THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF VETERINARY AND BIOMEDICAL SCIENCES

CHARACTERIZATION OF THE ER STRESS SENSOR IRE1α IN MOUSE AND HUMAN KERATINOCYTES EXPRESSING RAS

ALAYNA BRENNA CRAIG-LUCAS
SPRING 2015

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree in Toxicology
with honors in Toxicology

Reviewed and approved* by the following:

Adam Glick
Associate Professor of Molecular Toxicology and Carcinogenesis
Thesis Supervisor

Gary Perdew
John T. and Paige S. Smith Professor in Agricultural Sciences
Honors Adviser

* Signatures are on file in the Schreyer Honors College.

ABSTRACT

The Endoplasmic Reticulum (ER) is the site of protein synthesis for secreted and membrane bound proteins. When a cell accumulates incorrectly folded proteins it leads to a condition called "ER stress". ER stress is caused by a variety of disease states including cancer and metabolic imbalance. This stress triggers a variety of responses cumulatively called the "Unfolded Protein Response" (UPR). In eukaryotes the three signaling pathways that activate this response are mediated by the ER associated proteins IRE1α, PERK, and ATF6α. For the purpose of this thesis the focus will be the IRE1α pathway primarily because its role in cancer is not well understood.

IRE1α is a bi-functional protein with kinase and ribonuclease activities. Upon activation by autophophorylation, the IRE1α nuclease splices a 26 nucleotide sequence from the mRNA of x-Box-binding-protein1 (XBP1), creating the transcript for XBP1s, a transcription factor. The spliced protein helps reduce ER stress by upregulating the expression of genes that encode ER chaperone proteins as well as proteins in the ER-Associated Degradation (ERAD) pathway. This pathway is responsible for delivering misfolded proteins to the proteasome for degradation. The IRE1α nuclease also degrades other ER associated mRNAs in a process called "Regulated IRE1α-Dependent Decay Pathway (RIDD)." This pathway is independent of XBP1s and has been found to increase changes in numerous cell processes including inflammatory responses, apoptosis, growth, metabolism, and macroautophagy.

In studies of cellular response to ER stress, the IRE1α nuclease has been found to determine cell fate as it is believed that XBP1s promotes cell survival while the RIDD pathway promotes apoptosis. With low stress, cell survival is mediated by IRE1α nuclease favoring XBP1 splicing

while at higher stress levels RIDD activity causes cells to die. Here we have worked to determine if similar opposing effects of IRE1α nuclease regulate cellular response to Ras oncogene activation that occur at the early stages of cancer. The Ras oncogene is an important growth regulatory gene that is mutated in many human cancers. For these studies we have introduced a viral Ras oncogene into primary mouse keratinocytes as well as normal human epidermal keratinocytes to determine the effect on IRE1α pathway activation.

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ACKNOWLEDGEMENTS

Through the journey of writing this thesis there are a great many people who I would like to give thanks to for their instrumental role in helping me to achieve all that I have through college. I would first like to thank Dr. Adam Glick for providing me the incredible opportunity to conduct research with his laboratory for the past two and a half years. His continued guidance and support not just in research, but in my academic career as a whole has provided me with invaluable skills that I look forward to bringing forward and expanding upon in my future. I would also like to thank my mentor, Dr. Nicholas Blazanin, for his crucial role in my development as a researcher and also as a person. His direction in the laboratory has lead me to gain a deep and profound love for learning and taught me to enjoy working through the many struggles often encountered before discovery. He has continued to remain patient through my sometimes hectic schedule and more difficult days and for that I cannot thank him enough. Additionally I would like to provide thanks to all other members of the Glick lab: Mike Podolsky, Jeongin Son, Deirdra DeFranco, and Carrie Oaks for their ability to make every conversation light and enjoyable.

Finally, I would like to thank my family and friends for the support system that they have continuously offered over the course of many many years. Without their sincere reassurance that every obstacle would be overcome, I cannot imagine having completed this thesis. Their open hearts and understanding attitudes for every late night spent working or Saturday visits to the lab, have made this journey only that much more enjoyable. A special thank you to my mother, Laura Craig, Father, Wayne Lucas, and Brother, Garrett Craig-Lucas, for motivating me to do my best every day in every task that I set out to accomplish.

Chapter 1

Introduction

1.1 Overview

This overview will include information regarding ER stress and the functions and signaling of the Unfolded Protein response (UPR). Primary focus has been given to IRE1α which is the major topic of interest for this thesis. An explanation of ER stress and the UPR as well as the role of IRE1α in cancer progression will be provided as well as a general discussion of the role that oncogenic RAS plays in the skin carcinogenesis. The overarching goal of this thesis is to evaluate the role that these pathways play in the early stages of cancer progression in Normal Human Keratinocyte (NHEK) cells to determine if similar mechanisms occur compared to that which has been characterized in Primary Mouse Keratinocytes.

1.2 Endoplasmic Reticulum Stress

Eukaryotic cells have evolved a variety of mechanisms to evaluate the quality and movement of proteins within the endoplasmic reticulum before they can be relocated to other areas of the cell. If protein folding or maturation is disrupted in any way, unfolded proteins can accumulate within the ER and lead to a condition termed ER stress. The cell is conditioned to respond to this stress through a set of complex signaling pathways known as the unfolded protein response (UPR) which aims to reestablish ER homeostasis (1). However, if this stress is ongoing

the cell is pushed towards an apoptotic response. Because of the duel mechanism of the UPR it is necessary to further evaluate its mechanisms in hopes of finding potential selective modulators for specific pathway targets.

