The Effects of Rapamycin on the Unfolded Protein Response in Relation to Healthy Aging

by
Stephanie Anne Zhao

A THESIS

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Biology
(Honors Scholar)

Presented May 13, 2016
Commencement June 2016

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Abstract approved:

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Viviana Perez

The loss of proteostasis is the mechanism for maintaining properly folded proteins and degrading misfolded ones. One protective mechanism against loss of proteostasis is the Unfolded Protein Response (UPR), a chaperone mediated folding system in the Endoplasmic Reticulum (ER). The UPR communicates with the Nrf2 pathway, a transcription factor, and rapamycin, a drug that increases health span in several mammalian models.

We hypothesized that the protective effects of rapamycin can be mediated by activation of Nrf2 to regulate UPR pathways. Nrf2 WT and KO mouse embryonic fibroblast cells were pretreated with rapamycin under positive stressors, and key UPR chaperone protein levels and activation were measured by standard Western blot and polymerase chain reaction protocol.

In general, rapamycin pretreatment protected against Thapsigargin most efficiently in WT cells. Nrf2 KO cells showed overall lower levels of chaperone protein activation, indicating Nrf2 deficiency increasing cell susceptibility to ER stress. The data also indicated that Nrf2 has important buffering capacity in certain UPR pathways, as variation of protein expression changed in only WT cells. Therefore, our hypothesis seems to be supported by the data and we further hypothesize that rapamycin's effects would decrease protein aggregation and increase health span through regulating the UPR response.

Key Words: health span, rapamycin, unfolded protein response, Nrf2

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Introduction

Aging as a Factor of Protein Homeostasis

Aging is an observable time-dependent decline that is experienced by all species, including humans (Lopez-Otin et al., 2013). The rising interest in an aging population results from an increase of around thirty years in life span in developed countries (Brown & Naidoo, 2012). This extension in life span occurred over the last decade and is projected to continue increasing throughout the century. As a result of a growing elderly population, disease epidemiology now faces an increase in the number of age-related diseases, i.e., Dementia and Alzheimer's cases are expected to rise to 66 million in 2030 and to over 115 million in 2050 (Alzheimer's Association, 2012). Other common diseases associated with aging include metabolic disorders and Type 2 diabetes mellitus, atherosclerosis, and some cancers (Brown & Naidoo, 2012).

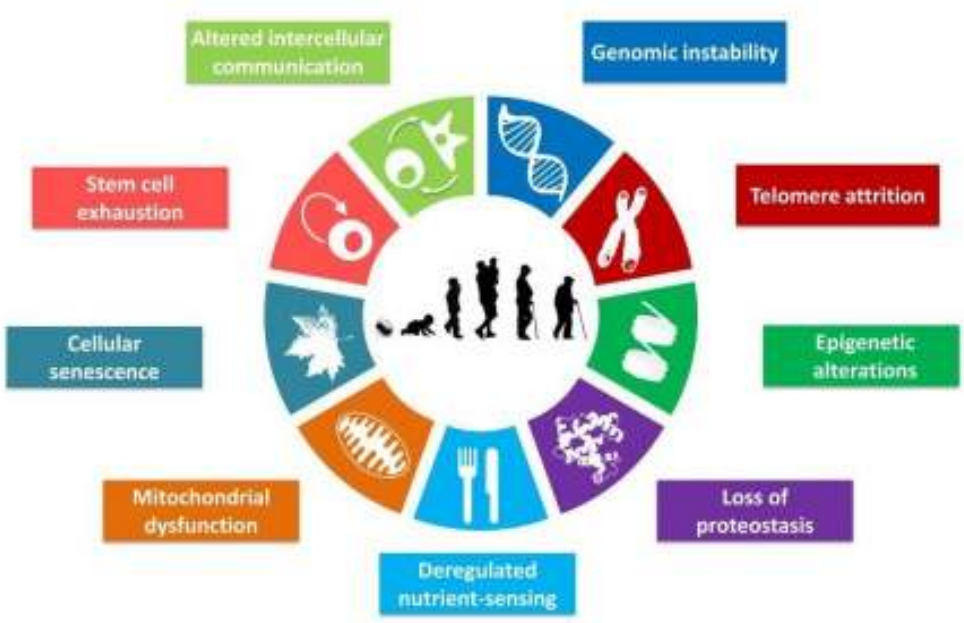

Figure 1: Hallmarks of Aging (Lopez-Otin et al., 2013)

There are a number of hallmarks developed to explain how aging occurs, as shown in Figure 1. In our lab, we focus primarily on the loss of proteostasis. Proteostasis is defined as a mechanism for stabilizing proteins in their correct conformations and degrading those that are not (Ron & Walter, 2007). In many age-related diseases, normal soluble proteins are converted to insoluble

fibrils or aggregates that can accumulate in different organs. Aggregates are toxic to cells, difficult to degrade, and present in neurodegenerative diseases such as Alzheimer's, Parkinson's, Amyotrophic Lateral Sclerosis (ALS), and Huntington's (Brown & Naidoo, 2012).

Protein homeostasis is maintained by chaperone protein systems (heat shock proteins and the Unfolded Protein Response), proteasomal degradation, and autophagy. Perturbations, such as oxidative or nutritional stress, disrupt proteostasis and activate protective responses in the cell. Chaperone proteins help to refold misfolded proteins to prevent protein aggregation under low levels of perturbations. However, if perturbations are high, these proteins can be degraded by the Ubiquitin-proteasome system. Larger scale degradation of aggregated proteins and cell organelles is achieved through autophagy (Lilienbaum, 2013). These disturbances can result in Endoplasmic Reticulum (ER) stress, which increases the amount of misfolded proteins and formation of toxic aggregates. The Unfolded Protein Response (UPR) is a protective mechanism to prevent this by working to preserve protein homeostasis in the ER. Consisting of three signal pathways, the UPR upregulates chaperone proteins to prevent the formation of protein aggregates, and ensure that proteins reach their correct conformations.

