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Stress-induced Proteolysis of FKBP10: Mutational Analysis and Functional Implications

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Abstract:

Cellular signaling is a complex system of communication that regulates cell function through a variety of molecular messengers. When cell survival is threatened by changes in environment or by malfunction of internal regulatory pathways, signals are initiated to restore homeostasis. Deficient protein processing within the endoplasmic reticulum (ER) causes an accumulation of misfolded proteins and stress signaling. The resulting stress signaling includes both adaptive signals (unfolded protein response) and in cases of severe ER stress apoptotic signals induced via the mitochondria.

FKBP10 is a key ER luminal peptidy-prolyl isomerase (rotamase) that mediates protein folding. The production of chaperone proteins and foldases typically increases following the activation of ER stress signaling. However, within 6 -12 hours of ER stress induction, FKBP10 protein levels decrease dramatically. Cell fractionation studies revealed FKBP10 fragments to appear in the cytosol during ER stress. Imaging confirmed decreased ER localization following ER stress. Both calpain and the proteasome were shown to be active in our cells and required for the rapid proteolysis of FKBP10. These data lead us to a model of FKBP10 destruction in which FKBP10 or its cleavage product is retrotranslocated from the ER to the cytosol as it is destroyed.

An examination of the structure of the FKBP10 protein revealed two calcium binding EF-hand domains near the C-terminus. As the ER lumen is a critical site of cellular calcium storage and ER calcium stores are known to decrease with ER stress, we have chosen to examine the importance of these EF-hand domains in FKBP10 stability by mutagenizing the putative calcium binding amino acids in a recombinant FKBP10 clone. Mutant constructs have been generated and calcium binding dependence of FKBP10 has been analyzed. The destruction of FKBP10 is interesting because it may identify a unique cellular strategy for repairing/restoring ER function following ER stress.

Introduction:

Cellular signaling is a complex system of communication that plays a role in regulating the processes required for life. Molecular messenger signals help the cell to maintain homeostasis, respond to stress, and regulate growth via intra- and extra-cellular signaling pathways. These pathways are essential for restoring normal cellular function and coordinating cellular activities. Pathways that are malfunctioning can cause a variety of disease phenotypes. Improper signaling can lead to uncontrolled cell growth resulting in cancer or apoptosis, which can lead to Alzheimer's or Parkinson's disease. A better understanding of these signaling pathways may help to restore the functionality of defective pathways with the goal of overcoming the abnormal cellular signals and resulting disease phenotypes. Cell signaling and apoptosis research has increased substantially in the past several decades. In this investigation, I studied particular cellular signaling pathways, specifically adaptive signals resulting from an accumulation of proteins in the endoplasmic reticulum. This study presents a mutational analysis of an endoplasmic reticulum foldase that is rapidly cleaved and destroyed following endoplasmic reticulum stress.

The endoplasmic reticulum (ER) is a cellular organelle responsible for protein and lipid synthesis as well as the folding and transport of proteins within the cell. The ER is composed of an organized network of tubules, vesicles, and cisternae that carry out the major functions of this organelle. These functions include post-translational modification of nascent proteins, sequestration of calcium, and production and storage of macromolecules. The ER is capable of initiating adaptive or apoptotic cascades via complex signaling from luminal and membrane bound proteins. Improperly processed proteins accumulate in the ER lumen as a result of improper post-translational activity. These unfolded or misfolded proteins lead to ER stress

which initiates survival and/or death signals from the ER. ER stress threatens cell viability by interfering with normal protein synthesis and proper protein processing. If homeostasis is not restored, the cell will ultimately activate apoptotic death by means of signals originating in the ER (Nakatsukasa and Brodsky 2008).

As a reaction to deficient protein folding, an adaptive cellular stress response is produced by the cell known as the unfolded protein response (UPR). The UPR is induced to alleviate ER stress and maintain survival by restoring normal cellular processes and ER functionality by arresting protein translation. In addition, the UPR activates signals that upregulate transcription of ER chaperone proteins that play a role in protein folding. This occurs through activation of the three main UPR pathways: protein kinase-like ER kinase (PERK), inositol-requiring enzyme (IRE1), and activating transcription factor 6 (ATF6) (Rutkowski and Kaufman 2004). These pathways are dependent on active glucose regulating protein 78 (Grp78; also known as binding immunoglobulin protein: BiP), an ER localized chaperone protein. Grp78 preferentially binds to misfolded proteins as a response to ER stress. In binding misfolded proteins, Grp78 is forced to forfeit its interactions with PERK, IRE1 and ATF6 (Ma and Hindershot 2001). (Fig. 1)

Disassociation of Grp78 from the luminal domains of the ER transmembrane proteins IRE1 and ATF6 initiates signaling cascades that initiate the transcription of ER resident chaperones (Kaufman et al. 2002; Harding et al. 2002). The first of these signaling cascades involves IRE1 forming a homodimer which becomes activated as a ribonuclease. This ribonuclease then splices a primary transcript of X-box DNA-binding protein (XBP1) mRNA. This processed XBP1 mRNA is then translated, creating a newly synthesized protein that migrates to the nucleus where it promotes transcription of essential UPR proteins (Rutkowski and Kaufman 2004). The second of the signaling cascades involves ATF6. ATF6 leaves the ER

membrane when not bound to Grp78 and travels to the Golgi apparatus to be activated by cleavage. Activated ATF6 is a 50 kDa form which translocates to the nucleus where it binds ER stress response element (ERSE). The ATF6/ERSE complex promotes transcription of ER localized molecular chaperones (Rutkowski and Kaufman 2004).

The third major pathway of the UPR involves PERK. PERK activates itself by forming homodimers and subsequently phosphorylating eukaryotic translation initiation factor 2 (EIF2-α). Phosphorylated EIF2-α prevents initiation of translation by inhibiting formation of the mRNA translation machinery, specifically the 80S ribosome initiation complex (Kaufman et al. 2002; Harding et al. 2002). Translation of most cellular proteins is arrested under these conditions but some mRNAs are preferentially translated when EIF2-α becomes phosphorylated. For example, under normal conditions ATF4 (activating transcription factor 4) is transcribed but not translated until EIF2-α is phosphorylated. If ER stress is sustained for a long period of time, ATF4 upregulates the transcription of CHOP, leading to pro-apoptotic signals, specifically the activation of the apoptotic executioner protein, caspase 3 (Ma and Hindershot 2001).

During prolonged periods of ER stress, UPR signals fail to alleviate the problem of misfolded proteins and apoptosis is initiated via the activation of irreversible self destruction pathways. The exact mechanism of the transition between the UPR and apoptosis is unclear; however, calcium release may play a key role in this transition (Scorrano et al. 2003). Ca^{2+} is an important molecular messenger in many cellular processes including apoptotic signaling. Ca^{2+} is stored in the ER in up to millimolar concentrations and is tightly regulated by pro- and anti-apoptotic members of the Bcl-2 protein family (e.g. Bak, Bax, Bok). Cytosolic homeostasis is disrupted when Ca^{2+} is released from the ER and cell death is activated through a mitochondrial dependent pathway (Anken and Braakman 2005). After Ca^{2+} is released into the cytosol, this

signals mitochondrial membrane proteins to complex to form a permeability transition pore (PTP) resulting in the release of cytochrome c from the intermembrane space of the mitochondria (Scorrano et al. 2003). Upon binding Apaf-1 and procaspase-9, cytochrome c induces the proteolytic cleavage of procaspase 9 into caspase 9 and subsequent activation of the executioner caspases -3, -6, and -7 (Li et al. 1997; Saleh et al. 1999).

Another key mediator of the transition between ER stress and apoptosis is localized within the ER lumen and membrane. As previously mentioned, IRE-1 acts as a ribonuclease, splicing mRNA of a UPR-activating transcription factor. IRE-1 also displays kinase activity which has been linked to activation of JNK stress-activated protein kinase (SAPK) to induce apoptosis (Urano et al. 2000). Apoptosis signal-regulating kinase 1 (Ask1) is known to activate apoptosis through the IRE1-TRAF2-ASK1-JNK pathway and Ask1 is found to be a key element in ER stress induced cell death for this reason (Nishitoh et al. 2002). While many of the pathways of apoptosis have been elucidated and are currently being investigated, the sequence of events leading to programmed cell death is yet to be determined.

Previous work in our lab (Esch 2008) involved an attempt to clarify apoptotic signaling pathways resulting from ER stress via a mass screening of changes in protein expression. A PowerBlot™ assay involving a complex western blot system was used to screen whole cell lysates for changes in protein expression as a result of ER stress. Experimental lysates of tsBN7 cells, derived from BHK21 baby hamster kidney fibroblasts, were generated in the laboratory and sent to Becton Dickinson Biosciences Pharmigen in San Diego. The tsBN7 cell line is known to display a mutant phenotype resulting from a temperature-sensitive point mutation in the DAD1 gene which is part of the ER oligosaccharyltransferase (OST) enzyme (Sanjay et al. 1998). Loss of OST function at the restrictive temperature (39.5˚C) induces a rapid loss of N-

linked glycosylation which leads to ER stress (Niederer et al. 2005). Within 36 hours of being placed at the restrictive temperature of 39.5˚C, cells demonstrate trademark features of apoptosis including membrane blebbing, nuclear fragmentation, chromatin condensation, and cell shrinkage (Niederer et al. 2005). Temperature shifted tsBN7s can be rescued with cyclosporin A (CsA), a blocker of the PTP, indicating a role of cytochrome c in temperature shift induced apoptosis (Niederer et al. 2005).

Lysates were also generated using a second stressor, tunicamycin, which activates ER stress by blocking N-linked glycosylation by inhibiting the synthesis of the mannose-rich oligosaccharides used by the OST complex (Lehle and Tanner 1976). This inhibition results in a buildup of unfolded proteins, UPR activation, and finally apoptosis (Niederer et al. 2005). Both temperature restricted tsBN7s and permissive temperature tsBN7s treated with tunicamycin experience ER stress through similar mechanisms, while apoptosis induced from these treatments occur through distinctly different signaling pathways. The Powerblot™ western array employed in this study offers an effective method of identifying commonalities as well as points of divergence in these pathways. The Powerblot™ array utilized over 1,000 antibodies to determine interesting protein expression changes and identify proteins as targets for further investigation. Among these proteins to be further studied were two members of the immunophilin family, FKBP12 and FKBP10.