1.3 Endoplasmic Reticulum

The Endoplasmic Reticulum (ER) is a large multifunctional cellular organelle that contains structurally distinct domains including the Rough and Smooth Endoplasmic Reticulum as well as its nuclear envelope. The ER functions in a wide variety of roles including translocation of proteins across the membrane, integration of proteins into membranes, protein modification and folding in the lumen of the ER, phospholipid and steroid synthesis on the cytosolic side of the membrane, and calcium ion storage and regulation of its release (5). Due to its multi-functionality, the ER has been found to associate with almost all other organelles and thus its role in many diseases is crucial (1). One of the most important functions of the ER is proper protein folding and posttranslational modifications (1). In order for proper folding to occur the ER contains many chaperone proteins that help a cell respond to stresses and other environmental cues that attenuate proper protein folding. General chaperones in the Endoplasmic Reticulum include GRP78/BiP, GRP94, and GRP170 while non-classical molecular chaperones include HSP47 and ER29 and folding chaperones include protein disulfide isomerase (PDI), ERp57 and peptidyl propyl- cis-trans-isomerase (PPI). In addition to these chaperones additional mechanisms are in place that only allow properly folded proteins to be packaged for secretion into extracellular space (2,3). Should misfolding occur the proteins remain in the ER and are then translocated to the cytosol where they

are degraded by the 16S proteasome (4). This process is known as ER-associated degradation (ERAD) and is required for misfolded protein removal.

1.4 Overview of the Unfolded Protein Response

When proteins are misfolded, amino acid residues are exposed in a way that they should not be. This exposure can result in inappropriate cellular integrations that can lead to aggregation of proteins and eventually insoluble formations. These insoluble protein complexes have been linked to a number of different diseases. For example, the formation of disordered aggregates has been noted in rhodopsin in autosomal retinitis pigmentosa, amyloid fibrils have been seen in Alzheimers, and nonamyloid fibrils have been noted in the alpha1-antitrypsin deficiency (4). ER stress is caused by a variety of disease states including cancer and metabolic imbalance and has been noted in neurodegenerative diseases such as those listed above as well diseases such as diabetes, renal failure, and atherosclerosis (4). To combat the effects of this stress the ER has adapted to create complex network of responses known as the Unfolded Protein Response. The three major branches of the UPR that have been characterized are comprised of three ER's transmembrane proteins: inositol-requiring-enzyme-1α (IRE1α), activating transcription factor 6 (ATF6), and Protein Kinase R – like endoplasmic reticulum kinase (PERK) (6). Together, these three proteins work to increase folding capacity and decrease the folding the demand in the ER. To increase the folding capacity of the ER, the UPR upregulates synthesis of chaperones and folding enzymes as well as promotes an increase in ER size (1). The folding demand is decreased by downregulating transcription and translation of proteins as well as increasing the clearance rate

of the unfolded proteins by ERAD (1). The following section will further explain the Unfolded Protein Response as well as it's pathways in greater detail.

1.5 Unfolded Protein Response (UPR)

The UPR is an adaptive mechanism that contains at least three components: transcriptional induction of ER chaperones, a decrease in translation to prevent further accumulation of proteins, and ER- associated degradation (ERAD) to remove misfolded proteins in the ER (6). These responses are carried out following the induction of multiple genes as well as a decrease in translation and a shift in the mRNAs that are being translated (6). The response can be either protective or toxic to the cell depending on how far progressed the damage to the cell has become. If the ER stress is prolonged and the cell is not able to restore homeostasis via the UPR, the cell will eventually undergo cell death (1).

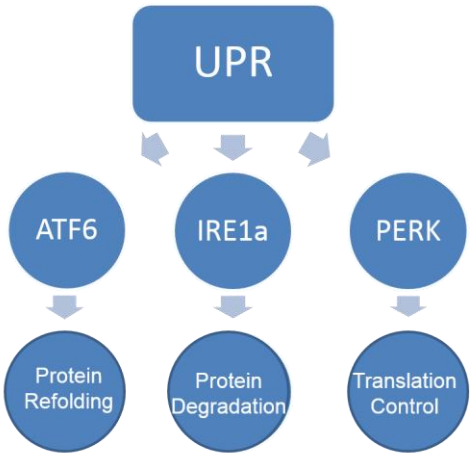

Figure 1: Map of Unfolded Protein Response pathways

The UPR is activated upon protein misfolding in the ER. This misfolding could result in various physiological conditions such as glucose deprivation, ischemia, infections, pH changes,

hypoxia and nutrient deprivation, all of which lead to the induction of the UPR (1,6). The three branches of the UPR: IRE1α, PERK, and ATF6 all interact with each other and of the three, IRE1α is the most conserved (figure 1) (7). All three of the transmembane proteins are dependent on BiP for their activation. BiP is the most abundant chaperone protein found in the lumen of the ER and interacts with each protein. Its role is essential to translocation as it binds proteins as they are moved into the ER and prepared for further folding and oligomerization (8). While its levels remain high during almost all growth conditions, it appears to be upregulated when the accumulation of unfolded proteins occurs in the ER. Thus, BiP is frequently considered a marker for diseases resulting from protein misfolding (8).

ATF6

ATF6 is an ATF/CREB basic-leucine zipper DNA-binding protein family member and acts as a transmembrane transcription factor located in the endoplasmic reticulum. Its ability activate GRP78, a potent ER chaperone, and other genes induced by the ER stress response has long implicated it in the ER Stress response pathway. The protein is 670 amino acids long and roughly 90 kDa. Haze *et al.* found a 50-kDa ER-stress-induced cleavage product of ATF6 which is believed to be the active form of the transcription factor. Upon stress induction ATF6 is translocated from the ER to the Golgi where it can be processed to its active form following cleavage of the N-terminal cytoplasmic portion via site 1 protease (S1P) and site 2 protease (S2P) (1). Shen *et al.* found that BiP/GRP78 is constitutively bound to ATF6 until ER stress causes dissociation (6). This loss of binding correlates to ATF6 translocation to the Golgi where ATF6 cleavage occurs. The resulting N-terminal domain translocates to the nucleus where it functions as transcription factor that recognizes the ER Stress Element (ERSE) in promoter regions of genes

that regulate the Unfolded Protein Response including BiP, PDI and those induced in ERAD (9, 10).