My project focuses on the UPR, one of the cell's quality control mechanisms to combat ER stress and maintain proteostasis. Polypeptides destined for the membrane or for secretion are synthesized, modified, and folded in the ER (Brown & Naidoo, 2012). The UPR resides in the ER and has three key functions: induce chaperone translation, attenuate general protein translation, and degrade misfolded and unfolded proteins to maintain cell homeostasis. This is especially important because protein folding is a relatively inefficient process, with almost a third of all proteins not reaching full conformation (Romisch, 2004). During the aging process, it is observed that the protective component of the UPR declines while the apoptotic outcome is more favored (Naidoo, 2009). The observation that chaperones are increasingly oxidized with time contributes to this functional decline. Therefore, the decrease in critical ER chaperones and folding enzymes, as seen with aging, can negatively affect correct protein folding and UPR response.

The Unfolded Protein Response (UPR)

The Unfolded Protein Response (UPR) is a stress response that is activated by many stressors including, but not limited to, energy depletion, nutrient depletion, calcium disturbances, ischemia, viral infections, and redox status (Kaufman et al., 2002). Disrupting ER homeostasis can lead to an

accumulation of unfolded proteins (Ballou & Lin, 2008) and an upregulation of several signaling pathways to either activate ER chaperones, attenuate protein translation, or send signals to degrade misfolded proteins. An ER chaperone functions to promote proper folding and prevent aggregation (Ron & Walter, 2007), and the progressive failure of these chaperone systems is a mechanism used to explain the relationship between aging and cell function decline (Brown & Naidoo, 2012).

Restrictions on these quality control mechanisms prevent misfolded or incomplete proteins from leaving the ER to downstream sites (Brown & Naidoo, 2012). Chaperones detect exposed hydrophobic regions, unpaired cysteines, and aggregates, and bind to them until the target proteins are refolded or degraded. During transient stress, chaperones hold onto these proteins to allow for folding or refolding until stress is resolved (Lopez-Otin et al., 2013). With aging, the efficiency of these chaperones is significantly diminished, leading to an increase in misfolded proteins.

Stress signals are received and transduced across the ER membrane by three integral transmembrane proteins of the UPR: Activating Transcription Factor 6 (ATF6), PKR-like ER kinase (PERK), and Inositol Requiring Element-1 (IRE-1). These are bound to a chaperone protein known as Binding immunoglobulin protein (BiP), and held in an inactive state (Prischi et al.,

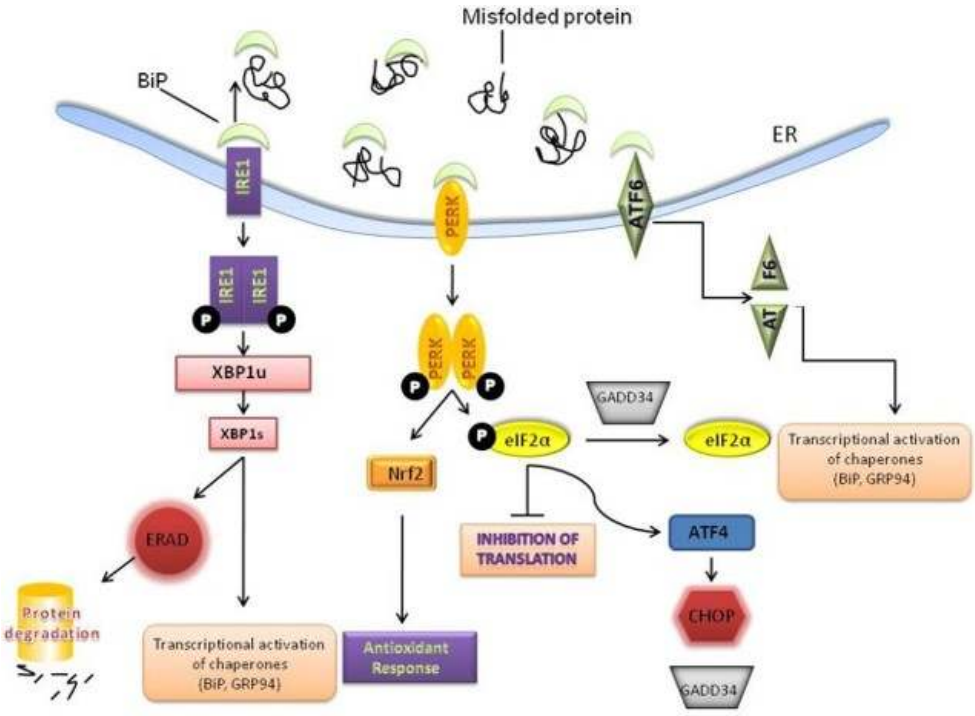

Figure 2: The Unfolded Protein Response Pathways (Brown & Naidoo, 2012)

2014). These pathways will determine the final outcome depending on the overall balance of unfolded to folded proteins: cell survival or cellular death.

Activating Transcription Factor 6 (ATF6)

As seen in Figure 3, Activating Transcription Factor 6 (ATF6) is released from the ER membrane and is activated by posttranslational modifications. After being transported and translocated into the Golgi apparatus, ATF6 is cleaved by site-1 protease (S1P) and site-2 protease (S2P) (Yoshida et al., 2003) and released to the cytosol as a 50 kilo Dalton (kDa) fragment (Ron & Walter, 2007). This ATF6 fragment (ATF6f) translocates into the cell nucleus to change gene expression (Yoshida et al., 2003) of ER chaperone coding genes to increase their transcription levels and upregulate the UPR.