Immunophilins are found in every cellular compartment and are characterized by peptidyl-prolyl *cis-trans* isomerase (PPIase) activity. In addition, they have an affinity for immunosuppressant ligands (FK506, rapamycin, or cyclosporin A (CsA); Schreiber 1991). All FK506 binding proteins (FKBPs) have an affinity for FK506 and rapamycin, but not CsA. To date, 13 FKBPs have been discovered, and the list continues to grow. Many immunophilins have

been implicated in protein folding and chaperone activity due to the PPIase motifs. PPIases catalyze the interconversion of peptide bonds from the trans conformation to the cis conformation in proteins. Thus, PPIases are a category of foldase proteins which aid in the proper folding of polypeptides. This function is vital for protein folding as the conversion is the rate limiting step in the folding process. In addition, this folding process is essential in that most denatured proteins exist in the trans conformation while folded proteins exist in a mixture of cis and trans.

FKBP22 is an ER localized folding protein that is known to associate with Grp78, the major Hsp70 chaperone protein in the ER (Tremmel and Tropschug 2007). FKBP13 is upregulated by an accumulation of unfolded proteins within the ER and shares considerable homology with the misfolded protein response element of Grp78 (Bush et al. 1994). FKBP10 shares 46% homology with FKBP13 suggesting a possible role in ER protein folding as well (Coss et al. 1995). Two other FKBPs have been found to localize to the ER, FKBP23 and FKBP60 (Shadidy et al. 1999), and it is suggested that they may play a role in general protein folding or chaperone activity.

The PowerBlot™ screen led to the interesting discovery of the ER stress-induced destruction of the ER protein, FKBP10. The FKBPs have recently been renamed and instead of being numbered based on molecular weight they are now named as one of 13 FKBPs. FKBP10 was previously referred to as FKBP65. FKBP10 disappears rapidly following the onset of ER stress and this was confirmed with western blots after its discovery in the PowerBlot™. Usually foldase production increases as an adaptation to ER stress. While FKBP10 protein levels decrease, mRNA levels remain constant so we know that it is not a question of half-life or transcription but instead an effective mechanism to actively get rid of this foldase. The unique

story of the rapid destruction of FKBP10 may identify a novel mechanism of ER stress signaling (Fig. 2).

While a highly conserved PPIase domain is common to all FKBPs, FKBP10 uniquely contains four catalytic PPIase domains and is the only member of its family to possess this unique characteristic which suggests an important role in the folding of proteins (Coss et al. 1995). In addition to the four PPIase domains, FKBP10 possesses two EF-hand domains, an N-terminal amino acid sequence which serves to target the protein to the ER, and a C-terminal ER retention HEEL sequence (Fig. 3A). The HEEL domain is a modified HDEL domain which is a known ER retention sequence and in some proteins the HEEL domain is an improved ER retention signal (Derkx and Madrid 2001). ER localization of FKBP10 has been confirmed through immunofluorescence staining in published data (Patterson et al. 2000). Patterson's lab used subcellular fractionation and Triton X-114 phase separation to further localize FKBP10 to the ER lumen (Patterson et al. 2000).

Elaine Davis' lab studies FKBP10 localization and they found that the structural protein, tropoelastin, co-localized with FKBP10 in the secretory pathway of the ER. Tropoelastin is a proline rich protein that contains 12% proline residues (Patterson et al. 2005). These residues are crucial for proper folding of the protein, and must remain in the trans conformation for proper processing (Davis et al. 1998). This association indicates that FKBP10 may play a role in ER protein folding and trafficking. It is highly unlikely that tropoelastin is the only target cargo for FKBP10 and further evidence of FKBP10 as a chaperone or folding enzyme is illuminated by its association with hsp90, an ER chaperone (Coss et al.1995). The exact physiological function of FKBP10 remains vague. FKBP10 has recently been linked to colorectal cancer and is considered an early marker of this cancer type (Olesen et al. 2005).

EF-hand calcium binding domains have been proven to be important for the stability of many ER localized proteins (Asselt and Dijkstra 1999). The EF-hand is a helix-loop-helix structural domain found in calcium binding proteins. The short loop region, which usually consists of only about 12 amino acids, is the region responsible for binding calcium ions. Calcium ion binding at the EF hand domain leads to a conformational change in the EF hand motif which opens a binding site for a target protein. Calcium ions are bound by six or seven oxygen atoms from all different sources but are bound strongest by oxygens of amino acid side chains (specifically negatively charged amino acids like aspartate and glutamate) through electrostatic interactions (Kesvatera et al. 2001). Aspartate and glutamate have a negative charge on the oxygen atom in the side chain which interacts with the positive charge of the calcium ion. Aspartate and glutamate are bidentate residues and play an important role in calcium binding because they use both of the oxygens of the side chain and the carboxyl group to bind calcium. The conserved aspartate at the beginning of the calcium binding domain and the glutamate at the end (position 12) provide negatively charged oxygen atoms that are critical for the coordination of the calcium ion to bind (Babu et al. 1992). The amino acid sequence for the calcium binding domains of FKBP10 is unique in that it has a glutamate at the twelfth position but an additional glutamate in the eleventh position as well. The unique double glutamate at the end of the calcium binding domain donates twice as many oxygen atoms which further strengthens the calcium binding ability (Fig. 3).

EF-hand calcium binding domains are important for the stability and proper functioning of many ER localized proteins. When ER calcium decreases and is released into the cytosol, there is less calcium available to bind to FKBP10. Calcium binding within the EF-hand domain

of FKBP10 may play an important role in regulating the stability of the protein following ER stress.

In this study, we report the apparent destruction of FKBP10 following induction of apoptosis and propose a model for its destruction via retrotranslocation. An inhibitor profile is used to analyze the extent to which calpain and proteasome inhibitors block the destruction of FKBP10. We propose that when FKBP10 is not bound to calcium, it is shuttled out of the ER by means of retrotranslocation to be destroyed by both the proteasome and calpain. In addition, calcium binding plays an important role in the stability of many ER proteins and might be important in the story of FKBP10 destruction as a result of ER stress. By using mutagenesis to alter the calcium binding domains and essentially affect FKBP10's ability to bind calcium and perform normal protein functions, we attempted to determine if FKBP10 stability is calcium dependent.

Materials and Methods:

tsBN7 cell culture

The tsBN7 cell line, derived from BHK21 baby hamster kidney fibroblasts, was provided by Dr. Claudio Basilico (NYU Medical School) and was maintained in a humidified, 5% CO_2 incubator at 32.5°C (unless indicated under TS-treatment at 39.5°C, 5% CO_2). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS), 5% of penicillin-streptomycin, and 5% non-essential amino acids. Cells were passaged every 3 days by washing with Dulbecco's Phosphate-Buffered Saline (DPBS), detaching cells from the culture dish with .05% trypsin-EDTA, adding the cells to prepared media, then dispersing the cells amongst new culture dishes and returning them to the designated incubator.

RFL6 cell culture

The RFL6 (rat lung fibroblast) cell line was purchased from American type culture collection (ATCC) and was maintained in a humidified, 5% CO_2 incubator at 37°C. Cells were grown in F12 nutrient medium with 20% FBS, 5% penicillin-streptomycin, and 5% non-essential amino acids. Cells were passaged every 4 days by washing with DPBS, detaching cells from the culture dish with .05% trypsin-EDTA at 37°C, then dispersing the cells amongst new culture dishes and returning them to the designated incubator.

Protein isolation/immunoblots

Cells were seeded in 100mm tissue culture plates 24 hours prior to the beginning of an experiment. To isolate proteins for immunoblot analysis, cells were washed gently with DPBS

and lysed with 150µL SDS-lysis buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 7% glycerol, 95˚C). Plates were scraped with cell lifters and lysates were transferred to a 2mL microcentrifuge tube, heated at 95˚C for 5 minutes, triturated with a 21 gauge syringe, and stored at -20˚C.

A Pierce BCA™ Protein Assay Kit (Rockford, IL) was used to determine protein concentrations and 30µg of each sample was loaded into a pre-cast 4-20% gradient agarose gel (Pierce; Rockford, IL) or 100 µg onto a polyacrylamide SDS-PAGE gel (10 or 15%). Proteins were transferred to a nitrocellulose membrane (0.45 µm) at 400 mA/25 V overnight. Even loading was confirmed by visual inspection following staining with 0.1% Ponceau S solution. The membrane was incubated with a filter sterilized block (1 % BSA, 0.01% Na Azide) for 1 hour followed by incubation with primary and secondary antibodies according to the manufacturers' protocol. A horseradish peroxidase conjugated secondary antibody was detected by a luminol solution and x-ray film.

Site-Directed Mutagenesis

Three plasmids were used as the mutagenesis templates. These three plasmids were obtained from Origene Technologies, Inc. (Rockville, MD). A precision shuttle vector (pCMV6-AC) with an FKBP10 open reading frame (ORF) clone for *Mus musculus* (mouse) was tagged with three different proteins to obtain the three different plasmids. All three tags are C-terminal and include a Myc epitope, Green Fluorescent Protein (GFP), and Turbo GFP (tag GFP is monomeric GFP while Turbo GFP requires dimerization for fluorescence). Six primer pairs designed to mutate different motifs of the FKBP10 gene were synthesized by Sigma-aldrich (St. Louis, MO). Plasmids were transformed into chemically competent JM109 cells according to

manufacturer's protocol to amplify and isolate large quantities of plasmid DNA (Promega; Madison, WI).

Mutagenesis of the four mutant sites on each plasmid was performed using the Stratagene Quikchange Lightning site-directed mutagenesis kit (Stratagene – Agilent Technologies; La Jolla, CA). This procedure employs the double stranded vector with the cDNA of interest and two oligonucleotide primers containing the desired mutation. In a thin-walled PCR tube, 50 ng of plasmid DNA was combined with 125 ng of each forward and reverse primer containing the desired mutation, 5 µL of 10x reaction buffer, 1 µL dNTP mix, 1.5 µL Quiksolution reagent, 1 µL Quikchange Lightning enzyme, and nanopure water to a total of 50 µL reaction volume. Thermal cycling in a PCR machine was used to denature the DNA template, anneal the mutagenic primers containing the desired mutation, and extend and incorporate primers using Stratagene's *Pfu*-based DNA polymerase blend. Thermal cycling involved preheating the reaction mixture to 95°C for 2 min, followed by eighteen cycles of 95°C for 20 sec, 60°C for 10 sec, 68°C for 4 min and 15 sec, and a final cycle of 68°C for 5 min.