PERK

PERK is a transmembrane kinase responsible for phosphorylation of elF2a, eukaryotic translation initiation factor 2A (5). Under normal physiological conditions PERK is bound to BiP as a monomer. However, when placed under stress PERK oligomerizes and autophosphorylates causing activation and phosphorylation of elF2a (1). This process results in a decrease in mRNA translation and thus a decrease in the proteins that enter the ER (1).However, there is a specific increase in translation of the transcription factor ATF4 which regulates genes involved in oxidative stress, amino acid metabolism and protein folding (11).

IRE1α

IRE1α is a critical sensor of misfolded proteins that have accumulated in the ER lumen. This protein is found in two isoforms, IRE1α and IRE1β and is a type 1 transmembrane Ser/Ther kinase that is unique in that it also has endoribonuclease activities (1). IRE1α's functions were first discovered in yeast where it was found that the unfolded protein response is dependent only on IRE1ρ, a transmembrane endoribonuclease that begins the splicing of HAC1 mRNA, a yeast analogue of XBP1 that acts as a transcription factor (10). HAC1 mRNA splicing results in a new HAC1 mRNA encoding the active HAC1 transcription factor. HAC1 induces expression of proteins that are necessary for ER-associated degradation (ERAD). As discussed, in mammals the unfolded protein response is much more complex and IRE1α is only one of the three necessary signaling molecules (10). Similarly to PERK, IRE1α is initially found as a monomer bound to BiP in its non-active state (12). However, upon stress it forms dimers that undergo transautophosphoryation resulting in activation of the RNase domain and splicing of a 26

nucleotide sequence from the XBP1 mRNA (6). XBP1 (Xbox-binding protein 1) was discovered while in search for regulators of MHC class II gene expression. It is a member of basic region leucine zipper family of transcription factors. It has been found in all adult tissues but expressed preferentially in fetal exocrine glands, osteoblasts and liver making knockouts embryonic lethal as XBP1 is necessary for liver development (10). IRE1α splices the 26 base pair fragment from the mRNA, and the resulting altered reading frame encodes a 371 amino acid protein named XBP1s. This protein is a transactivator that translocates to the nucleus where it binds to its target sequence and helps to regulate unfolded protein response gene expression. It has been characterized that XBP1 regulates a wide variety of genes that involve processes such as protein folding, entry of proteins into the endoplasmic reticulum, ER-associated degradation (ERAD), autophagy, lipid biogenesis, glycosylation, redox metabolism and vesicular trafficking (13).

IRE1α is also involved in Regulated IRE1 Dependent Decay (RIDD) which is responsible for reducing protein loading into the ER (12). Several recent studies indicate that the RIDD function of IRE1α may play a role in the induction of the apoptotic response through degradation of mRNAs involved with growth promoting proteins thus reducing the overall number of proteins entering the ER (2). Recently, it has been shown that unspliced *Xbp1* mRNA is translated into the protein XBP1U which is interestingly acts as a chaperone to its own unspliced mRNA (14).

Initially activation of the UPR results in upregulation of mechanisms that increase cell survival. When ER stress overwhelms the ER, though, the cell commits to apoptosis through pathways involved in UPR (22). IRE1α can play a role in cell fate determination in response to different levels of ER stress. If the level of ER stress is only slightly increased, IRE1α drives XBP1 mRNA splicing which leads to adaptive mechanisms. If the levels of ER stress become overwhelming and the cell can no longer adapt, IRE1α transautophosphorylation and

oligomerization will result in a shift of the pathway towards RIDD mechanisms involving the degradation of mRNAs, some of which are important in regulating apoptosis

IRE1α involvement in cancer

Because the tumor microenvironment predisposes the cell to ER stress, it is no surprise that the Unfolded Protein Response plays a strong role in cancer development. However, because it is not currently understood exactly how the UPR balances the cell-survival and cell-death signals its ultimate role in cancer has not been completely characterized (7). There are several studies showing that XBP1 is over expressed in many cancer types including breast, liver, and colon (10). However, because RIDD has not been studied to the same extent, its role in cancer progression is not currently well understood.

1.6 Ras

Members of the Ras family of oncogenes are mutated in 30 percent of human cancers. These GTPase proteins associate with the plasma membrane and transmit signals from transmembrane receptors to downstream targets that regulate a wide variety of biological functions such as apoptosis, differentiation, survival, proliferation and immune regulation (13) Ras is activated by GTP binding which then allows association with other cellular protein such as the Raf kinases. The GTPase activity of Ras converts GTP to GDP and turns off Ras activity. Mutations in Ras convert it to a constitutively active form that functions in the absence of signals from surface receptors. The conformational change that results allows for a high affinity binding sites to be displayed. Because nearly 30% of all human cancers display a Ras mutation it is essential to understand how this oncogene functions to modulate cellular functions (13).

Ras and senescence in primary cells

Preliminary discovered showed that Ras has the ability to transform immortalized cell lines or work with other oncogenic factors to transform primary cells, inducing only oncogenic Ras into primary cells results in irreversible growth arrest known as senescence (14). This form of growth arrest is a dominant survival mechanism that many premalignant and benign tumor types utilize as a way to prevent development to malignant phenotype (15). At physiological levels, the expression of the Ras oncogene yields transformation and cell proliferation, senescence is not observed without the addition of other oncogenes (16, 17). This indicates that at low levels, the oncogene can bypass the senescent response.