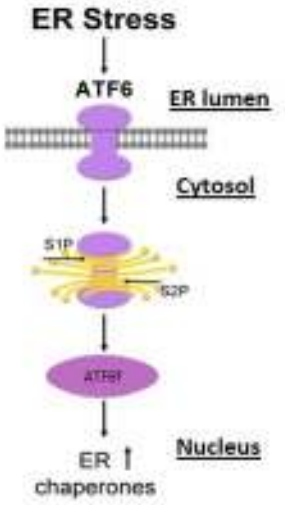

Figure 3: ATF6 Pathway (Fazio, Jiang, and Lajoie, 2014)

PKR-like ER Kinase (PERK)

Figure 4 shows PKR-like ER Kinase (PERK), a Type 1 transmembrane serine threonine kinase that auto-phosphorylates upon activation (Harding et al., 2000). The cytoplasmic portion of PERK contains a protein kinase domain that phosphorylates the alpha subunit of eukaryotic translation initiation factor-2 (eIF2a) (Ron & Walter, 2007). Phosphorylated eIF2a (P-eIF2a) causes a general decrease in protein translation, with the exception of certain ER chaperone proteins (Brown & Naidoo, 2012). P-eIF2a also

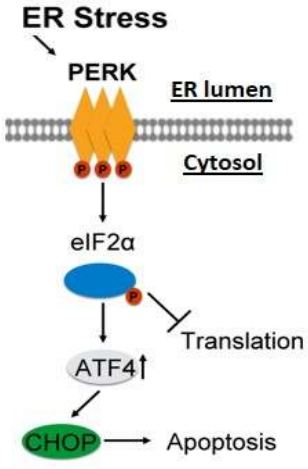

Figure 4: PERK Pathway (Fazio, Jiang, and Lajoie, 2014)

functions to activate ATF4 (Li et al., 2014), a transcription factor that upregulates autophagy genes (B'chir et al., 2013)

Inositol Requiring Element-1 alpha (IRE-1a)

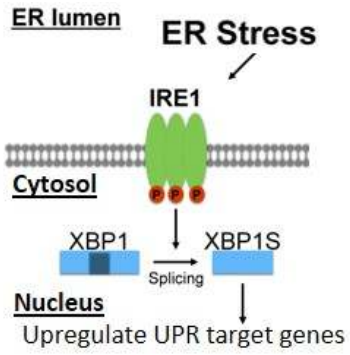

Figure 5: IRE1 Pathway (Fazio, Jiang, and Lajoie, 2014)

Figure 5 shows a diagram of Inositol Requiring Element-1 alpha (IRE-1a), an auto-phosphorylating kinase and endoribonuclease (Ron & Walter, 2007). When activated by an accumulation of misfolded proteins in the ER, the luminal domain dimerizes to position the cytoplasmic kinases close enough to allow auto-phosphorylation to occur (Prischi et al., 2014). The endoribonuclease domain of IRE-1a excises an intron from mRNA encoding X-box binding protein-1 (XBP-1), a UPR-targeted transcription factor (Yoshida et al., 2003). Splicing a 26-nucleotide sequence from the XBP-1 mRNA encodes for a transcriptional activator that increases chaperone protein levels (Ron & Walter, 2007).

Rapamycin

Rapamycin is a macrocyclic antibiotic produced by the bacterium *Streptomyces hygroscopicus.* Rapamycin is found to increase both life span and health span in mice (Miller et al., 2011). Health span is defined as the number of healthy years lived, whereas lifespan is the total number of years lived. Rapamycin pretreatment buffers against cell death from apoptosis (Kapuy et al., 2014), while rapamycin-treated mice show delayed development of many age-related pathologies (Wilkinson et al., 2012). Rapamycin is an allosteric inhibitor of the Mammalian Target of Rapamycin (mTOR) (Ballou & Lin, 2008). mTOR is a protein kinase involved in cell growth, proliferation, and survival in the presence of adequate energy and nutrients. Under plentiful conditions, mTOR inhibits autophagy (Kapuy et al., 2014). However, mTOR also plays a pro-apoptotic role during extreme stress, feeding into the activation of downstream pathways of IRE-1a and PERK of the UPR. The UPR is also suggested to have upstream control of mTOR activation, signifying a feedback loop that ultimately determines cell survival or death (Kapuy et al., 2014). Mutations that decrease mTOR activity are

observed to increase lifespan in flies, nematodes, and yeast. Mice with mutated genes for Growth Hormone (GH) and Insulin-like Growth Factor (IGF-1), or calorie restricted diets, show decelerated aging and diminished mTOR activity (Wilkinson et al., 2012). In these experiments, we will be pretreating with rapamycin to see how it influences the activation of certain UPR pathways under ER stress. In this study, we will be measuring the levels of these proteins and other key chaperones in their downstream pathways to determine whether there is activation of the UPR.

Nuclear Factor Erythroid 2-related Factor (Nrf2)

Nrf2 is a transcription factor that regulates several hundred molecules, including molecular chaperones, and plays a protective role in the cell (Lewis et al., 2015). Known as the master regulator of ER stress, Nrf2 helps to slow the onset of age-related diseases and extend maximum lifespan potential (MLSP) (Bruns et al., 2015). After activation by phosphorylation, Nrf2 travels to the nucleus and upregulates transcription of protective proteins involved in proteostasis and degradation by binding to antioxidant response elements (AREs) on target genes. Nrf2 is regulated by Kelch-like ECH-associated Protein-1 (Keap1), which binds and targets Nrf2 for ubiquitination by the proteasome in the cytosol. In long-lived species, Keap1 is downregulated, resulting in higher levels of Nrf2 expression (Lewis et al., 2015). Rapamycin is observed to interact with Nrf2 and its signaling pathway, with upregulated Nrf2 protein and transcript expression in fibroblast tissue induced by chronic rapamycin treatment (Bruns et al., 2015). UPR machinery under stressful ER conditions can also induce the Nrf2 pathway (Digaleh et al., 2013). Nrf2 activation and downstream signaling is upregulated in long-lived species in both stressed and non-stressed conditions (Lewis et al., 2015). Nrf2 KO animals are also observed to have a slower recovery from age and acute stress events (Muthusamy et al., 2012) compared to WT animals. Preliminary data from our laboratory shows that pretreating with Rapamycin increases Nrf2 protein and transcription levels. Previous literature shows that Nrf2 modulates ER stress, which activates the UPR, and is also upregulated itself by the UPR signaling cascade. We expect that Nrf2 KO cells will have a deficient UPR response due to the lack of Nrf2 protein and that the upregulation of these protective functions by rapamycin will decrease protein aggregation and ultimately slow the aging process. Therefore, we hypothesize that the protective effects of rapamycin in health and aging are mediated by activation of Nrf2 to regulate UPR pathway activity to decrease protein aggregation and slow the effects of aging.