Following the cycling reaction, 2 µl of *Dpn*I restriction enzyme was added to each PCR reaction tube, mixed well and incubated at 37°C for 5 minutes to digest the parental DNA. Two microliters of the final PCR products were transformed into 45 µl of XL10-Gold ultracompetent cells (as per the Quikchange transformation protocol) and 50 µl volumes were spread on LB agar plates containing ampicillin (100 µg/ml). Five clones were isolated and amplified in 3 ml of LB medium containing ampicillin (100 µg/ml). Plasmids were isolated and purified using the Qiagen EndoFree Plasmid Maxi Kit (Qiagen Inc.; Valencia, CA). To confirm the introduction of desired mutations, plasmid DNAs were sent out to the sequencing department at Retrogen to be

sequenced (Retrogen, Inc.; San Diego, CA). (Sequencing Primers were also synthesized at Sigma-aldrich).

The following is a direct report of the mutagenesis from Stratagene: The oligonucleotide primers are each complementary to opposite strands of the double stranded vector and are extended during temperature cycling by *PfuUltra* HF DNA polymerase. Extension of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with *Dpn* I. The *Dpn* I endonuclease (target sequence: 5′-Gm6ATC-3′) is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. (DNA isolated from almost all *E. coli* strains is dam methylated and therefore susceptible to *Dpn* I digestion.) The nicked vector DNA containing the desired mutations is then transformed into XL10-Gold ultracompetent cells.

Immunocytochemistry/Plasmid Transfection/Imaging

Cells were plated at a density of 1.25×10^5 cells/cm^2 on glass coverslips in a 24 well tissue culture plate. After 24 hours, the cells on the coverslips were transfected with the DNA plasmid using Lipofectamine 2000 (Invitrogen Corporation; Carlsbad, CA). Transfection of ER specific-RFP (pDsRed2-ER: Clontech; Mountain View, CA) along with our GFP-tagged plasmids was performed as per the following protocol: 0.8 μg DNA was diluted in 50 μL DMEM without serum per coverslip to be transfected. Additionally, for each coverslip, 2 μL of Lipofectamine was diluted in 50 μL DMEM without serum. Each dilution mixture was mixed well and allowed to stand at room temperature for 5 minutes. Immediately after the 5 minutes, the DNA solution and Lipofectamine solution were mixed gently and allowed to stand for

20 minutes at room temperature to allow the DNA–Lipofectamine 2000 lipoplexes to form. After 20 minutes, 100 μL of the transfection complex was added to each well containing a coverslip with adherent cells and 400 μL of DMEM. Cells were incubated at appropriate growth temperature for 4 hours. After the four hours, transfection medium was replaced with serum containing medium and cells were allowed to grow for an additional 36 hours.

After the 36 hours cells were fixed using 400 μL of 4% para-formaldehyde and allowed to sit overnight at 4°C. Following the fixation period, cells were washed twice with DPBS and then 300 μL of immunocytochemistry – Permeabilize/Block solution (5% BSA, 0.1% Sodium Azide, 0.002% Triton x-100 in DPBS) was add to each coverslip and incubated at 4°C for one hour. Primary antibody was diluted in 300 μL in immunocytochemistry antibody solution (2% BSA, 0.1% Sodium Azide, 0.002% Triton x-100 in DPBS) (1:500 antibody dilution). Following the permeabilization step, antibody solution was added to coverslips and allowed to sit overnight at 4°C. Cells were then washed five times with DPBS to remove excess primary antibody and 300 μL of a secondary antibody solution was added (300 μL DPBS, secondary anti-mouse fluorescent green conjugate at 1:500 dilution, and DAPI at 1:400 dilution). Secondary antibody was allowed to sit for 2 hours in the dark before the coverslips were mounted. Coverslips were mounted using Fluoromount G (Electron Microscopy Sciences) with 0.1% Phenylenediamine and stored at -20°C in the dark until ready for imaging.

Images were captured using a Nikon TE 2000 inverted microscope and Metamorph Imaging software.

Subcellular Fractionation

The subcellular fractionation of tsBN7 cells was performed according to the protocol of the Qproteome™ cell compartment kit (Qiagen Inc.; Valencia, CA). Differential-speed centrifugation of the lysates obtained from 5×10^6 cells with a series of buffers was implemented to obtain cytosol, membrane and nuclear fractions. The supernatant from the 1000 x *g* centrifugation with extraction buffer CE1 contained cytosolic proteins, the supernatant from the 6000 x *g* centrifugation with extraction buffer CE2 contained membrane proteins, and the supernatant from the 6800 x *g* centrifugation with extraction buffer CE3 contained nuclear proteins. The remaining pellet was resuspended in extraction buffer CE4 and contained cytoskeletal proteins.

A direct report from the Qiagen handbook on how the company defines the protocol follows: Extraction Buffer CE1 is added to cells and selectively disrupts the plasma membrane without solubilizing it, resulting in the isolation of cytosolic proteins. Plasma membranes and compartmentalized organelles, such as nuclei, mitochondria, and the endoplasmic reticulum (ER), remain intact and are pelleted by centrifugation. The pellet from the first step is resuspended in Extraction Buffer CE2, which solubilizes the plasma membrane as well as all organelle membranes except the nuclear membrane. After solubilization, the sample is centrifuged. The supernatant contains membrane proteins and proteins from the lumen of organelles (e.g., the ER and mitochondria). The pellet consists of nuclei. In the next step nuclei are solubilized using Extraction Buffer CE3 in which all soluble and most membrane-bound nuclear proteins are extracted. Addition of Benzonase® Nuclease allows the release of proteins tightly bound to nucleic acids (e.g., histones). After another centrifugation, Extraction Buffer

CE4 is used to solubilize all residual — mainly cytoskeletal — proteins in the pellet. Fractions 1 to 3 contain proteins in their native state.

Marker proteins for each fraction were used to analyze the amount of cross-contamination from one fraction to another and to verify the integrity of the fractions (Fig. 4B). For each fraction, protein concentration was measured by the Micro BCA™ Protein Assay kit (Pierce) and the protein fractions were analyzed by Western-blot analysis as previously described.

Inhibitor Profile

Cells were seeded in 100 mm tissue cultures dishes 48 hours prior to experiment. 6 hours prior to protein isolation medium was removed and replaced with 10 mL of medium and inhibitor to specified concentration: 100 μM Calpeptin (CalBiochem; La Jolla, CA), 10 μM Calpain inhibitor III (CalBiochem), 10 μM Calpain inhibitor IV (CalBiochem), 5 μM MG132 (CalBiochem), 5 μM Lactacystin (CalBiochem), 5 μM Proteasome inhibitor II (CalBiochem). Cells were placed in 32.5°C for 6 hours and then proteins were isolated immediately. Protein isolation, protein assay, and immunoblotting were performed as previously described in *Protein isolation/immunoblots.*

Proteasome Luminescence Assay

In vitro proteasome activity was measured using the Proteasome-Glo Chymotrypsin-Like Cell-Based Assay (Promega; Madison, WI). The luminogenic substrate used for the proteasome activity is Suc-LLVY-aminoluciferin. The substrate is cleaved by the proteasome which

generates a “glow-type” luminescent signal. The signal is proportional to the amount of proteasome activity.

Cells were seeded in a 96 well black-walled, clear bottom, fluorescence tissue culture plate with 100,000 cells/mL and 100 µL of cell solution in each well. 10 mL of Proteasome Glo Based Buffer was added to Luciferin Detection Reagent and then 50 µL of substrate was added to the Luciferin solution and allowed to sit at room temperature for 30 minutes. 24 hours after seeding the 96 well plate, proteasome luminescence readings began to be measured. At time=0, MG132 proteasome inhibitor was added to inhibitor wells at a concentration of 5 µM. At each time point, add 100 µL of Proteasome-Glo/Luciferin reagent. At time=0 read first unihibited and inhibited wells to obtain control readings as well as blank readings (blank = media and Proteasome-Glo/Luciferin reagent). A Thermo Scientific Fluoroskan Ascent FL plate reader was used to measure luminescence signal and was set to shake for 2 minutes at 720 rpm, incubate for 10 minutes at 39.5°C and then read luminescence at each time point. Time points were read for uninhibited and inhibited data every hour for 8 hours. ANOVA and Tukey all pairs post hoc statistical tests were performed to determine any statistical significance between each timepoint and between unihibited and inhibited data points.

Antibodies and Reagents

100 µM Calpeptin (CalBiochem; La Jolla, CA), 50 µM Caspase Inhibitor I (Z-VAD-FMK; CalBiochem), 5 µM MG132 (CalBiochem), 50 µM 2-aminoethoxyphenylborate (2-APB) (Sigma), 10 µM Calpain inhibitor III (CalBiochem), 10 µM Calpain inhibitor IV (CalBiochem), 5 µM Lactacystin (CalBiochem), 5 µM Proteasome inhibitor II (CalBiochem), 1 µM Tunicamycin (CalBiochem), 5 µM Brefeldin A (CalBiochem).

Primary antibodies: The following primary antibodies were obtained from BD Pharmingen (San Diego, Ca): FKBP12, FKBP10, and Calreticulin. The following primary antibodies were obtained from Cell Signaling Technology (Danvers, MA): β-Actin, m-Calpain, Lamin, GFP, and Myc. The following primary antibodies were obtained from R & D Systems (Minneapolis, MN): FKBP13 and FKBP38. (Primary antibodies were used at a concentration of 0.5 μg – 1 μg per mL).

Secondary antibodies: Stabilized Peroxidase conjugated Goat Anti-Mouse and Stabilized Peroxidase conjugated Goat Anti-Rabbit (1:3000 dilution) (Thermo Scientific; Waltham, MA).

Results:

Previous work in the lab (Esch 2008) involved the discovery of FKBP10 destruction in tsBN7 cells and to our knowledge, this was the first study looking at FKBP10 under ER stress. Each of the interesting proteins (FKBP10, FKBP12, Rock II, KRIP-1, and p130Cas) found in the Powerblot™ has been implicated in cancer or other disease phenotypes. Understanding the role these proteins play in survival and/or apoptotic signaling may further elucidate their function within the cell and ultimately contribute to a greater understanding of the diseases they are involved in. For this reason we wanted to take a closer look at FKBP10 localization to the ER and analyze it in greater depth under ER stress conditions.

