domains that a kinase usually consists of and thus lacks catalytic function used as a readout. It functions by allosterically binding or unbinding a target to modulate the activity of the proteins around it [4]. Because TRB3 function is based on the proteins it interacts with, the TRB3 interactome was interrogated. Notably, we found that a large part of the interactome consisted of both ER and mitochondria proteins as well as proteins localized to the MAMs. One of these proteins include glucose-regulated protein 75, or GRP75, a protein localized to the MAMs that acts as the link to sustain calcium transfer between the ER and mitochondria. The significance of this calcium transfer mechanism cannot be overstated. Calcium is typically stored in high concentrations within the ER (100-500 μM) due to the high demand of the Ca^{2+} ions needed to act as the cofactor for protein folding chaperones [18]. Typically, calcium is released from the ER in puffs by the inositol 1,4,5-trisphosphate receptor, or IP3R, on the ER membrane. Calcium then diffuses across the MAMs and is taken up via voltage-dependent anion channel, or VDAC, on the mitochondrial membrane [11,1]. Mitochondria utilize calcium for dehydrogenases that are critical in metabolic pathways but are very sensitive to calcium levels and have much lower internal concentration of calcium (100-200 nM) [24]. It does not store large amounts of Ca^{2+} and uses the ER as a source of calcium [18,21].

When calcium crosstalk is not properly regulated, proper protein management by chaperones in the ER to combat the accumulating misfolded and unfolded proteins is inhibited and ER stress is unable to be mediated [6]. The unregulated mitochondrial calcium intake may also lead to permeabilization of the mitochondria and activation of cell death [13]. By interacting with GRP75, TRB3 alters the volume of calcium transferred from ER to mitochondria. In this study, we identified the interactome and the goal is to understand the function of TRB3. Our results will show that TRB3 plays a major role in communicating ER stress to the mitochondria. We hope to better understand the mechanism by which TRB3 regulates these processes by taking a closer look at its function within the MAMs.

MATERIALS AND METHODS

Reagents

Antibodies used in Western blotting procedures include rabbit anti-Flag Tag (14793S), rabbit anti-IP3R (8568S), rabbit anti-GRP75 (3593S), rabbit anti-VDAC (4661S), and rabbit anti-Cytochrome C (11940S) from Cell Signaling in Beverly, MA. Western blotting included use of HRP-conjugated secondary antibodies from Jackson ImmunoResearch Laboratories (West Grove, PA) and was detected using SuperSignal West Pico PLUS and Dura (Thermo Scientific, 34577, 34075). Thapsigargin was obtained from EMD Biosciences (San Diego, CA).

Cell Culture

Min6 cells (AddexBio, CA, C0018008) (passage 16-18) were grown in DMEM (Thermo Fisher, CA, 12100046) supplemented with 1.5g sodium bicarbonate, 4% heat-inactivated FBS (Gemini, CA, 100-508) and 50μm β-mercaptoethanol (Sigma). Transfection and infections were performed using Lipofectamine 3000 (Thermo Fisher, L3000015) as per manufacturer instructions.

Plasmids and Constructs

GFP- and Flag-TRB3-adenovirus are used for control and TRB3-overexpression in Min6 cells in samples prepared for mass spectrometry processing and treatment for MAM preparations. HA-tagged TRB3 wild-type and Δ94AA, ΔN-terminus, and ΔC-terminus truncated mutant constructs are used for pulldown in co-immunoprecipitation assays. GFP-GCamP (Genetically encoded Calmodulin-M13-myosin light chain kinase Plasmid) construct Mito-CH-GECO, is used to observe levels in the mitochondria over a period.

MAM Fractionation

Cell fractionation for the MAMs was performed by adapting the following procedure [19]. ER and crude mitochondria were isolated with a Percoll gradient. Crude mitochondria was further fractionated into pure mitochondria and MAMs by centrifugation at 95,000g. The post-MAM-mitochondrial supernatant was used to isolate ER by centrifugation at 100,000g.

Mass Spectrometry Protein Quantification

Mass Spectrometry was performed on the samples at University of California, San Diego's Biomolecular/Proteomics Mass Spectrometry Facility. Data obtained was analyzed using Cytoscape version 3.8.2, an open source software for visualization of networks. Only proteins that displayed a two-fold or higher rate of recovery after correcting for false discovery using the SEAQUEST algorithm provided by the facility in the presence of TRB3 were considered.