Material and methods

Cell Culture

Nrf2 wildtype (WT) and knockout (KO) mouse embryonic fibroblast (mef) cells were cultured using Iscove's Modified Dulbecco's Medium (IMDM) High Glucose, 10% fetal bovine serum, and 1% penicillin or streptomycin media in 6-well culture dishes. Cells were obtained from laboratory stock used in previous experiments. Upon reaching 80% confluency, half these cells were pretreated with 25 nM rapamycin for 24 hours, the optimal time and dosage as determined from previous experiments. Next, selected wells were treated with two known ER stress activators: 3 mM Thapsigargin and 10 mM of Tunicamycin for 6 hours before harvest. Thapsigargin functions to inhibit ER calcium ATPase, which decreases calcium levels. Calcium-dependent chaperone activity is attenuated and unfolded proteins accumulate (Oslowski & Urano, 2013). Tunicamycin inhibits the first step in glycoprotein biosynthesis, resulting in an accumulation of unfolded proteins in the ER (Oslowski & Urano, 2013). These positive control time and dose courses were determined to be optimal by previous experiments in the laboratory. Thus, our experiments have a total of six treatments as follows: Basal control (no treatment), rapamycin, Thapsigargin, Thapsigargin plus rapamycin, Tunicamycin, and Tunicamycin plus rapamycin.
To harvest for Western blot experiments, the cells were washed with warm Dulbecco's phosphate-buffered saline (DPBS) and treated with a lysis buffer (Radioimmunoprecipitation assay (RIPA) buffer containing phosphatase inhibitor, and protease inhibitor) and frozen in -80 °C for 20-30 minutes. This solution is centrifuged for protein isolation and extraction. To harvest cells for Polymerase Chain Reaction (PCR) experiments, cells are washed with DPBS and then treated with warm trypsin and Fetal Bovine Serum (FBS).

Western blot

Western blots were used to measure the levels of chaperones IRE-1a, PERK, ATF6, eIf2a, and P-eIF2a. Actin was used as a loading control. The Bradford method was used to find protein concentration of each sample supernatant to normalize for Western blot analysis. Samples were run on a 4-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After running the gel electrophoresis and transferring onto a nitrocellulose blotting membrane, the membrane was blocked with 5% non-fat milk solution in Phosphate Buffered Saline with Tween® 20 (PBS-T) to prevent non-specific

binding of antibodies. The membrane was then incubated with an appropriate primary antibody overnight at 4 °C. The following antibodies were used: ATF6 monoclonal anti-mouse from Imgenex (1/5000 dilution), PERK monoclonal anti-rabbit from Cell Signaling Technology (1/5000 dilution), eIF2a polyclonal anti-rabbit from Cell Signaling Technologies (1/5000 dilution), P-eIF2a polyclonal anti-rabbit from Cell Signaling Technology (1/5000 dilution), and IRE-1a anti-rabbit antibody from Cell Signaling Technology (1/5000 dilution). Next, the membrane was washed with PBS-T and incubated with the corresponding secondary antibody for an hour before imaging with the BioRad ChemiDoc Imaging System.
Protein analysis of the activity of each pathway was conducted by measuring the protein levels with band densitometry using image lab software (BioRad). Quantification was done by normalizing the band for each protein with respect to the loading control Actin. WT control values at basal state were set at one, and the rest of the treatments were calculated relative to the control.

Polymerase Chain Reaction (PCR)

To determine whether the XBP-1 pathway is activated, PCR was used to measure splicing activity. The cells are lysed with lysis buffer given in *PureLink® RNA Mini Kit from Life Technologies* and 2-mercaptoethanol, then processed with a number of washes and RNA isolation steps. The RNA is then converted to cDNA with the 1st Strand Synthesis Kit, and amplified through PCR at 35 cycles. Once amplified, this cDNA was run through an agarose gel and imaged by the BioRad ChemiDoc Imaging System. Un-spliced XBP-1 is 171 base pairs (bp) and spliced XBP-1 (XBP-1s) is 145 bp long.

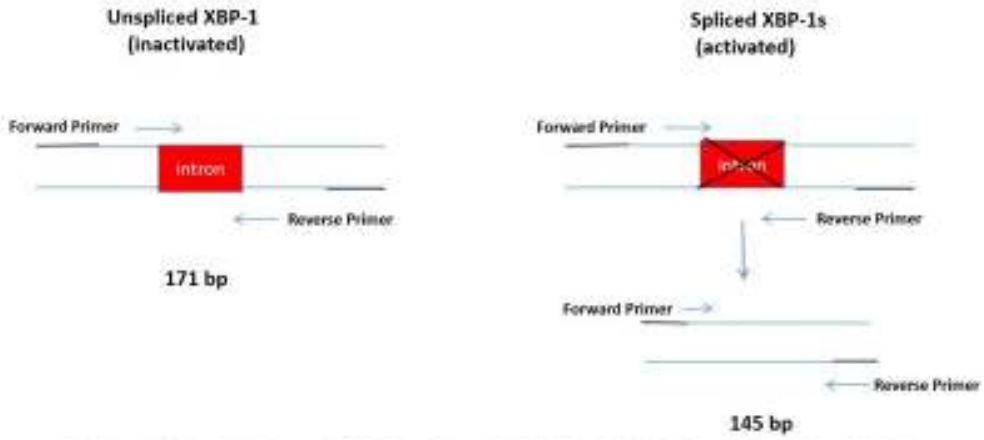

Figure 6: Activation of XBP1. Inactive XBP1 is 145 bp and active XBP1 is 171 bp. These two bands can be measured by PCR

Data Analysis

Unless specified, all data were expressed as mean ± standard error of the mean (SEM), using an n = 3-4 of independent experiments.

Results

Western blots

To investigate whether rapamycin was able to modify the ER stress response in WT and Nrf2 KO cells, we looked at the levels of key proteins that participate in each of the pathways described above. The first pathway investigated was the ATF6 pathway, and as seen in Figure 7, we measured the level of its activated form – a 50 kDa fragment (ATF6f).