Immunocytochemistry

FKBP10 is a protein that is localized to the ER because of the tetrapeptide HEEL ER retention sequence. This was confirmed with immunocytochemistry images. Fig. 4A contains images of tsBN7 cells that were transfected with a plasmid containing an ER specific Red Fluorescent Protein (RFP) and FKBP10 was detected with antibodies. These images confirm that FKBP10 is localized to the ER in non-stressed cells. In cells that were stressed for 12 hours at 39.5˚C, the FKBP10 signal decreased in intensity and ER localization was diminished. This leads to a conclusion that FKBP10 (or a possible cleavage product) is shuttled out of the ER to get destroyed as a response to ER stress.

Fractions

ER stress was activated in tsBN7 cells by temperature shifting them for 6 and 12 hours and subcellular fractionation was performed. Western immunoblotting of the proteins from the

fractions demonstrated a loss of full length FKBP10 (72 kDa) in the membrane fractions following ER stress. A cleavage product of about 30 kDa appeared and began accumulating in the cytosolic fraction as full length (72 kDa) FKBP10 disappeared from the membrane fraction. The cleavage product in the cytosol strengthens the model that we have proposed which states that retrotranslocation of FKBP10 from the ER to the cytosol occurs as a result of ER stress (Fig. 4B).

Effects of Inhibitors

Despite our conceptual model that FKBP10 is being destroyed as it is retrotranslocated from the ER, the mechanism for FKBP10 destruction remains unknown. Previous work in our lab prompted the idea that the proteasome and calpain may be involved. It was demonstrated that the proteasome can be inhibited and that inhibition is sufficient enough to block FKBP10 destruction. The calcium dependent enzyme called calpain can also destroy FKBP10. Purification of FKBP10 via immunoprecipitation was performed and active calpain enzyme was added. When active calpain was added, cleavage of FKBP10 was observed. These data give us reason to believe that calpain plays a role in cleavage of FKBP10 similar to the proteasome. One question that we wanted to answer was the extent to which each of several calpain and proteasome inhibitors was able to rescue FKBP10 destruction. Experiments confirmed that FKBP10 localization to the ER deteriorates as the protein is destroyed, but it was still uncertain if the inhibition of the proteasome or calpain could effectively block FKBP10 destruction.

tsBN7 cells were treated with an array of different inhibitors as we induced ER stress in these cells by temperature shifting them. Whole cell lysates were obtained and a western blot for this inhibitor profile was executed. Each of the three calpain inhibitors used: Calpeptin, Calpain

inhibitor III, and Calpain Inhibitor IV, were able to somewhat block the destruction of FKBP10 (Fig. 5A). Higher levels of FKBP10 protein are seen in samples containing calpain inhibitors than in the uninhibited termperature stressed sample. All three of the proteasome inhibitors applied: MG132, Lactacystin, and Proteasome inhibitor II, were able to effectively block FKBP10 destruction as well. Some proteasome inhibitors are known to also inhibit calpains which suggested the use of lactacystin as a proteasome inhibitor because it exclusively inhibits the proteasome and not calpain. Even with lactacystin, a protection of FKBP10 against destruction was observed.

Proteasome Assay

Since inhibiting the proteasome was sufficient to block FKBP10 destruction, we hypothesized that the proteasome might be involved in this part of the ER stress story. We predicted that proteasome activity would increase immediately following ER stress because it is responsible for actively destroying most of the unfolded proteins within the ER This question was addressed by measuring the proteasome activity in cells experiencing ER stress using a proteasome luminescence assay. Fig. 5B shows a graph of the data from the replicates of the proteasome assays and from this graph we can conclude that the proteasome is indeed active in our tsBN7 cells. A lack of statistical significance between timepoints of cells experiencing ER stress leads us to conclude that proteasome activity does not immediately increase with temperature stress exposure. Proteasome activity remained relatively constant as exposure time to ER stress increased. We also verified that proteasome activity can be effectively inhibited in our tsBN7 cells with statistical significance indicating that the proteasome is undeniably active in our tsBN7 cells. The proteasome assay data along with the inhibitor profile western blot from

Fig. 5 demonstrate that the proteasome and calpain are operating in our cell line and that the use of inhibitors sufficiently blocks FKBP10 destruction.

Mutagenesis Design

Mutagenesis of each calcium binding domain with the EF-hand domains was proposed in an attempt to eliminate calcium binding to FKBP10. When calcium gets released from the ER as a part of the ER stress response, there is less calcium available to bind to FKBP10. When FKBP10 is not bound to calcium, we hypothesized that it is shuttled out of the ER by means of retrotranslocation to be destroyed. I proposed that a mutation within the calcium binding domain would strongly affect the stability of the protein. Three different tagged vectors with the FKBP10 ORF were obtained to be employed in this study.

After a widespread study of the ORF nucleotide sequence, I discovered that there were no unique restriction enzyme cut sites near the targeted calcium binding domains to perform a simple deletion. Instead, a site directed mutagenesis procedure from Stratagene called Quikchange Lightning was utilized. The mutagenesis strategy aimed at creating three mutant constructs per plasmid. Some modifications to the cDNA constructs were necessary before mutagenesis of the calcium binding domains could be performed.

FKBP10 possesses an ER retention HEEL domain at the C-terminus of the protein. The constructs that were purchased all contain a tag that is fused to the C-terminus which could have potentially disrupted this important ER retention signal. A HEEL domain was inserted into each of the three plasmid constructs at the very C-terminus of the nucleotide sequence coding for the tag. This would ensure that the HEEL ER retention signal that is crucial for keeping FKBP10 inside the ER lumen would not be disrupted by being at the C-terminus of the tagged version of

our DNA sequence.

A short linker consisting of only the smallest of amino acids (glycine and serine) was inserted to separate the target protein from the tag. A four amino acid sequence is proposed to be long enough to put space between the proteins but to also maintain protein function of both the ORF and the tag. A GSGS flexible linker insert was the second mutagenesis reaction that was done and it was performed on the HEEL mutant species of our three plasmids. These two mutagenesis reactions were performed in order to generate plasmid constructs containing our FKBP10 cDNA that would produce protein that behaves exactly like the native protein. By inserting a HEEL domain at the C-terminus to keep the protein in the ER and inserting a GSGS linker between the ORF and the tag that would aid in maintaining proper protein function, we expected that ectopically expressed FKBP10 would perform in a similar manner to native protein when exposed to ER stress. When both the HEEL and GSGS were introduced into our three plasmids, we considered these our new wild-type plasmids.

After extensive analysis of the nucleotide sequence, it was determined that both calcium binding domains in FKBP10 have double glutamates at the end of the sequence. This double glutamate, as described in the introduction, strengthens calcium binding. In order to diminish calcium binding, the glutamates from the calcium binding domains need to be mutated to ensure that calcium binding has been eliminated. We know that the glutamates play a crucial role, if not the most important role, in calcium binding to FKBP10. I proposed to replace the glutamates of each calcium binding domain with lysine (involving a replacement of adenine for guanine in the first position in all glutamate coding codons). Lysine is a positively charged amino acid due to the amino group in the side chain. In lysine, the critical oxygen of the carboxyl group is missing, which has an affect on calcium binding. Additionally, of the positively charged amino acids it is

the closest in size to glutamate. The positive charge reduces the charge interactions between the amino acid side chain and the calcium ion, resulting in a decrease for calcium affinity to the calcium binding site and reduced calcium association rate (Kesvatera et al. 2001)

The mutagenesis involved mutating each wild-type plasmid containing the HEEL and GSGS mutations three times. The first mutagenesis involved mutating the first calcium binding domain occupying amino acid sites 509-520 (of the 581 amino acids in FKBP10). Specifically, replacement of the glutamates at 519 and 520 to Lys519 and Lys520 generated the EF-hand 1 mutants. The second mutagenesis involved mutating the second calcium binding domain occupying amino acid sites 554-565. The mutants generated in this mutagenesis were named EF-hand 2 mutants with Lys564 and Lys565. Finally, double mutants were created that we called EF-hand 1/EF-hand 2 mutants. This mutagenesis was done by taking the EF-hand 2 mutants and mutagenizing the calcium binding domain of EF-hand 1with the primers containing the EF-hand 1 mismatches using Quikchange Lightning. Upon completion of the mutagenesis reactions for each individual EF-hand mutant and the double mutant, 12 mutant plasmids had been generated and these are illustrated in Fig. 6.

Preliminary Analysis of Mutant Constructs

It was essential to confirm that our wild-type constructs were behaving similarly to our native FKBP10 in terms of instability under ER stress and localization to the ER. The first preliminary study involved confirming ER localization of our wild-type plasmids. The monomeric GFP tagged plasmid containing the HEEL and GSGS mutants was transfected into tsBN7 cells along with an ER specific RFP plasmid and imaging was performed. Our images confirm that ectopically expressed FKBP10 is localized to the ER as direct colocalization with

ER specific RFP can be observed. As a control image, non-specific GFP was transfected into tsBN7 cells along with ER specific RFP. These second images show GFP present throughout the cell and ER specific RFP only present in the ER. The two different plasmids are expressed in different places which aids in the confirmation that our FKBP10-GFP plasmid is localized to the ER like native protein**.** (Fig. 7A)

The second study utilized western blotting to analyze the stability of the FKBP10 tagged constructs when exposed to ER stress conditions. Fig. 7B illustrates a western blot of whole cell lysates that were transfected with the Turbo and monomeric Gfp tagged wild type plasmids and then temperature shifted to induce ER stress. A band of 72 kDa disappears in the nontransfected lanes as expected and a band at 72 kDa, the size of the native FKBP10, can be seen in the transfected lanes as well that behaves the same way. Additionally, a band that is 27 kDa bigger than the native FKBP10 band is present in transfected lanes. This band at 99 kDa is expected in transfected lanes as this band confirms the presence of a protein that is the estimated size of our tagged version of FKBP10. This full length protein is getting destroyed at about 12-24 hours as indicated by the accumulation of cleavage products.