1.7 Aim of paper:

Due to the lack of understanding of the duel functionality of IRE1α this thesis aims to continue the currently unpublished research of the Glick lab. The research was conducted in an in vitro skin carcinogenesis model to evaluate the contrasting roles of the IRE1α RNase abilities. The research results concluded that IRE1α activity resulting in Xbp1 splicing lead to cell proliferation dependent on ER stress as well as determining that RIDD promoted via IRE1α lead to senescence independent of Xbp1. The lab also determined that Id1 was an important mRNA target of IRE1α. The goal of my research was to determine if oncogenic Ras caused similar effects in human keratinocytes. The continuation of this research has strengthened the findings and hopes for a therapeutic agent capable of targeting this pathway.

Chapter 2

Materials and Methods

2.1 Cell Culture and Reagents

Primary mouse keratinocytes from FVB/n or C57/Bl7 mouse strains were isolated from 1-3 day old newborn mice following documented protocols (18). Primary keratinocytes were cultured using Eagle's minimal essential medium (Formula # 06-174G, Lonza) containing 8% chelexed FBS, 18.3 I.U/ml penicillin, 18.3 μg/ml streptomycin and 0.2 mM Ca^{2+}. Day 2 post plating media was changed to 0.05 mM Ca^{2+}.

Normal Human Epidermal Keratinocytes (NHEK) cells were obtained from Harvard Dermatology Core Facility. These cells were plated in GIBCO keratinocyte serum-free medium (K-sfm) (Life Technologies catalog #10724-011) containing 5mL of a 100X penicillin/streptomycin stock (10,000 units/mL of pen and 10,000 ug/mL strep), 25ug/mL bovine pituitary extract (BPE), 0.2 ng/mL Epidermal Growth factor, 0.3 mM CaCl2 (from a 1000X stock).

Squamous Cell Carcinoma (SCC-13) cells were obtained from the Harvard Dermatology Core Facility. These cells were plated in GIBCO keratinocyte serum-free medium (K-sfm) (Life Technologies catalog #10724-011) containing 5mL penicillin/streptomycin at 100X stock, 25ug/mL bovine pituitary extract (BPE), 0.2 ng/mL Epidermal Growth factor, 0.3 mM CaCl2 (from a 1000X stock).

SCC-13 and NHEK cells were grown to confluence and passaged at a 1:10 dilution. Cells were washed 2 times with sterile PBS and trypsinized for 5 minutes at 37 degrees Celsius, the cells

were collected in 10 mLs of DMEM to inactivate the trypsin and spun down at 800 RPM for 5 minutes. The pellet was suspended in 10 mLs of KSFM and then one mL of the resuspension was added to 9 mLs of KSFM for plating. If cells were not to be plated the resulting cell suspension would not be diluted to 1:10 but instead, 10% DMSO would be added to the solution to allow for cryo preservation. Frozen cells were cryopreserved and stored in a nitrogen tank for future use.

2.2 Virus production and infection

Ecotropic Ras retrovirus was generated from psi2 producer cells following protocol generated by Roop *et al*, and an amphoteric virus was created by infecting PA317 cells, an NIH3T3 based packing cell line that produces amphoteric virus, with the ecotropic virus. 5 days after Ras infection the supernatant was collected, filtered and frozen in liquid nitrogen. SCC-13 cells were seeded at 200,000 cells per 6 well tissue culture tray and infected with a 1, 1:3, and 1:6 virus dilution to determine with would allow for best infection rate (figure 2). NHEK cells were seeded at 200,000 cells per 6 well tray and infected after 2 days of culture with the amphoteric Ras retrovirus in a 1:3 dilution. On day 3 post infection the cells were treated with 4ug/mL and on day 5 they were harvested for protein and RNA isolates.

2.3 Western blot

Protein Isolation:

Total cell lysates were obtained in RIPA lysis buffer (50mM Tris- HCL, pH7.4, 150mM NaCL, 1% IGEPAL, 0.5% Sodium Deoxycholate, 0.1% SDS, 2mM EDTA, 1μg/mL Aprotinin, 1μg/mL Pepstatin, 1mg/mL leupeptin, 5mM NaF, 1mM PMSF, 2mM β-glycerophosphate, 2mM

sodium orthovanadate), rotated for 1 hr at 4 degree C and centrifuged at 12,000 rpm for 15 min to remove cellular debris. Protein concentrations were determined using BCA protein assay (Pierce). The kit involved preparing standards with provided BSA (2mg/mL). 50 μL of standard was placed in duplicate in a 96 well BD falcon plate. Unknowns were prepared by adding 48uL of 0.1N NaOH to each well followed by 2 μL of unknown sample. The dye was prepared based on number of samples in a 50:1 dilution of part A reagent to part B reagent provided from the Thermo Scientific Pierce BCA protein Assay Kit, 200 μL was added to each well. The plate was allowed to incubate in at 37C for 30 minutes and cooled down to room temperature before being red in the plate reader at 570nm.

20 μg of protein was separated on 8%, 10% or 15% SDS-Page based on the antibody that was used to analyze the protein. The gels were all transferred to a sheet of nitrocellose by using a Bio-Rad trans-blot TURBO transfer system according to provided instructions from the manufacturer and then blocked with a 5% milk blocking solution for 1 hour at room temperature. The blot was rinsed several times with TBS-T and then incubated with the primary antibody in a 1:1000 dilution overnight. The following day the blot was washed for fifteen minutes, 3 times, with TBS-T and then incubated with the secondary antibody in a 1:5000 dilution or 1:1000 dilution for actin, for 1 hour at room temperature. Following a quick rinse with TBS-T, ECL or DURA were used to develop blots. The blots were incubated in ECL (Pierce) or DURA (Pierce) for 2 minutes prior to exposure. Antibodies used for incubation: ID1 (Santa Cruz), XBP1s- (Biolegend), ERK, p-ERK, IRE1α- (Cell Signaling), β–actin (Mill Pore).