RESULTS

TRB3 Interactome

Due to the lack of TRB3 intrinsic activity and lack of a direct functional readout, it is difficult to study the mechanism by which it functions. TRB3 functions by modulating the action of proteins it interacts with. For this reason, we used an empirical approach of identifying the TRB3 interactome using co-immunoprecipitation followed by mass spectrometry analysis of interacting proteins [12]. This type of analysis reveals protein-protein interactions and provides clues toward understanding the function of a pseudokinase like TRB3. Understanding the protein interaction provides insight into the mechanism of its molecular action [3].

Min6 mouse insulinoma cells were infected with a Flag-TRB3-adenovirus followed by a co-immunoprecipitation on the cellular lysates. GFP-infected cells were used as controls. The immunoprecipitates were submitted for mass spectrometric analysis to the Biomolecular and Proteomics Mass Spectrometry Facility at University of California, San Diego. The resulting data was analyzed and visualized in Figure 1 using open source network visualization software, Cytoscape Version 3.8.2. Proteins/peptides with a two-fold or higher recovery rate in the presence of TRB3 after correction for false discovery using the SEAQUEST algorithm from the facility were grouped by color based on its localization within the cell. Protein clusters within the cytoplasm (mustard), nucleus (black), endoplasmic reticulum (blue), MAMs (yellow), and mitochondria (blue) were shown. As shown in Figure 1, the TRB3 interactome reveals a high representation of proteins localized to mitochondria, ER and MAMs. Key proteins in the interactome include ER and mitochondrial chaperone proteins and are depicted with a square-shaped node in the figure.

Specifically, TRB3 interacts with mitochondrial chaperone protein HSPA9, also known as GRP75. TRB3 also interacted with channel proteins on the outer mitochondrial membrane including VDAC2 and VDAC3. Both are key components of the calcium transfer complex IP3R-

GRP75-VDAC that is critical in regulating ER-mitochondria calcium transfer [18]. TRB3 interaction with these proteins may indicate a role in modulating their activity and thereby altering calcium flow between the two organelles. The interactome thus can be used as a guide to direct exploration of TRB3 function.

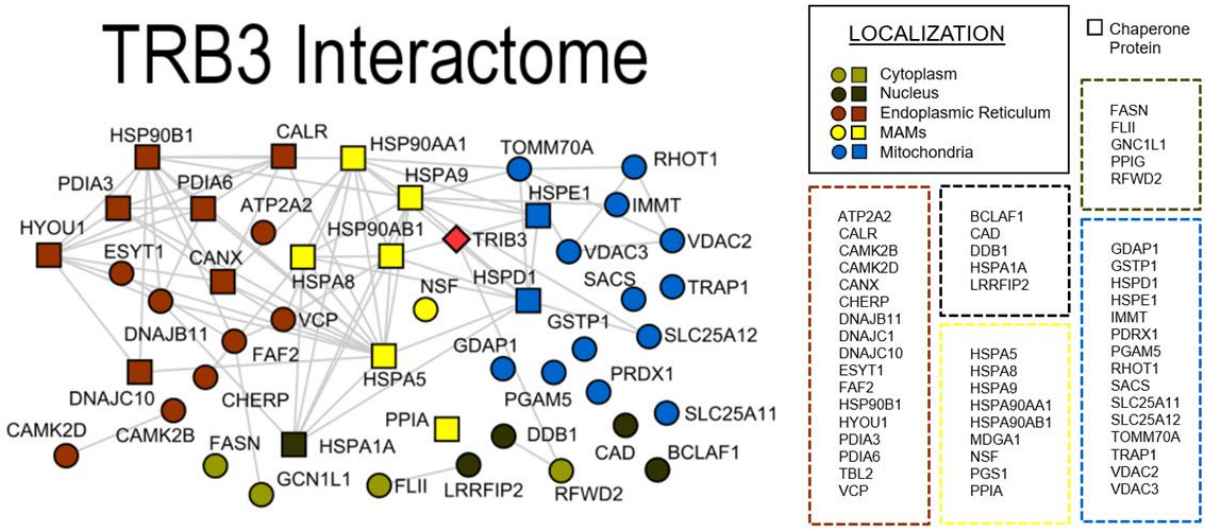

Figure 1: TRB3 Interactome. Min6 insulinoma cells were treated to induce ER stress. Using HA-tagged beads, a pulldown was performed in order to precipitate proteins bound only to TRB3 protein. The samples were sent for mass spectrometry analysis and the resulting data was analyzed. Proteins were grouped and distinguished by node color according to localization within the cell – cytoplasm (mustard), nucleus (black), ER (red), MAMs (yellow), and mitochondria (blue). Chaperone proteins are depicted by a square node shape. All visualization of string network was created using the string app of using Cytoscape 3.8.2.