FKBP10 in RFL6 cell line

The RFL6 rat lung fibroblast cell line is known to express tropoelastin (Mariani et al. 1998). As stated earlier, FKBP10 is known to associate with tropoelastin lung cells and has been found to behave similarly in these cells. As an introductory study, ER stress was induced in these cells and FKBP10 levels were examined. The western blot in Fig. 8 shows a decrease in FKBP10 levels with tunicamycin treatment. This observation ensures that we are seeing the same phenomenon of ER stress induced destruction of FKBP10 in these cells as we see in tsBN7

cells. In the future, tropoelastin antibodies and western blot analysis will be used in an attempt to pick up tropoelastin in these cells to understand how its levels are affected by ER stress and provide insight into the tropoelastin-FKBP10 interaction.

Discussion:

Over the past several decades, the search for a greater understanding of changes in gene and protein expression and how that affects normal cellular processes has broadened the possibilities and implications for medical advancement. Ultimately, cellular signaling is carried out by proteins, and it is the understanding of protein-protein interactions that will lead to the greatest advancements in modern medicine. A powerful way to analyze the link between genotype and phenotype in genetics is to manipulate gene expression, which essentially alters the gene product. The Powerblot™ data ultimately led our lab to look at the role of the ER localized foldase, FKBP10, in stress signaling. This study investigates the rapid decrease in levels of this essential ER foldase in tsBN7 cells and proposes a potential model for the destruction of FKBP10 via retrotranslocation from the ER and cleavage by the proteasome and calpain. In addition, this study utilizes the overexpression of FKBP10 wild-type protein and FKBP10 with mutant calcium binding domains to analyze the protein's stability.

tsBN7 cells treated at the restrictive temperature initiate stress signaling pathways and eventually cell death if ER stress does not subside. At the restrictive temperature, there is a destabilization of Dad1 from the oligosaccharyltransferase (OST) complex and subsequent depressed N-linked glycosylation. This important posttranslational modification, N-linked glycosylation, and the loss of Dad1 function results in ER stress signaling and ultimately activates apoptosis.

FKBP10 is an ER folding protein that is rapidly destroyed and shuttled out of the ER following ER stress. It is imperative to understand the mechanism for destruction and removal of this foldase from the ER. A consideration of the structural motifs of FKBP10 and their effect on protein stability should help to elucidate the significance of this protein in the cell. The

reason for the rapid destruction of an ER localized foldase upon the onset of ER stress remains a mystery. It seems counterintuitive that a cell, whose survival is threatened by an accumulation of misfolded proteins, would destroy important folding machinery. Many other chaperone proteins are upregulated following ER stress so the question of what distinguishes FKBP10 from these other enzymes is raised. The rapid disappearance of FKBP10 following ER stress suggests that the cell is not attempting to destroy all proteins in a self destruction mechanism as the cells progresses towards apoptosis but rather that the cell is deliberately attempting to rid the cell of this particular foldase.

The abrupt nature of the destruction of FKBP10 suggests that the interesting phenomenon taking place is a proteolytic cleavage of the protein instead of a natural protein turn over rate. Previous data in the lab suggested the possibility that FKBP10 is being proteolytically destroyed by a member of the Ca^{2+} dependent protease family of calpains. An array of protease inhibitors specific to either the proteasome or calpain was used on our tsBN7 cells and it was found that all of the inhibitors were sufficient to block FKBP10 destruction. This suggests that not only does calpain play a role in FKBP10 destruction, but so does the proteasome. Proteasome and calpain assays demonstrated that while the proteasome and calpain are not acutely induced over the timecourse of FKBP10 destruction, they are indeed active in our tsBN7 cells.

Immunocytochemistry and subcellular fractionation demonstrated decreased ER localization of FKBP10 following ER stress. The cleavage product that accumulated in the cytosol gives us reason to propose a model for retrotranslocation of the protein from the ER to the cytosol as a result of ER stress. FKBP10, or its cleavage product, is shuttled out of the ER after being signaled for destruction.

Calcium release from the ER is pivotal in terms of apoptotic signaling. In this study, we question whether the stability of FKBP10 is contingent on calcium binding. We wanted to study the behavior of FKBP10 when calcium binding is eliminated. With ER stress, we believe FKBP10 is shuttled out of the ER to be destroyed and we questioned whether this was calcium binding dependent. This specific characteristic of FKBP10 was studied using mutagenesis to alter the calcium binding domains and essentially affect FKBP10's ability to bind calcium and perform normal protein functions.

Once the mutant constructs were generated, preliminary analysis was performed using western blotting and immunocytochemistry. The western blot of transfected cell lysates demonstrates that the tagged version of our protein is behaving like native FKBP10. Full length FKBP10 disappears following ER stress like the native and a larger band which we presume to be tagged FKBP10 also appears to be destroyed. We think that a complete destruction of this version of the protein is not seen because the protein is being really overexpressed in our cells. In addition, there are some very large bands at the top of the gel image that we predict to be ubiquitinated versions of our protein. Further experiments may involve looking at ubiquitination levels and how FKBP10 stability is affected.

We suggest a mechanism of destruction for FKBP10 as a possible proteolytic destruction by calpain and the proteasome. This study emphasizes a possible model for retrotranslocation of FKBP10 from the ER as a means of signaling the destruction of this protein and its possible cargo. FKBP10 is known to carry cargo, including tropoelastin. We have proposed a model that FKBP10 carries its cargo with it as it gets destroyed as a means to get rid of tropoelastin. Tropoelastin might be risky for the cell if it spontaneously forms insoluble fibrous networks within the ER. This is a common occurrence under ER stress conditions and the proposed model

suggests that ER stress signaling rapidly destroys proteins like tropoelastin that form aggregates. The ER destroys cargo proteins of protein carriers (like FKBP10) by rapidly removing the protein/carrier complex (Fig. 9).

Recent preliminary studies in RFL6 cells have attempted to investigate the FKBP10/tropoelastin association. FKBP10 has been successfully detected with an antibody in these cells and it has been found to decrease with ER stress similar to the behavior in tsBN7 cells. In future studies, we hope to detect tropoelastin in these cells and observe the behavior of tropoelastin expression as a response to ER stress. We are interested in the sequence of events as well as the biological significance of the proteolytic destruction of FKBP10 and its cargo proteins.

In the future, we hope to analyze the 12 EF-Hand mutant constructs and observe the effect on protein stability and FKBP10 destruction when calcium binding is eliminated. Subcellular fractionation of cells transfected with mutant plasmids would confirm co-localization to the ER and the behavior of the mutants under ER stress conditions. In addition, coimmunoprecipitation studies to find other potential cargos of FKBP10 is a priority in order to help us understand the reason for FKBP10 destruction in the cell.

In summary, FKBP10 is localized to the ER in unstressed cells but cleavage products accumulate in the cytosol following ER stress. We propose that FKBP10 is being cleaved by the proteasome and/or calpain as a means for the cell to destroy FKBP10's cargo proteins. Finally, 12 mutant constructs were generated to eliminate calcium binding in order to use an overexpression experiment to observe the effects of the mutants on the phenotypes. This study is essential for understanding the role calcium plays in FKBP10's instability as a result of ER stress.

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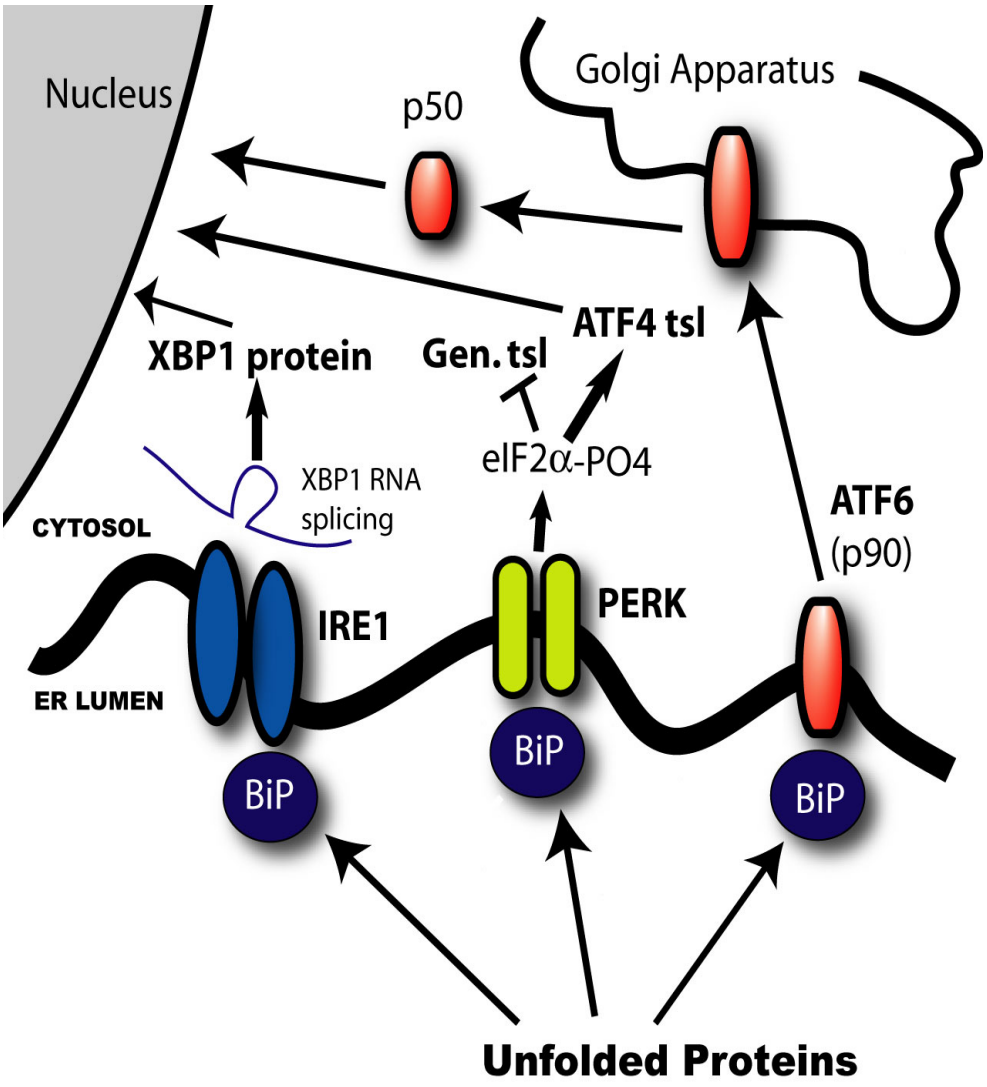


Fig. 1 **Unfolded Protein Response Pathways**
GRP78, or BiP, dissociates from IRE1, PERK, and ATF6 and associates with unfolded proteins as they accumulate in the ER following stress. This initiates UPR stress signaling, an attempt to rescue the cell from death. If ER stress persists the cell initiates apoptosis. IRE1, ATF6, and PERK are three transmembrane proteins that sense and respond to the protein-folding needs of the ER. Each of these molecules activates a specific transcription factor to initiate a distinct UPR signaling branch. The unfolded protein response upregulates the transcription of ER resident foldases.