2.4 Phos-Tag SDS-PAGE

Phos-Tag SDS-PAGE was utilized to produce a clearer image representing separation of proteins when using IRE1Aα antibody while preforming western blots. This method allows for differential retardation of phosphorylated proteins in the gel to allow for the separation of the protein into phosphorylated and unphosphorylated forms of the same protein. It allows for the detection of both forms using an antibody against total protein of interest. Briefly, 5% Phos-tag copolymerized SDS-PAGE gels for IRE1α (Cell Signaling) were done with the following running conditions: 100 V for 3 h using 25 μM Phos-tag. Vinculin (Cell Signaling) was used as a loading control.

2.5 RNA isolation and q-PCR

Total RNA was isolated using TRIZOL following washing the cells two times with ice cold PBS. Cells were pipetted several times to dislodge any cells that remained attached and the trizol homogenate was transferred to labeled 1.7 mL microcentrifuge tubes. Tubes were vigorously shaken and allowed to incubate for 5 minutes for room temperature. 80μL of chloroform was added to each tubes and once again shaken and allowed to incubate for 5 minutes at room temperature. Following incubation, the tubes were spun at 12000 rpm for 15 minutes at 4C. 200μL of the aqueous phase was removed and transferred to a new labeled microcentrifuge tube. An equivalent volume of isopropanol was added to each tube and mixed by inversion. The RNA was allowed to precipitate over-night and then spun as 12000 rpm for 15 minutes at 4C to pellet the RNA. Isopropanol was decanted and the RNA pellet was washed with 70% ethanol and spun

again at 12000 rpm for 15 minutes. The ethanol was carefully removed via pipet and allowed to air dry for 20 minutes. The remaining RNA pellet was resuspended in DEPC water.

cDNA was created using a lab generated kit that contained the following reagents: M-MLV RT enzyme (Promega #M1705, 50, 000U), 5X RT Buffer (Promega #M5313), Random Primers (Promega #C1181, 20ug), dNTPs (Denville Scientific #CB4421-4, 100 mM), RNAsin (Promega #N2515, 10,000U). 25μL reactions were planned using 2.5μg total RNA giving a final concentration of 100ng/uL. The mastermix was created and 12.5μL was added to a total of 12.5 μL of totally RNA + water. The reaction was run in a thermocycler using the following reaction conditions: 25C for 10 minutes, 42C for 2 hours, 85 C for 4 minutes. Reverse transcription and quantitative PCR (qPCR) was performed using intron-spanning primers. All mRNA levels were normalized to glyceraldehyde 3-phosphate (*Gapdh*) or 18s rRNA levels. The relative levels of each mRNA was determined by q-PCR using a standard curve with a 5 fold dilution series.

The following primers were used for qPCR analysis: *Xbp1u* forward primer: 5'-AGTCCGCAGCACTCAGACTAT-3'; *Xbp1u* reverse primer: 5'-TGAAGAGGCAACAGT GTCAGA-3'; *Xbp1s* forward primer: 5'-CTGAGTCCGCAGCAGGTG-3'; *Xbp1s* reverse primer: 5'-TCTGAAGAGGCAACAGTGTCA-3'; *Ire1α* forward primer: 5'-TGTTTGTCTC GACCCTGGATG-3'; *Ire1α* reverse primer: 5'-CGTTGTTCTTGCCTCCAAGTG-3'; *Hgsnat* forward primer: 5'-AGCGCTGATTACCAACCAGAA-3'; *Hgsnat* reverse primer: 5'-AAACCATGGGAAGACGAGGTC-3'; *Pmp22* forward primer: 5'-TGGCAGAA CTGTACCACATCC-3'; *Pmp22* reverse primer: 5'-ACGCTG AAGATGACAGACAGG-3';

Chapter 3

Results

3.1 Oncogenic v-Ras activates MEK/ERK kinase activation as well as XBP1 Splicing

To determine how the IRE1α pathway was regulated, freshly isolated primary mouse keratinocytes, indicated as MEK (Mouse epidermal keratinocytes) in all preceding figures, were infected with an ecotropic Ras retrovirus that was created by infecting psi2 producer cells with Ras. The cells were treated at a multiplicity of infection (MOI) of 1-3 in order to determine the effect on ER stress and the UPR. An MOI is the ratio of viral particles to target cells and as the MOI increases, the number of target cells that is infected with at least one viral particle should increase as well. Protein was isolated at 2, 4 and 5 days following retroviral transduction and activation of pathways downstream from Ras and the IRE1α pathway were measured by immunoblotting with specific antibodies. Figure 2 shows that there was a significant increase in the levels of MEK and ERK kinase activation at day 2, 4, and 5 following the retroviral infection. This is represented by upregulation of the phosphorylated forms of MEK and ERK (Figure 2A).