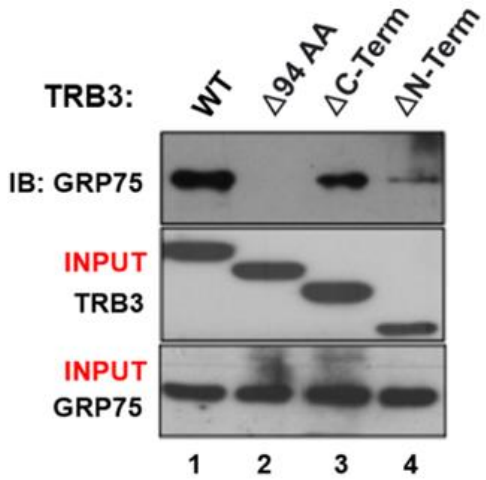

Figure 2: Verification of TRB3-GRP75 interaction by Co-immunoprecipitation. Co-immunoprecipitation assay was performed to confirm that TRB3 and GRP75 are indeed interacting to verify the interactome. TRB3 wild-type and truncated deletion mutants were expressed in Min6 cells with endogenous GRP75 being pulled down.

TRB3 Localizes to MAMs and Interacts with GRP75

We focused on GRP75 and verified protein-protein interaction with TRB3 using co-immunoprecipitation assays. Before pulldown, Min6 cells were transfected with HA-tagged TRB3 wild-type and truncated mutants, Δ94AA, ΔC-term, and ΔN-term constructs to observe if TRB3-GRP75 interaction is occurring and where on TRB3 it occurs. As shown in Figure 2, endogenous GRP75 comes down with the TRB3 wild-type and ΔC-term constructs, confirming that GRP75 and TRB3 do indeed interact and that it most likely binds the n-terminus of TRB3.

GRP75 localizes to the MAMs acting as a tether to connect IP3R to VDAC and aligning calcium transport from the ER to the mitochondria by forming the IP3R-GRP75-VDAC complex [22]. The outer ER transmembrane surface channel, IP3R, releases calcium from the ER into the inter ER-mitochondrial space. Calcium passively migrates toward the mitochondria where it is taken up by VDAC on the outer mitochondrial membrane and imported by mitochondrial calcium uniporter, or MCU, on the mitochondrial inner membrane. GRP75 orients calcium transport to mitochondria by tethering IP3R to VDAC [12]. A depiction of this mechanism is shown in Figure 3A.

We next examined whether TRB3 is present in the MAMs. A high-speed MAM fractionation of Min6 cells expressing GFP- or Flag-TRB3 adenovirus was performed to examine whether TRB3 is indeed present in the MAMs. Cell fractions including pure mitochondria, crude mitochondria, ER, and MAM fractions from each set were isolated and resolved on SDS-PAGE gel followed by western blotting. The blot was probed with anti-Flag, anti-IP3R, anti-GRP75, anti-VDAC, and anti-Cytochrome C antibodies as shown in Figure 3B to show the purity of various fractions. IP3R, localized to ER, GRP75 and VDAC, localized to the outer mitochondrial membrane and Cytochrome C, an inner mitochondrial membrane resident protein, were used to show enrichment and purity of mitochondria and ER fractions.

Flag antibody confirms the presence of TRB3 in the samples and as seen in Figure 3, reveals that TRB3 is localized to the MAMs and ER (lanes 7 and 8). While it is present in the crude mitochondrial fraction (lane 6), the absence of TRB3 in pure mitochondria (lane 5) suggests that any TRB3 found in crude mitochondria can be attributed to MAMs present in the fraction. Thus the presence of TRB3 in the MAMs is consistent with the recovery of proteins in the TRB3 interactome, which are mainly localized to the ER/mitochondria and MAMs.