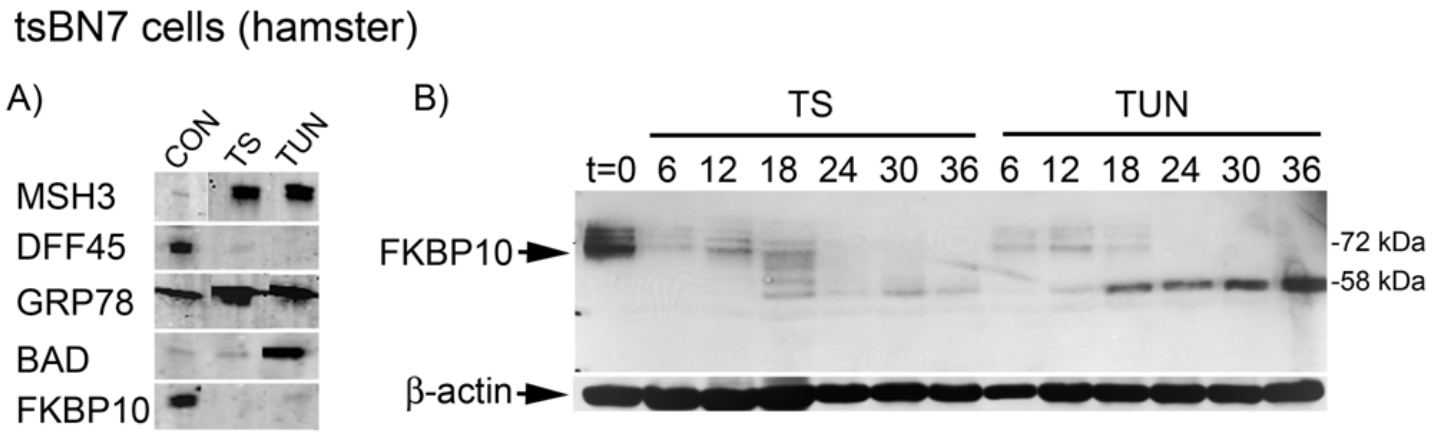
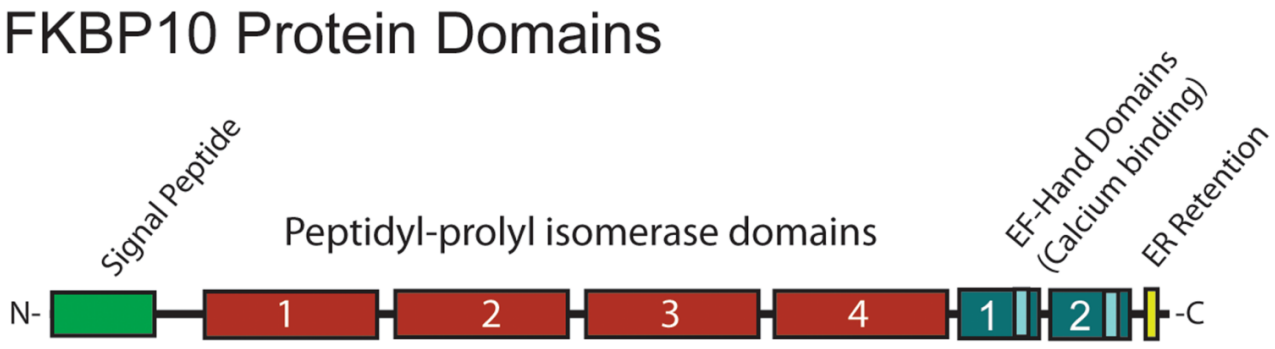


Fig. 2 **FKBP10 proteolysis in cells experiencing ER stress**
These images come directly from Emily Esch's Honors Thesis
(A) A summary of Powerblot™ targets that were chosen based on an interest in the function of the protein in ER stress response. Cells were exposed to control conditions (CON), 39.5°C (TS) or tunicamycin (TUN) for 24 hours. These results are consistent with known results for how the top four proteins behave with exposure to ER stress. DFF 45 gets cleaved in apoptotic cells, GRP78 levels go up with apoptosis (it is an ER protein that mediates protein folding). This is the western blot where the rapid destruction of FKBP10, another ER folding protein, was discovered. (B) Western blot to confirm the rapid destruction of FKBP10 that was found in the Powerblot™. In order to characterize the time course of FKBP65 destruction, cells were stressed with either tunicamycin (1 μM) or temperature shift and harvested at the hour time points indicated. Within 6 hours of induced ER stress, full length FKBP65 is significantly diminished, and by 24 hours it has completely disappeared. An accumulation of a low molecular weight protein, presumably the underglycoslylated form, is also noted between 18 and 36 hours. Folding proteins are usually found to increase as an adaptation to ER stress. It is intriguing that we see a rapid decrease in this foldase.

A)



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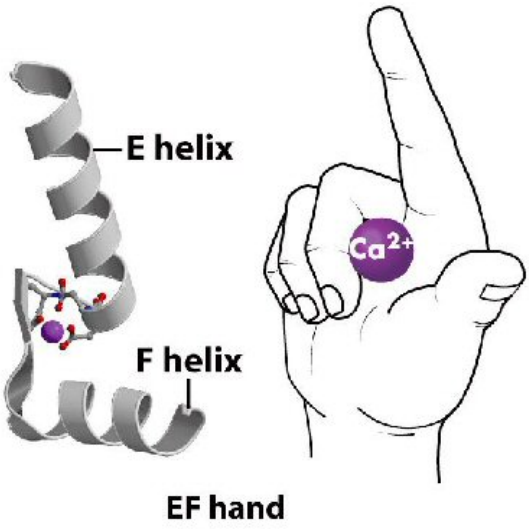

University of Maine, Chemistry Dept.

Fig. 3 **Functional domain organization of FKBP10**
(A) FKBP10 is a 581 amino acid sequence that possesses four PPIase domains and two EF-hand calcium binding domains. An N-terminal signal that targets the protein to the ER and a C-terminal HEEL ER retention signal are among the motifs present in FKBP10. (B) EF-hand domains are found in calcium binding proteins and possess calcium binding domains that are usually 12 amino acids long. EF-hands are a helix-loop-helix structure responsible for sequestering and storing calcium or for using this calcium to bind other proteins. Calcium binds in the loop portion of this site by electrostatic interactions with oxygen atoms of amino acid side chains. Negatively charged amino acids like aspartate and glutamate have a negatively charged oxygen that interacts with the positive charge on the calcium ion.

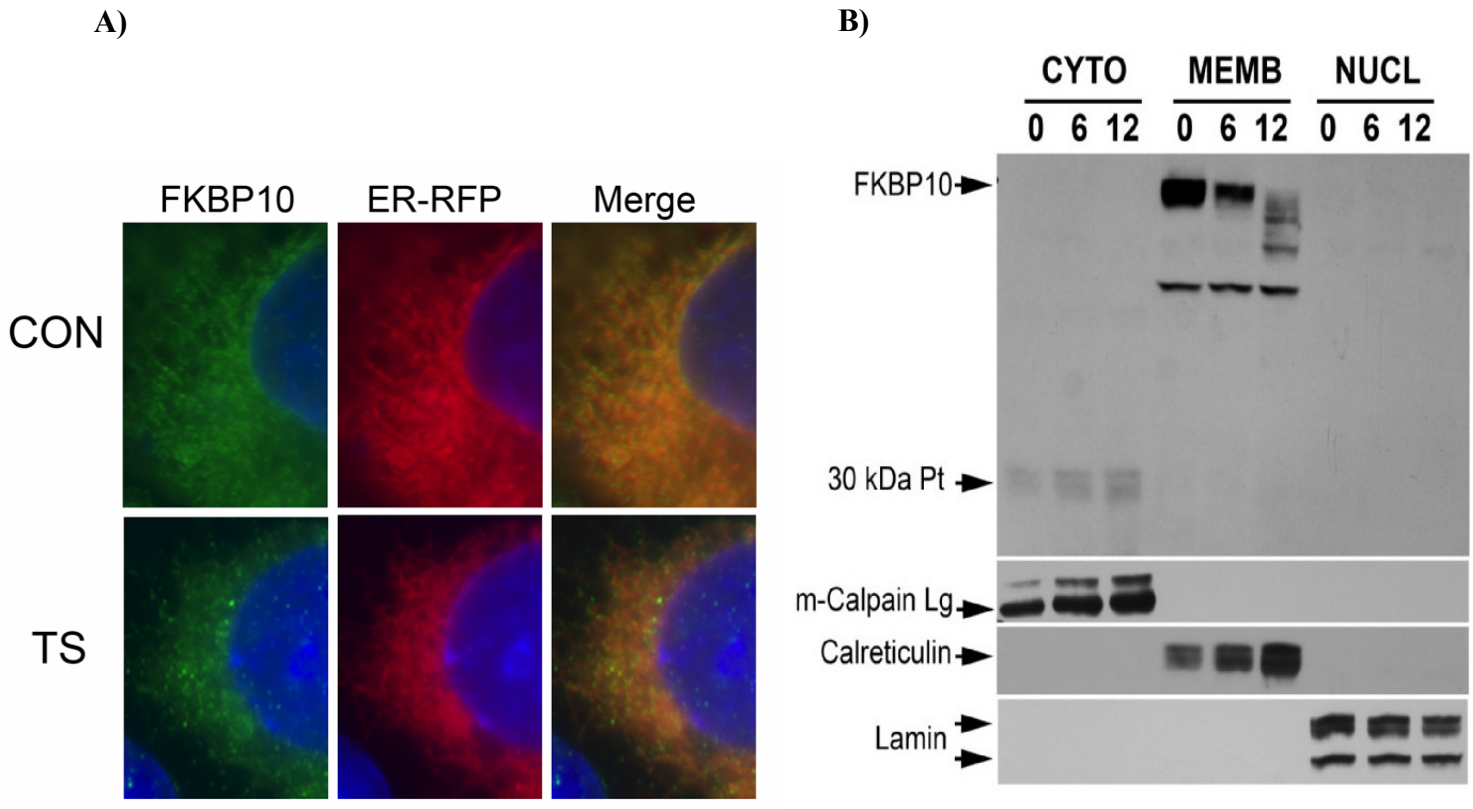

Fig. 4 **ER stress diminishes FKBP10 co-localiztion with the ER.**
(A) tsBN7 cells were transfected with pER-RFP (Invitrogen) to label the ER with RFP. Transfected cells were stressed for 12 hours at temperature-shift (TS) conditions – 39.5ºC and analyzed for FKBP10 localization using an antibody and immunocytochemistry. FKBP10 showed diminished signal intensity and became less ER localized following ER stress. (B) Subcellular fractionation was performed on tsBN7 cell lysates exposed to TS for 0, 6, or 12 hours resulting in cytosolic, membrance (ER, mitochondria), and nuclear fractions. The integrity of each fraction was determined by examining marker for each cellular compartment (cytosol, calpain; ER, calreticulin; nucleus, lamin). Following ER stress, full length 72 kDa FKBP10 localization within the ER is maintained diminishing in intensity while a 30 kDa cleavage product accumulates within the cytosol. The accumulation of a 30 kDa cleavage product is consistent with a model in which FKBP10 or its cleavage products are retrotranslocated from the ER following ER stress.