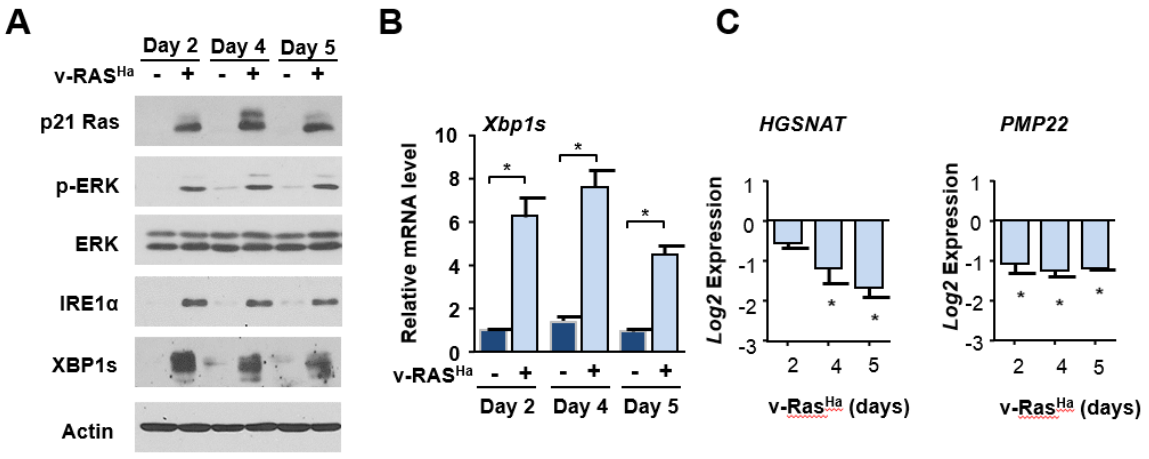

Figure 2 Oncogenic v-Ras activates MEK/ERK kinase activation as well as XBP1 Splicing (A)Western blotting was used to determine relative protein levels of IRE1α, p21 RAS, p-ERK, ERK, and actin control in MEK and V-RAS cells (B) q-PCR of MEK and Ras cells was conducted under control or Ras infection to quantify relative mRNA levels of *Xbp1s* (C) q-PCR of MEK and Ras cells was conducted under control or Ras to quantify relative mRNA levels of *Hgsnat*, and *Pmp22*

In addition to protein, mRNA was also isolated using TRIZOL to lyse cells and total RNA was purified following instructions as stated in the Materials and Methods chapter. Quantitative rt-PCR was run with the RNA samples obtained and it was determined that expression of oncogenic Ras in primary keratinocytes also resulted in an increase in the mRNA of the spliced form of XBP1 as indicated by the q-PCR data shown in figure 2B. Additionally, IRE1α upregulation also lead to activation of the "Regulated IRE1α Dependent Decay" (RIDD) pathway, which induces mRNA cleavage and degradation of proteins to decrease ER stress load. In order to determine if the RIDD pathway was activated in Ras transduced keratinocytes, RNA was isolated from v-Ras expressing mouse keratinocytes and mRNA expression of two ER-mRNAs previously determined to be IRE1α-RIDD cleavage substrates were also analyzed using quantitative q-rt-PCR (5). PMP22 is an Extracellular Matrix protein largely involved in the peripheral nervous system as it serves a major role in myelin composition. HGSNAT acts in degrading heparin sulfate and can be located in the membrane as a lysosomal transferase (5).

Comparing the standard to Ras infected keratinocytes showed that the transduced cells had downregulated *Pmp22* and *Hgsnat* mRNA levels within 2 days of infection (Figure 2C). Together, this data represents that oncogenic Ras activates the RNase capabilities of IRE1α in the primary mouse keratinocytes

3.2 MEK/ERK signaling regulates IRE1Aα activation in mouse epidermal keratinocytes expressing RAS

In order to understand if Ras induced IRE1α activation is MEK-ERK dependent, the primary and Ras induced keratinocytes were treated with U0126, a drug that inhibits MAPK (ERK 1/2) activation via the inhibition of MAP Kinase Kinase (MAPKK or MEK1/2) (Promega).

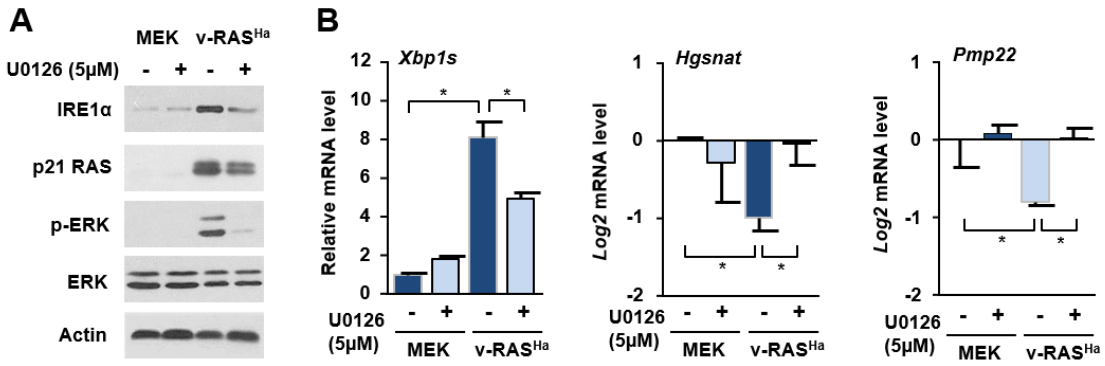

Figure 3 MEK-ERK signaling in mouse epidermal Keratinocytes (MEK) expressing the Ras oncogene is regulated by IRE1α activation (A) Western blotting was used to determine relative protein levels of IRE1α, p21 Ras, p-ERK, ERK, and actin control in MEK and Ras cells with control or U0126 treatment (B) q-PCR of MEK and Ras cells was conducted under control or U0126 treatment to quantify relative mRNA levels of Xbp1s (C) q-PCR of MEK and Ras cells was conducted under control or U0126 treatment to quantify relative mRNA levels of *Xbp1s Hgsnat,* and *Pmp22*.

As figure 3A indicates, UO126 resulted in an almost complete down regulation of ERK phosphorylation, and we can see that the IRE1α protein level is dramatically decreased when the Ras MEK cells are treated with the drug. Treatment also caused Xbp1 mRNA levels to decrease

in conjunction with *Pmp22* and *Hgsnat* mRNA levels showing a significant decrease (figure 3B). As a whole, figure 3 shows that IRE1α activation is regulated via the MEK- ERK signaling in mouse epidermal keratinocytes expressing Ras.