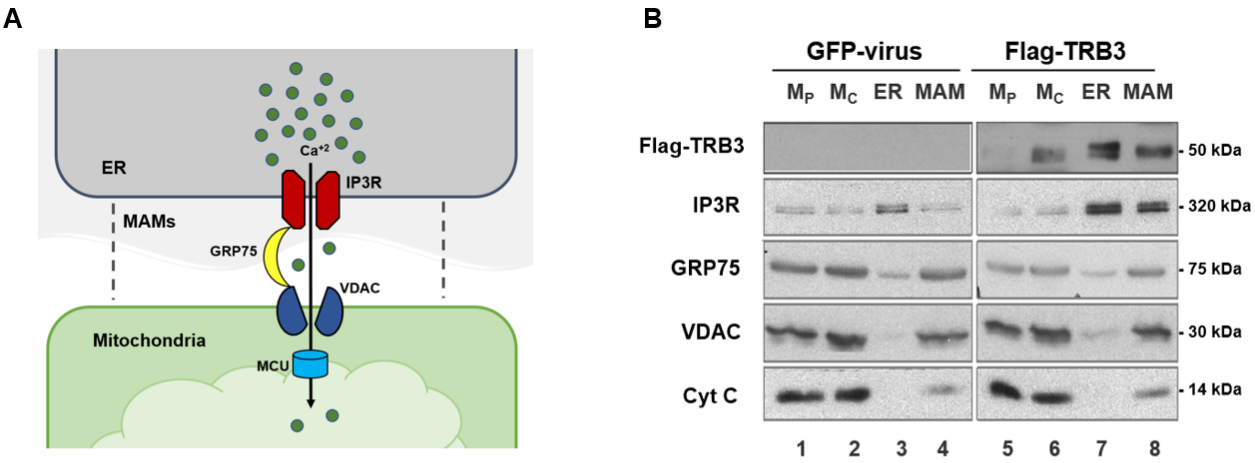

Figure 3: MAM fractionation in TRB3-expressing Min6 insulinoma cells.
A) Graphic of calcium flow via the IP3R-GRP75-VDAC complex across ER, MAMs, and mitochondria. **B)** Min6 cells were infected with GFP- and Flag-TRB3 virus overnight. MAM fractionation was performed to isolate pure mitochondria, crude mitochondria, ER and MAM fractions. Western blots were probed with anti-Flag-Tag, anti-IP3R, anti-GRP75, anti-VDAC, and anti-Cytochrome C antibodies to confirm shift in MAM-residing proteins as indicated in interactome data (Figure 1).

TRB3 Augments Calcium Transfer from ER to Mitochondria

Unexpectedly, data on the cellular subfractionations seen in Figure 3B, showed a clear increase in IP3R levels in both ER and MAM (lanes 7 and 8) fractions for TRB3-expressing cells while GRP75 and VDAC levels remain virtually unchanged. While the mechanistic basis of IP3R increase in the ER and MAM fractions of TRB3 expressing cells is unclear, our results closely resemble those seen under other pathologic conditions. For example, a similar increase in levels of IP3R in the MAMs is seen in liver fractions of mice fed a high-fat diet [17]. Furthermore, extracts of neuronal cells mimicking conditions seen in Alzheimer's disease also display IP3R in

MAM fractions. Such an increase in IP3R levels have been associated with increased calcium uptake into the mitochondria. Small increases in calcium levels have been associated with increased metabolic activity in the mitochondria [29]. However, persistent increase in mitochondrial calcium has been shown to induce formation of the mitochondrial transition pore (MTP), leading to a breach in mitochondrial outer membrane to trigger cellular apoptosis. It has been suggested that this could be a mechanism for ER stress dependent induction of apoptosis. Increased volume of calcium in the MAMs may create opportunities for increased ER-mitochondria calcium transfer via the VDAC protein.

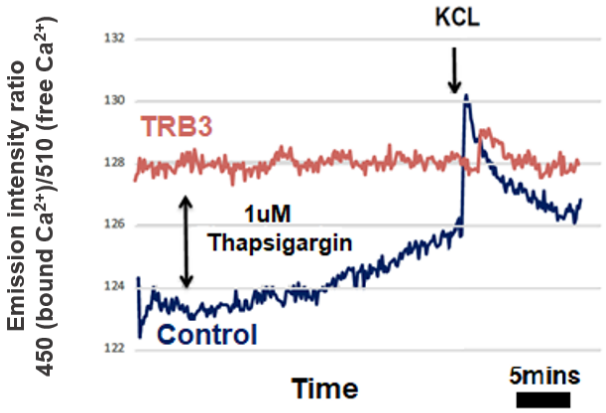

Figure 4: Mitochondrial Calcium Shift in TRB3-expressing Min6 cells
Control and Flag-TRB3 transfected Min6 cells were co-expressed with the GFP-GCamP construct Mito-CH-GECO to observe calcium levels in the mitochondria over the course of 30 minutes at 30 second intervals. 1uM thapsigargin treatment for 20 minutes followed by KCl was added to induce depolarization of the membrane. Values were quantified and graphed.