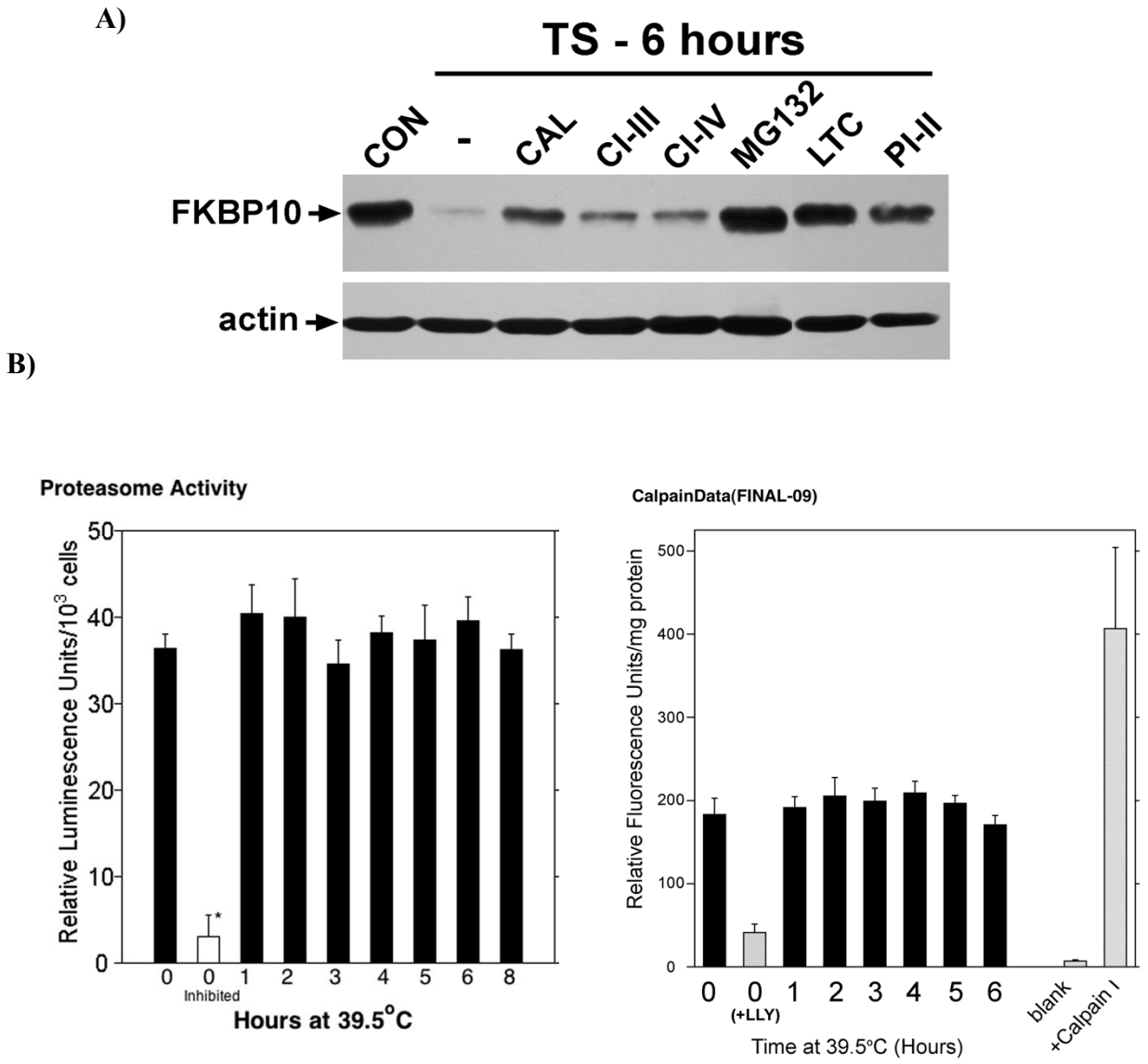

Fig. 5 **The proteasome and calpain are active in tsBN7 cells and the use of inhibitors sufficiently blocks FKBP10 proteolysis.**
(A) Proteasome and calpain inhibitors were utilized to determine if inhibition is sufficient to block FKBP10 destruction. tsBN7 cells were treated with an array of different inhibitors as ER stress was induced in these cells by temperature stressing them for 6 hours. Each of the calpain inhibitors (calpeptin (CAL), calpain inhibitor III and calpain inhibitor IV) was able to partially block the destruction of FKBP10. Each of the proteasome inhibitors (MG132, lactacystin (LTC), and proteasome inhibitor II) was highly effective at inhibiting FKBP10 proteolysiswas able to effectively block FKBP10 destruction. Some proteasome inhibitors also inhibit calpains but lactacystin only inhibits the proteasome and protection of FKBP10 destruction was observed. (B) The graph of proteasome activity demonstrates that the proteasome is not acutely induced over the timecourse that tsBN7 cells were exposed to ER stress and FKBP10 destruction is observed. While there is no obvious increase in proteasome activity, we can conclude from this graph that the proteasome is active in tsBN7 cells. Proteasome activity can be effectively inhibited in tsBN7 cells. (C) The same phenomenon that is seen with the proteasome assay was observed for a calpain assay performed by Emily Esch. There is no induction of calpain activity over the timecourse of FKBP10 destruction with ER stress and the use of inhibitor effectively inhibits calpain activity in tsBN7 cells

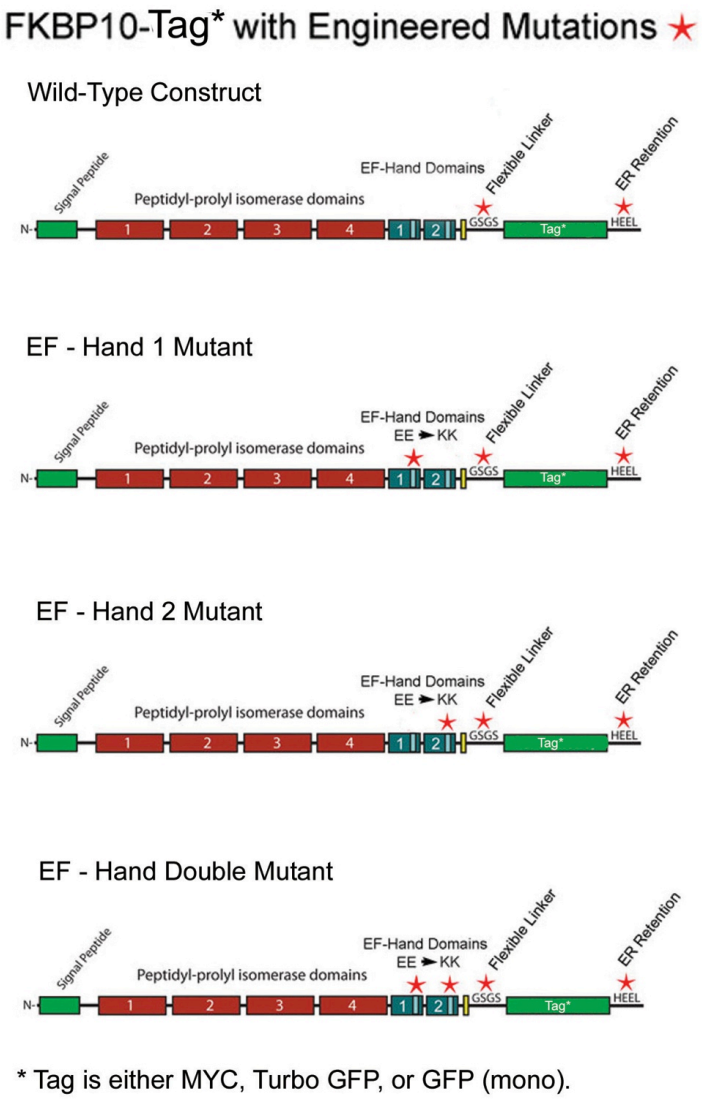

Fig. 6 **Mutagenesis generated 12 mutant constructs**
Each of the plasmids that was obtained from Origene contained the FKBP10 ORF and one of three tags: Myc epitope, GFP (mono), or Turbo GFP. The four mutant constructs illustrated here were generated for each of the three plasmids for a total of 12 mutants. The first mutant constructs to generate "wild-type" plasmid involved addition of a C-terminal HEEL ER retention sequence and a GSGS linker between the ORF and the tag. The EF-hand 1 mutant was made by mutating the calcium binding domain of the first EF-hand domain of FKBP10 (double glutamate was changed to a double lysine). The EF-hand 2 mutant was generated in the same manner for the calcium binding domain of the second EF-hand domain of FKBP10. The double mutant possesses mutated calcium binding domains within both of the EF-hand domains of FKBP10.