3.3 Test of amphotropic Ras virus production

In order to test whether oncogenic Ras could induce similar effects in human keratinocytes we generated an amphotropic retrovirus expressing Ras. To do this we infected PA317 cells, the amphotropic packaging cell line, with the ecotropic Ras retrovirus. The virus produced from this new packaging cell line was able to infect human keratinocytes. To determine if this was the case I infected a test human cell line, SCC-13, with the virus supernatant from the packaging cell line in dilutions of 1:6, 1:3, or "No Dilution". At each virus dilution protein was harvested in RIPA lysis buffer. After measuring protein concentrations using a BCA protein assay kit, SDS-PAGE was used to measure expression of the Ras protein by ECL.

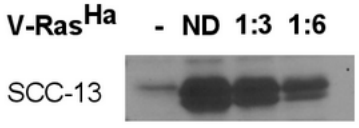

Figure 4 SCC-13 cells were infected with amphotropic Ras to test infective capabilities. The cells were treated with no virus, no dilution, 1:3 dilution, or 1:6 dilution

Figure 4 shows that with increasing dilution of the viral supernatant, there was reduced level of Ras expressions, but all dilutions were higher than the uninfected control cells.

3.4 Oncogenic RAS activates IRE1α in normal human epidermal keratinocytes

To determine if the MEK-ERK pathway had any relevance or significance in human cells, Normal Human Epidermal Keratinocytes (NHEK) were infected with the amphotropic Ras virus, created as described previously in the methods section. Protein was harvested using RIPA lysis buffer on day 5 and a BCA protein assay kit was used to determine concentrations. An SDS-PAGE was used to evaluate the RIDD pathway and analyze protein expression.

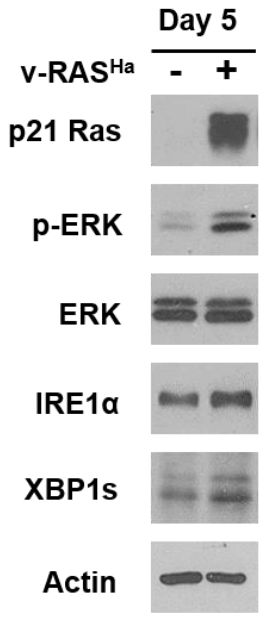

Figure 5 Immunoblot analysis was completed in normal and Ras induced NHEK cells to determine protein levels of p21 Ras, p-ERK, ERK, IRE1Aα. Actin was used as a loading control.

Results correlated as expected as seen in figure 5. Ras transduced NHEK cells showed an increase in P-ERK, IRE1α, and XBP1s signaling indicating that Ras is activating the IRE1α RNase activity in Normal Human Epidermal Keratinocytes (NHEK) similarly to its activation in Mouse Epidermal Keratinocytes.

Chapter 4

Discussion

4.1 Overview

Upon activation, the Unfolded Protein Response is initially serving as an adaptive measure to decrease the amount of stress the endoplasmic reticulum is experiencing by activating transcriptional and post-transcriptional mechanisms. These mechanisms increase the ER chaperones present and also work to hinder translation while increasing decay of mRNAs of proteins throughout the cell. Previous research involving chemical agents such as Dithiothreitol (DTT), Thapsigargin, and Tunicamycin have all lead to findings linking IRE1α to playing a role in the regulation of cell fate. These drugs were all found to upregulate ER stress via protein misfolding, thus activating the UPR (19). If these stresses become too great on the ER, though, the UPR can push the cell towards apoptosis through the RIDD pathway (21). Conversely, when *Xbp1* mRNA splicing is favored, XBP1s is generated and upregulates cell chaperones that regulate proper protein folding (20). Because of these diverging responses in the IRE1α pathway, the role of the protein is still not understood with regards to its role in cancer.

The goal of the research conducted here was to use an *in vitro* mouse model to determine how oncogenic Ras activated the UPR pathways as opposed to chemical ER stressors. This model has allowed us to replicate an early stage cancer with only one genetic change in a normal epithelial cell. It is more comparable to a physiologically relevant system and helps to determine how the UPR is actually activated in human cancers compared to chemical treatments with agents such as thapsigargan. While ER stress induced by chemical agents directly affects the protein folding, the ER stress induced by Ras activates signaling pathways that change cell phenotype (19). Here we

found that Ras induced ER stress and singularly activated IRE1α and not the ATF6 or PERK portions of the UPR pathway as opposed to the chemical stress models which showed all three UPR pathways to be activated (19). Rapid activation and up-regulation of IRE1α protein caused a direct increase in the *Xbp1* mRNA splicing and thus XBP1s protein levels. This IRE1Aα activation and protein increase differed from the chemical model of induced IRE1α which showed only an activation of the pre-existing protein (19). Additionally, the mRNAs encoding the membrane proteins PMP22 and HGSNAT were degraded by the IRE1α RNAse. When the Mouse Epidermal Keratinocytes were treated with U0126, a selective inhibitor of MEK1 and MEK 2, which is a type MAPK/ERK Kinase, we saw a down regulation in the amount of ERK phosphorylation as well as the amount of IRE1α. Additionally, we saw that *Xbp1* mRNA splicing was decreased and that *Pmp22* and *Hgsnat* degradation was almost completely attenuated. This indicates that the well characterized MAPK/ERK pathway, which is activated by the Ras oncogene, plays a role in the regulating IRE1α activation.

As a whole, this data further strengthens the hypothesis that early in cancer, Ras activation and activation of the MAPK pathway leads to proliferation followed by senescence as a cellular survival mechanism. We have indicated that this duel response of IRE1α may have a potential for therapeutic modulation as IRE1α acts as a conditional tumor suppressor. While XBP1 can actually promote tumor growth, regulation of the RIDD pathway has tumor suppressive qualities. For this reason it may actually be detrimental to entirely inhibit IRE1α function.