TRB3 Expression is Associated with Increased Levels of Mitochondrial Calcium

Having seen a TRB3-dependent increase of IP3R in the MAMs, we examined if TRB3 also led to an increase of mitochondrial calcium levels. To verify altered calcium levels in the mitochondria in the presence of TRB3, Mito-CH-GECO, a fluorescent calcium biosensor construct GFP-GCamP (Genetically encoded Calmodulin-M13-myosin light chain kinase Plasmid), was co-expressed using control or Flag-TRB3 in Min6 cells. The biosensor protein is

targeted to the inner mitochondrial matrix and has a range of detection commensurate with the range of calcium found in the mitochondria (5nM-5uM) [18]. Calcium dependent levels are tracked by quantifying fluorescence levels that are dim in the absence of calcium and bright when bound to calcium. The construct measures calcium dependent change in ratio of blue (400 nm) to green (510 nm) fluorescence when excited at 400 nm. Using this method, calcium levels within the mitochondria are mapped over a period of 30 minutes and by taking measurements in 30 second intervals. After baseline, fluorescence measurements were continued in the presence of 1uM thapsigargin for 20 minutes. Thapsigargin is a chemical agent that blocks calcium re-entry into the ER by inhibiting SERCA-1 activity which is instrumental in calcium reuptake from the ER-mitochondrial interspace into the ER. Inhibition of SERCA-1 results in a gradual increase in calcium in the ER-mitochondrial interspace resulting in a gradual increase in calcium uptake into the mitochondria [23]. As seen in Figure 4, addition of thapsigargin leads to a gradual increase in mitochondrial calcium in control samples over 20 minutes. Next, the Min6 cells were stimulated with 50uM KCl to induce closure of KATP channels on the plasma membrane. Closure of KATP channels lead to depolarization, followed by the opening of calcium channels, and influx of calcium into the cells. This influx of calcium leads to insulin secretion but also causes a sharp increase in calcium concentrations within the mitochondria [20,21] as indicated in the blue line of control samples in Figure 4.

As shown in Figure 4, the TRB3-expressing cells show an altered/higher baseline of calcium (red). Compared to the control, upon addition of 1uM thapsigargin the calcium levels in the TRB3-expressing cells failed to increase. Furthermore, the TRB3-expressing cells failed to produce a sharp increase of calcium compared to the control cells. The elevated initial calcium levels in the presence of TRB3 are consistent with a constitutively high calcium import into the mitochondria.

DISCUSSION

TRB3 is strongly induced in ER stress across several cell types including pancreatic beta cells, liver/HepG2 cells, cardiac cells, and neurons [15,17,30,7]. Due to the difficulties faced in studying a pseudokinase like TRB3, the functions by which it mediates the effects of ER stress were unknown. Several papers have suggested the ability of TRB3 to inhibit prosurvival kinase Akt leads to TRB3-dependent cellular damage to ER stress [8,31,32] and while it has been strongly linked to Akt signaling in the literature, a mechanism for this inhibition has still not been provided [33].

Using the interactome as a foundation, we have established that TRB3 localizes to the MAMs and is instrumental in increasing IP3R levels in the ER and MAMs. Increase of IP3R levels is also accompanied with increase in mitochondrial calcium levels which in turn is associated with altered metabolic function and cell death. Upregulation of ER-mitochondria calcium crosstalk by modulating GRP75 and VDAC of the IP3R-GRP75-VDAC complex suggests that TRB3 stabilization of calcium flow into the mitochondria. While damaging, TRB3 and ER stress presence encourages calcium crosstalk, suggesting that in the presence of ER stress, TRB3 plays a major role in modulating ER- mitochondria calcium crosstalk to augment mitochondrial function and gives a mechanism to how ER stress is communicated to the mitochondria. In the later part of UPR, ER stress on its own already sends out death signals but by augmenting mitochondrial function during ER stress, pro-apoptotic features are further activated to induce cell death.

IP3R induction in the presence of TRB3 upregulated in ER stress occurs across several cell types including beta cells, liver and neurons [27,29]. The parallels of this mechanism between several cell types indicates a common role of TRB3 across these systems. We speculate that TRB3 could be instrumental in ER stress-dependent mitochondrial damage in multiple cell types under similar disease conditions.

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