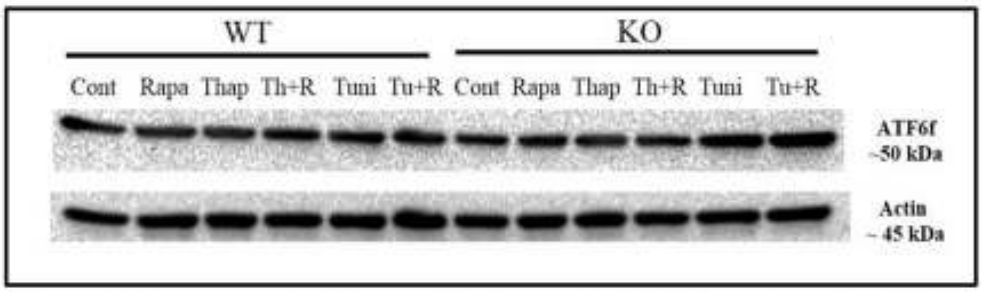

Figure 7: Representative Western blot image for ATF6f protein levels in Nrf2 WT and KO cells pretreated with Rapamycin (25 nM) and treated with Thapsigargin (3 mM) and Tunicamycin (10 mM) as positive stressors

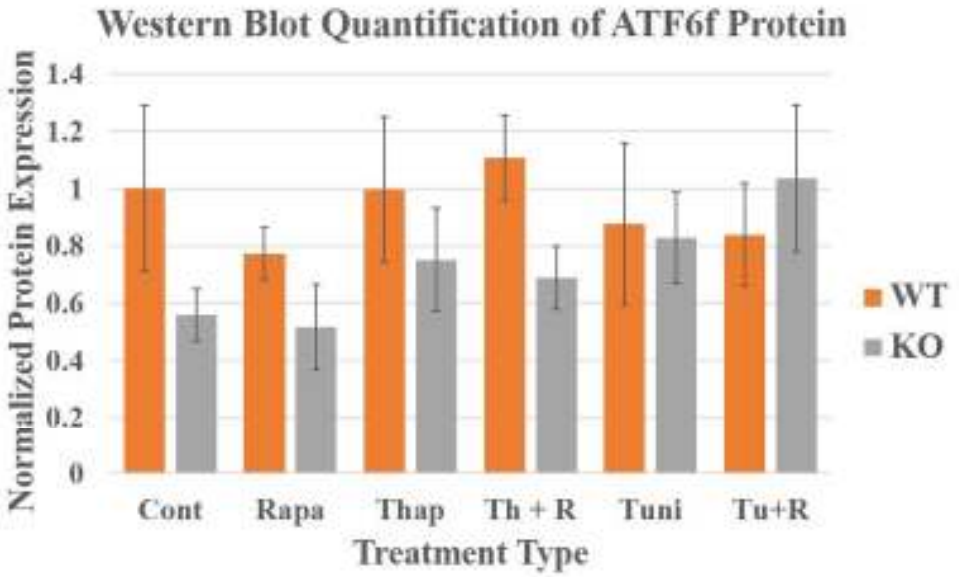

Figure 8: Levels of ATF6 in WT and Nrf2 KO mouse embryonic fibroblast cells. The levels of ATF6 were measured by Western blot as described in Material and Methods. Orange bars represent WT mouse cells and gray bars represent Nrf2 KO mouse cells. The data was normalized to WT control as a value equal to 1, and is represented as the mean ± standard error of n = 3-4 of independent experiments. Rapa=rapamycin, Thap=Thapsigargin, Tuni=Tunicamycin, Th+R=Thapsigargin and rapamycin, and Tu+R=Tunicamycin and rapamycin

Quantification analysis to determine changes in the ATF6 pathway was done using lab image densitometry analysis. Figure 8 shows the results of this using the basal, untreated control WT cells (value = 1) and quantifying the other treatments relative to this value. From Figure 8, we can observe that control KO Nrf2 cells showed lower ATF6f protein levels than the WT control. Rapamycin treatment yielded no difference in response from the controls. Thapsigargin treatment did not induce any change in response and pretreatment with rapamycin in Thapsigargin treated cells showed similar results. Tunicamycin treated cells also did not show a difference in response. However, Nrf2 KO cells treated with both rapamycin and Tunicamycin show a potential increase in response from the positive control treatment alone.

The second pathway investigated is initiated by the PERK chaperone, a 140 kDa protein, as seen below in Figure 9. The same method of analysis used for ATF6 was done to measure levels of PERK protein.

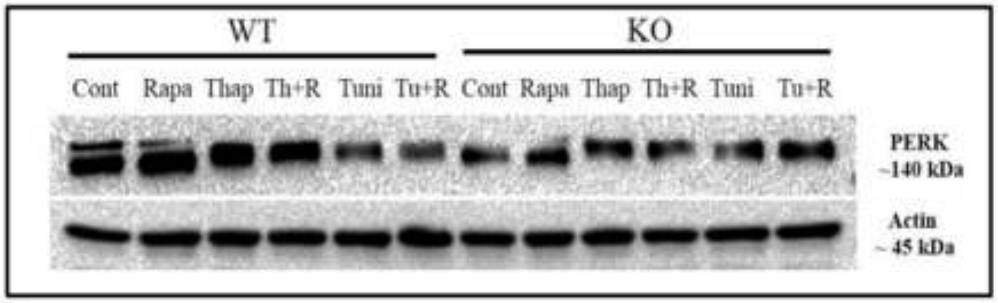

Figure 9: Representative Western blot image for PERK protein levels in Nrf2 WT and KO cells pretreated with Rapamycin (25 nM) and treated with Thapsigargin (3 mM) and Tunicamycin (10 mM) as positive stressors