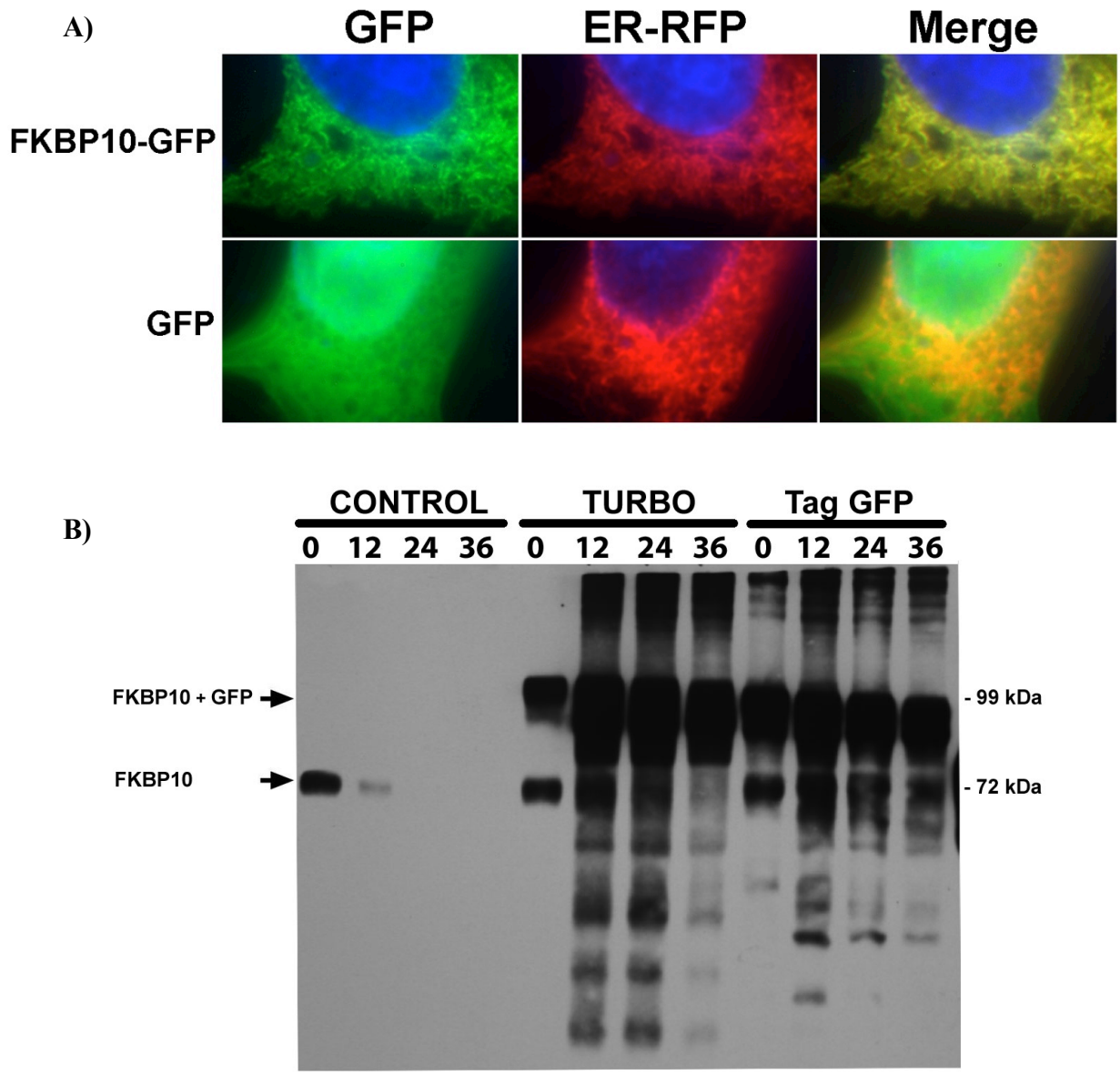

Fig. 7 **Preliminary analysis of wild-type mutant constructs**
(A) Confirmation of the stability of the tagged protein under ER stress and its localization to the ER was done with imaging. These microscope images show that the GFP(mono) tagged version of FKBP10 colocalizes to the ER. The second set of images illustrate a control of non-localized GFP and ER specific RFP. In these images, the RFP can be seen to fluoresce in the ER while GFP is present throughout the cell. (B) A western blot of transfected cell lysates (Turbo and monomeric GFP wild-type plasmids) illustrates a tagged protein that is behaving like native FKBP10. A 72 kDa band of FKBP10 is noted to disappear with ER stress similar to the native. A 99 kDa band (27 kDa bigger than the FKBP10) is seen in this western blot and is getting destroyed between 12 and 24 hours, as indicated by the accumulation of cleavage products. This size 99 kDa band is the expected size from the GFP tagged version of the protein.

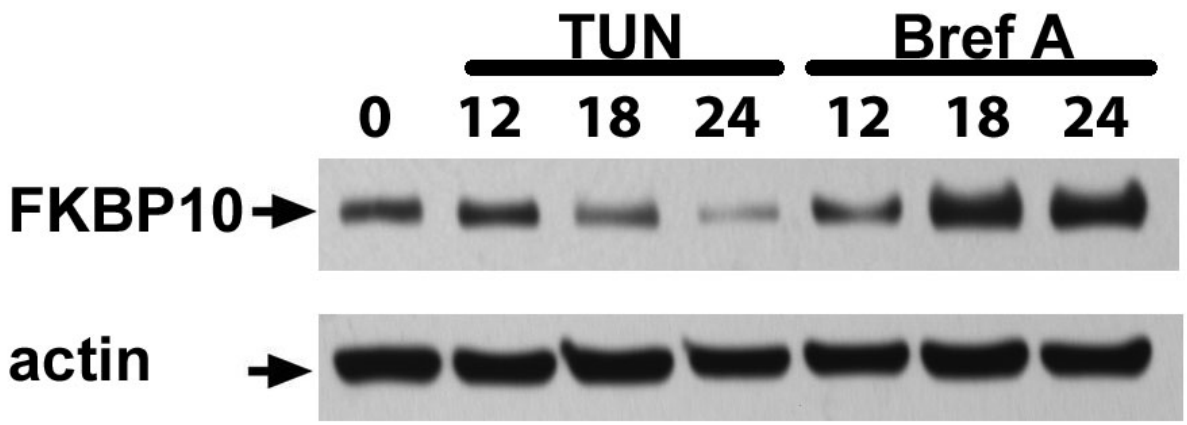

Fig. 8 **FKBP10 is rapidly destroyed in RFL6 cells**
A western blot on the rat lung fibroblast cell line (RFL6 cells). These cells are known to express tropoelastin. FKBP10 is known to associate with tropoelastin in these cells. This western blot analyzes ER stress in these cells induced with tunicamycin treatment and Brefeldin A treatment in which FKBP10 depletion is observed. This confirms that the same phenomenon of ER stress induced destruction of FKBP10 is occurring in the RFL6 cells similar to the tsBN7 cells.

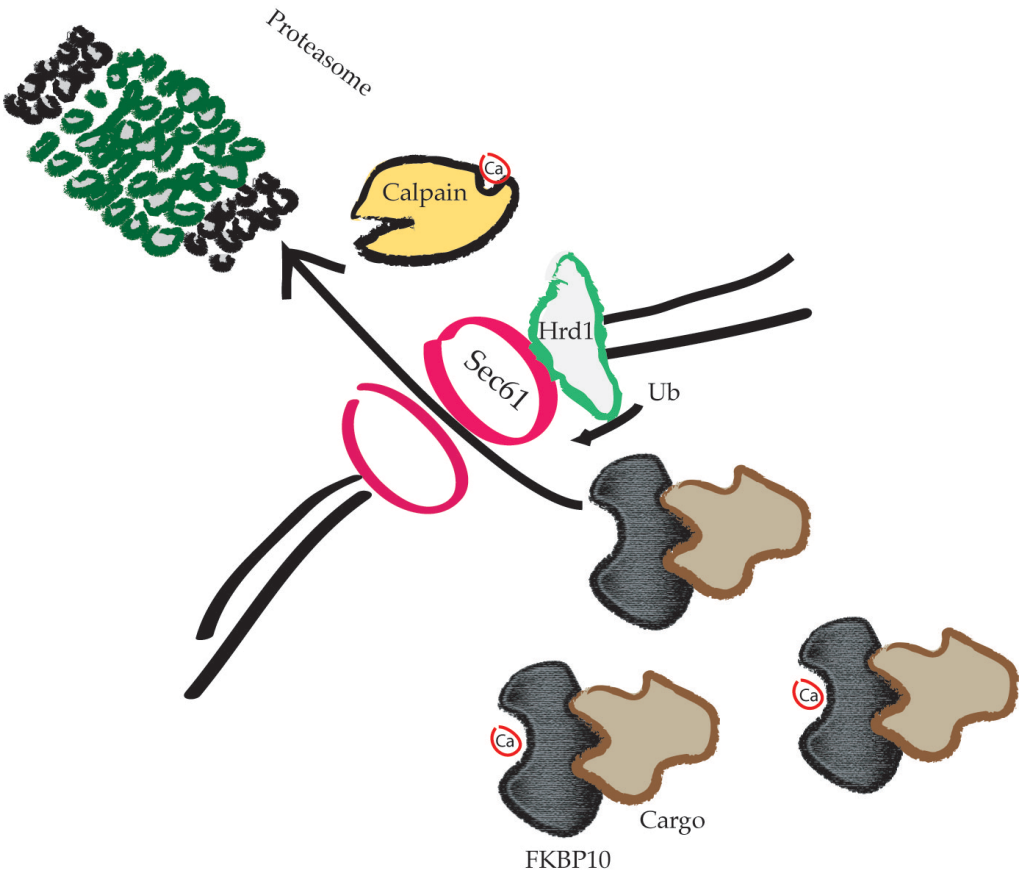

Fig. 9 **Model of FKBP10 retrotranslocation for destruction**
This study involves the characterization of the protein, FKBP10, that is known to carry cargo such as tropoelastin and other proteins involved in forming extracellular connective networks. This cartoon demonstrates a model that FKBP10 carries cargo with it, like tropoelastin. Tropoelastin is risky for the cell as it can spontaneously form insoluble fibrous networks with ER stress. This model suggests that the ER rapidly destroys proteins like tropoelastin that are at risk of forming aggregates inside the organelle by rapidly removing them and their carriers from the ER to the cytosol. This retrotranslocation might be associated with ubiquitination in which the protein is triggered to be destroyed by the proteasome and calpain. The studies discussed in this honors thesis have focused on calcium's role, especially in this retrotranslocation model.