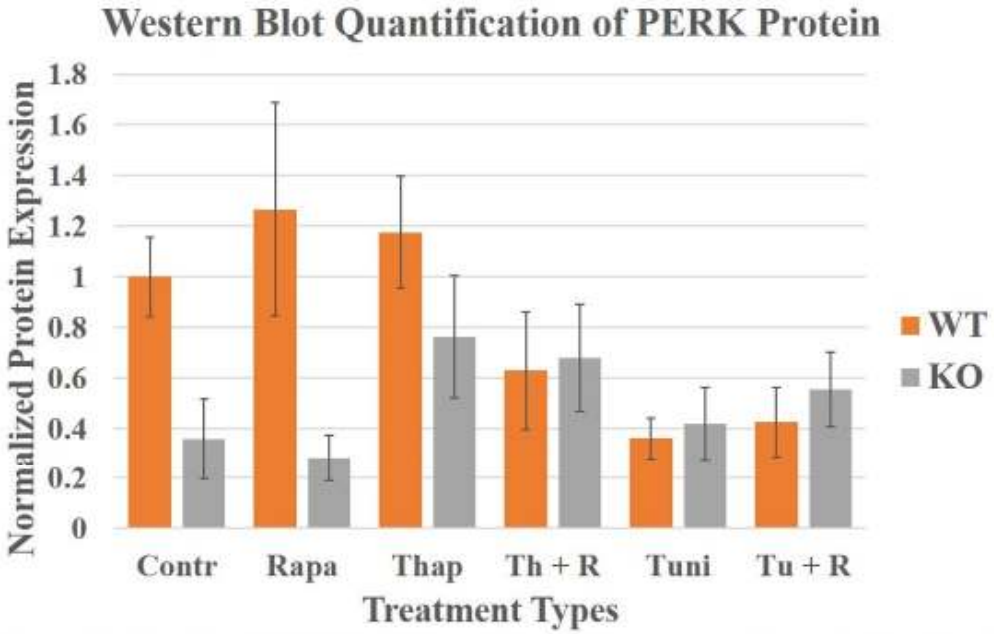

Figure 10: Levels of PERK in WT and Nrf2 KO mouse embryonic fibroblast cells. The levels of PERK were measured by Western blot as described in Material and Methods. Orange bars represent WT mouse cells and gray bars represent Nrf2 KO mouse cells. The data was normalized to WT control as a value equal to 1, and is represented as the mean ± standard error of n = 3-4 of independent experiments. Rapa=rapamycin, Thap=Thapsigargin, Tuni=Tunicamycin, Th+R=Thapsigargin and rapamycin, and Tu+R=Tunicamycin and rapamycin

After n=3 trials, we observe in Figure 10 that KO control cells have lower levels of PERK protein, a similar pattern that was seen in ATF6. Rapamycin treatment alone did not change any response levels. Thapsigargin treatment in KO cells showed a positive response, indicating that KO cells may be more susceptible to cell stress. Pretreating WT Thapsigargin cells with rapamycin showed a decrease in PERK protein levels, whereas this response was not observed in the KO counterpart. This suggests that Nrf2 has a role in

PERK protein levels. Tunicamycin and rapamycin pretreated Tunicamycin cells both showed similar levels of PERK protein.

Since PERK is at the beginning of the UPR signaling cascade, it is important to measure signals downstream to determine whether this pathway is activated. As a result, Western blot analysis was done on 38 kDa chaperone eIF2a, which is activated by PERK through phosphorylation to become P-eIF2a. Protein activation was then measured by levels of P-eIF2a with respect to eIF2a levels as seen in Figure 11.

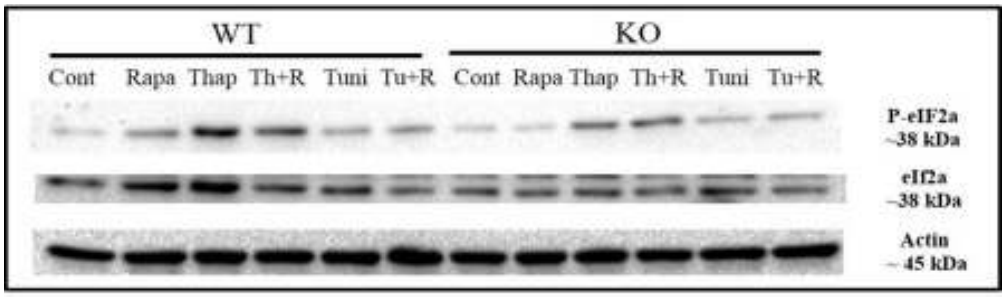

Figure 11: Representative Western blot image for phosphorylated eIF2a relative to eIF2a protein levels in Nrf2 WT and KO cells pretreated with Rapamycin (25 nM) and treated with Thapsigargin (3 mM) and Tunicamycin (10 mM) as positive stressors

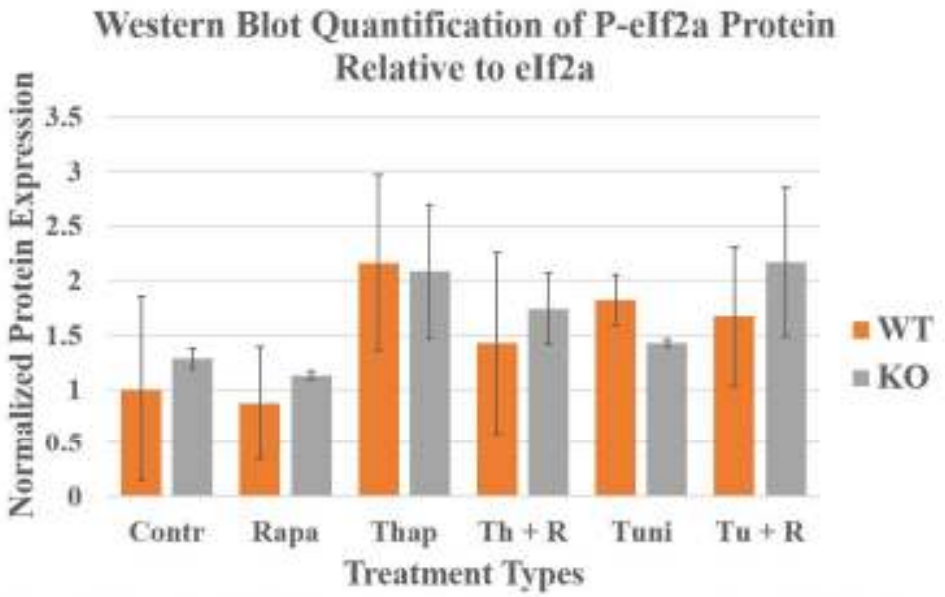

Figure 12: Levels of P-eIF2a in WT and Nrf2 KO mouse embryonic fibroblast cells. The levels of P-eIF2a were measured by Western blot as described in Material and Methods. Orange bars represent WT mouse cells and gray bars represent Nrf2 KO mouse cells. The data was normalized to WT control as a value equal to 1, and is represented as the mean ± standard error of n = 3-4 of independent experiments. Rapa=rapamycin, Thap=Thapsigargin, Tuni=Tunicamycin, Th+R=Thapsigargin and rapamycin, and Tu+R=Tunicamycin and rapamycin

Figure 12 shows the results of the P-eIF2a protein quantification analysis. In general, the data obtained from eIF2a protein activation corroborate with what we observed previously with PERK activation, with the exception of Nrf2 KO cell results. WT control cells were set to a value of one and the rest of the treatments were quantified relative to this value. We observe that Nrf2 KO cells actually showed similar or higher P-eIF2a protein levels than the WT control. Rapamycin treatment yielded similar P-eIF2a protein levels as the controls. Similar to its upstream signal PERK, Thapsigargin treatment had a more positive effect on KO cells than in WT cells, suggesting Nrf2 deficiency has an effect on ER stress susceptibility. Rapamycin pretreatment in Thapsigargin treated WT cells showed a possible increase in activation, a trend also seen in PERK. Tunicamycin control and Tunicamycin with rapamycin pretreatment showed similar activation levels.

The last pathway investigated, depicted in Figure 13, is the IRE-1a pathway, a 138 kDa chaperone protein.

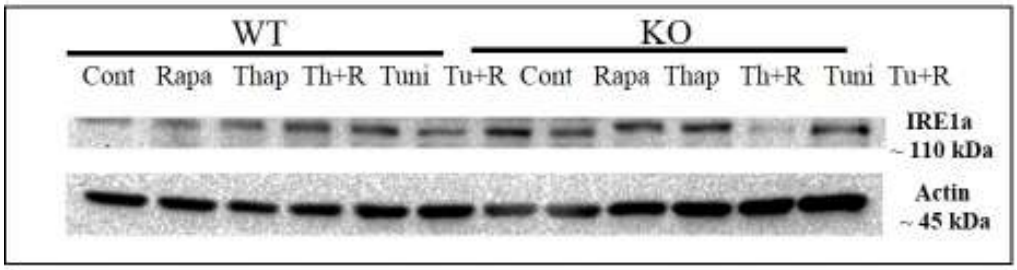

Figure 13: Representative Western blot image for IRE1a protein levels in Nrf2 WT and KO cells pretreated with Rapamycin (25 nM) and treated with Thapsigargin (3 mM) and Tunicamycin (10 mM) as positive stressors

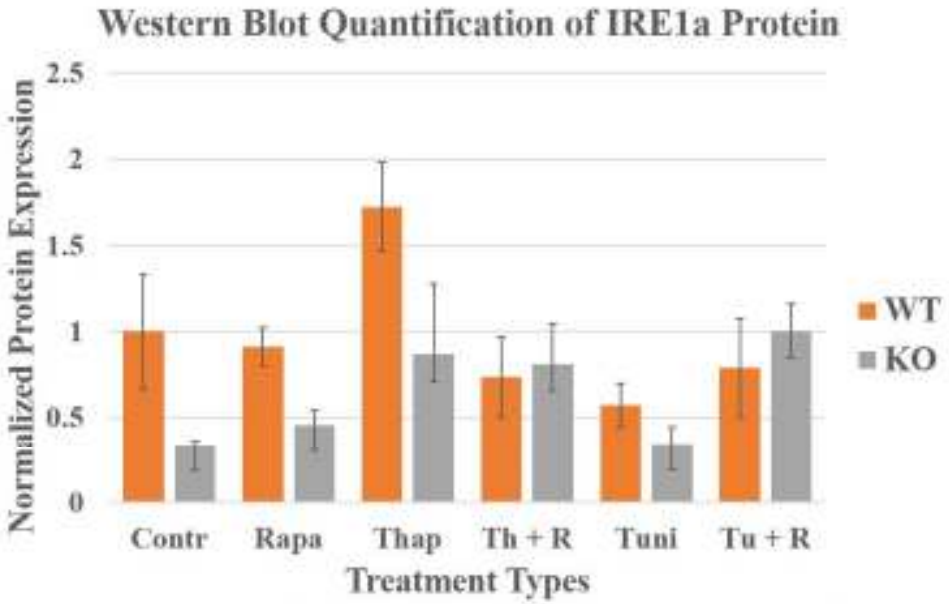

Figure 14: Levels of IRE1a in WT and Nrf2 KO mouse embryonic fibroblast cells. The levels of IRE1a were measured by Western blot as described in Material and Methods. Orange bars represent WT mouse cells and gray bars represent Nrf2 KO mouse cells. The data was normalized to WT control as a value equal to 1, and is represented as the mean ± standard error of n = 3-4 of independent experiments. Rapa=rapamycin, Thap=Thapsigargin, Tuni=Tunicamycin, Th+R=Thapsigargin and rapamycin, and Tu+R=Tunicamycin and rapamycin

Using the same analysis methodology as previously described, in Figure 14 we observe that Nrf2 KO cells had lower IRE-1a levels than WT cell levels, a trend that is observed for several of the pathways we have investigated so far. Rapamycin treatment showed no effect on IRE1a levels in WT and Nrf2 KO cells. However, Thapsigargin induced a positive response in both types of cells. Pretreatment with rapamycin in Thapsigargin treated cells showed a decrease in IRE1a protein levels in WT cells but not Nrf2 KO cells. This suggests that Nrf2 is involved in this pathway. Rapamycin pretreated Tunicamycin cells had an increase in IRE1a protein levels in KO cells compared to Tunicamycin control cells.

To determine whether this pathway was activated, the next step was to look further down the signal cascade. XBP-1s is a common marker for activation of the IRE-1a pathway. XBP-1 mRNA is spliced by IRE-1a and the translated product is an activated XBP-1s fragment (see Figure 6). However, the concentration of XBP-1s in the cell is too small to detect by Western blot methodology, so a Polymerase Chain Reaction was conducted at 35 cycles to amplify and detect spliced forms of XBP-1.

PCR

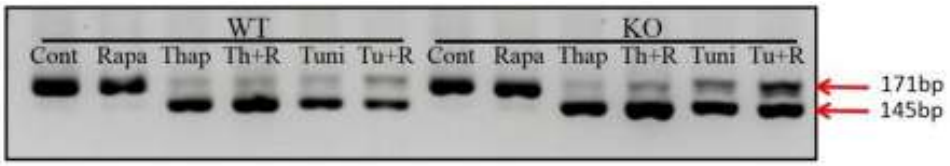

Figure 15: PCR analysis. Representative gel image for XBP1 cDNA levels in Nrf2 WT and KO cells pretreated with Rapamycin (25 nM) and treated with Thapsigargin (3 mM) and Tunicamycin (10 mM) as positive stressors. The top bands represent un-spliced levels and the bottom bands represent spliced levels of XBP1 cDNA

Cells were subject to the same treatment as described for the Western blots. As seen in Figure 15, the ratio of spliced XBP-1s (145 bp) over un-spliced XBP-1 (171 bp) cDNA was taken using the Chemidoc Imaging system to determine how much activation occurred with each treatment. As seen in Figure 15, WT and Nrf2 KO control cells showed similar results in the activation of this pathway. Rapamycin treatment yielded similar results to the basal controls. Thapsigargin treatment induced a positive response in both types of cells, especially in Nrf2 KO cells. Pretreatment with rapamycin increased response levels in both cells as well. Tunicamycin treatment in both cells also induced a positive response, and rapamycin pretreatment decreased response levels in both WT and Nrf2 KO compared to Tunicamycin positive control.

Discussion

The UPR is a protein chaperone system that is activated upon ER stress to prevent protein aggregation through maintenance of misfolded proteins. This system evaluates total cell response to stress and determines whether to initiate cell survival or apoptosis. Containing three main signal pathways (ATF6, PERK, and IRE1a), the UPR activates the upregulation of chaperone proteins and the general attenuation of protein translation to prevent ER machinery from being overwhelmed. Nrf2, known as the master regulator of aging, also has an integral role in determining whether the cell survives or undergoes apoptosis due to the overall stress load. Nrf2 is a transcription factor that regulates molecular chaperones that play a protective role in the cell. There appears to be crosstalk between the UPR and Nrf2 pathways, in which Nrf2 plays a role in changing the activation of certain parts of the UPR pathway under ER stress. This can be seen from the difference in protein levels and activation responses between WT and Nrf2 KO cells. We were also expecting that Nrf2 KO cells would have a deficient response to the UPR due to the lack of Nrf2 protein, but due to the large error bars, we cannot make firm conclusions about this yet. Central to this, the antibiotic rapamycin is

observed to mediate and influence both these pathways, as seen in the change of protein levels and their responses when comparing cells pretreated with rapamycin and their respective control cells. Rapamycin inhibits the mTOR pathway, which is involved in a positive feedback loop with the UPR. Nrf2 protein expression is observed to be upregulated by the application of rapamycin as well. In this project, we studied the interactions between these pathways.

In general, the positive controls did not work as well as we were expecting from our preliminary assay. In the beginning, we saw a difference with positive activation. However, as we did the rest of the experiments, there were large variations and error bars that compromised the statistical analysis and made data analysis difficult. The best response in WT cells was Thapsigargin in the IRE-1a experiment, and positive controls responded better in Nrf2 KO cells overall than in WT cells. A bigger N of samples will give us a better sample size to do statistical analysis and yield more reliable results.

With our current data, we observed no difference between the different treatments in the ATF6 signal pathway. This could suggest that rapamycin does not act through this pathway or that the time and dose courses were not optimal for its activation.

In the PERK pathway, we generally observed that rapamycin treatment failed to elicit a cellular response from this part of the UPR in WT cells. Treatment with Thapsigargin and Tunicamycin did not affect protein levels in WT cells as well. However, rapamycin and Thapsigargin treated WT cells showed a decrease in protein levels compared to only Thapsigargin treatment, which was not observed in KO cells. This suggests Nrf2 involvement in protecting against this type of cellular stress and upregulating PERK protein production. Nrf2 KO cells showed overall lower expression of PERK protein, suggesting that the absence of Nrf2 may play an important role in UPR response from this branch and that these cells are more susceptible to stress. Since PERK is at the beginning of the signaling cascade, we measured downstream P-eIF2a levels, the activated form of eIF2a, for activation of this UPR branch.

The protein expression of P-eIF2a (activated) was normalized to eIF2a expression levels. Rapamycin did not induce any change in protein expression, however rapamycin pretreatment showed a possible decrease of P-eIF2a levels in Thapsigargin treated WT cells. Since this decrease was not observed in KO cells, this indicates that Nrf2 plays a role in regulating the PERK and P-eIF2a pathway. This is the first pathway where there is little to no difference in protein expression levels between WT and Nrf2 KO cells. We expected that Nrf2 KO cells would also show lower eIF2a phosphorylation in correspondence to upstream signal proteins. One possible explanation is that Nrf2 upregulates the number of eIF2a phosphorylation per PERK molecule, with the concentration of proteins negligible in importance. Measuring other protein levels in this pathway would be the next step to determining how Nrf2 and the PERK pathway correlate.

The last pathway investigated was the IRE-1a signal pathway. Rapamycin treatment did not change IRE1a protein levels. Thapsigargin treatment showed

higher levels of IRE1a protein in both WT and Nrf2 KO cells, suggesting that this pathway is more sensitive to Thapsigargin than the others. However, it is well known that not all pathways are involved in ER stress response at the same time and dosages, so we would expect each pathway to respond differently. Rapamycin pretreatment in Thapsigargin treated cells decreased IRE1a protein levels in WT cells, and not in Nrf2 KO cells. This indicates that Nrf2 is involved in the IRE1a pathway. To measure activation of this pathway, we quantified for XBP-1s activation.

XBP-1s expression in rapamycin treated WT and Nrf2 KO cells are similar to the levels of the basal control cells. Both Thapsigargin and Tunicamycin induced a positive response, and a similar trend is observed with rapamycin pretreatment in both WT and Nrf2 KO positive control cells. However, the bands, as seen in Figure 15, were saturated and consequently, we need to perform more experiments at lower cycles and more N trials to refine these conclusions.

In general, these results have pretty high deviation. Therefore, the conclusions from these results must be made carefully and are minimized. However, our hypothesis that rapamycin's protective qualities can be mediated by activation of Nrf2 to regulate UPR pathways, seems to be supported by the data from these experiments.

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