Unpublished data that has not been shown here indicated the role of senescence in MEK cells. A ShRNA lentivirus was created for both IRE1α and XBP1 to allow knock down of their respective proteins. The IRE1α knock down resulted in a lessened response from Ras as well as decrease in p-ERK, and BiP levels. The XBP1 knock down MEK cells showed a rapid up-

regulation of p-ERK as well at p-IRE1α and total IRE1α. U0126 treatment of shXBP1 treated cells no longer showed signed of senescence indicating that the MAPK pathway was regulating senescence in XBP1 deficient Ras Keratinocytes. Treatment with U0126 on shXBP1 treated Ras induced mouse keratinocytes also inhibited phosphorylation of IRE1α as well as total levels of IRE1α. This led to the conclusion that it was in fact the IRE1α RNase action which was playing a role in the regulation of senescence in ShXBP1 treated Ras keratinocytes (Blazanin *et al* manuscript in preparation). The combined effects of the RNase activity have illustrated the duel functionality resulting in either Oncogenic or tumor suppressive roles.

To determine if the IRE1α pathway was activated in a similar manner to its activation in mouse keratinocytes, normal human epidermal keratinocytes were cultured to run a variety of experiments. Ras infected NHEK cells induced the IRE1α cells in a similar fashion to the MEK's. Many experiments still need to be conducted in order to conclude that the duel functionality of IRE1α is maintained in the human cell line.

4.2 Further Experiments

Further Experiments in NHEK Cells

The first steps that need to be taken in order to determine the role of this protein in human cells is to characterize the RIDD pathway. An experiment should be run that involves the infection of NHEK cells with amphotropic Ras virus to obtain RNA isolates in order to analyze the relative mRNA levels *of Xbp1s, Hgsnat*, and *Pmp22* as done in MEK cells. Additionally, human ShIRE1A and ShXBP1 lentivirus should be made to knock down the expression of these proteins and determine if the MEK/ERK signaling pathway as well as other RIDD targets that were observed

in MEK cells are also exhibited in NHEK cells upon pathway activation. Based on our results in MEK cells, it would be expected that the IRE1α activity free of *Xbp1*1 splicing would result in increased senescence.

There are still many unanswered questions revolving around the role of IRE1α. Because it is unknown if the RIDD targets are tissue specific or constant during cancer we should consider evaluating the selective activation of IRE1α in the model that has been used. This selectivity was discovered in both the MEK and NHEK cell lines.

Further experiments to characterize IRE1α activation

It will be necessary to characterize if IRE1α activation is dependent on Ras induced ER-stress. To do this the Ras transduced cells could be treated with a drug called 4-phenyl butyrate (4-PBA). This drug is a chaperone that is known to reduce the misfolded protein load in the ER lumen and thus increase ER function and reduce ER stress (22). In 4-PBA treated MEK cells it was seen that IRE1α phosphorylation was decreased, resulting with a correlating down-regulation of BiP, *Xbp1*s, *Hgsnat* and *Pmp22* (Blazanin *et al* manuscript in preparation**)**. It would be beneficial to see if this pathway remained activated in a similar fashion in 4-PBA treated NHEK's. Another potential drug that could be used similarly to 4-PBA is the ER stress inhibitor tauroursodeoxycholic acid, TUDCA, an ER resident chaperone. One study completed by Son *et al.* showed that the drug can protect against ER stress by inducing IRE1α knock down, however it would not rescue the cell from IRE1α-KD induced cell death by attenuating only ER stress (23). We would expect the TUDCA to decrease IRE1α phosphorylation and thus downregulate the major proteins and mRNA that we have been looking at (BiP, XBP1s, *Hgsnat* and *pmp22*).

Determining Phenotype in Human cell lines

Another potentially useful experiment would be to look at the phenotype of IRE1α expression in a human tumor. However, because it would be very difficult to prove the expression of a specific mRNA is due exclusively to RIDD, this may only be a descriptive study. To determine a more in depth understanding in vivo it would be beneficial to look at different tumor stages using tumor cell lines from malignant and premalignant human lesions. Experiments could be set up using these cell lines along with shIRE1α and shXBP1 lentiviruses to determine what protein expression is lost or enhanced with tumor progression. It would be useful to compare IRE1α activation, ER stress as well as the RNase outputs. This may help provide more insight into understanding at which stage the activation of IRE1α is occurring as well as how early to target its expression and other factors that could lead to a more accurate view of the signaling events occurring and potential therapeutic target possibilities.

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ACADEMIC **VITA**

Alayna Craig-Lucas
329 E. Beaver Ave. Apt 503, State College PA, 16801
116 Miles Street. Dalton, PA 18414.
570.780.4887. abc5216@psu.edu

Education:

Schreyer Honor's College at The Pennsylvania State University State College, Pennsylvania.
Major: Toxicology
Minor: Bioethics and Medical Humanities

Relevant Coursework:

Tumor Viruses and Oncogenes
Environmental Toxicology
Immunotoxicology
Molecular and Cellular Toxicology
Bioethics and Medical Humanities Capstone
Introduction to biostatistics
General Biochemistry
Organic Chemistry (+lab)
Biology: Molecules and Cells
Introduction to Molecular Pharmacology
Principles of Epidemiology
Principles of Toxicology
Mammalian Physiology
Values and Ethics in Human Development Professions
Introduction to Bioethics
Critical Issues in Reproductive Health.
General Chemistry (+lab)
Biology: Function & Development of organisms

Research Experience:

Summer 2013-Present: The Pennsylvania State University: Department of Veterinary and Biomedical Sciences: Cancer Mechanisms involved in ER Stress. Dr. Adam Glick, Principal Investigator.

Academic Distinctions:

2011-present: National Society of Collegiate Scholars
2013- 2014: College of Agriculture Undergraduate Research Grant
2012-2015: Dean's List

Technical Skills:

Virus production
qRT-PCR
Genotyping
Western blotting
Stable cell transformation
Agarose gel electrophoresis
Primary keratinocyte and immortalized cell culture
Transfection
Protein and mRNA isolation

Posters:

Divergent IRE1Aα endonuclease outputs dictate the senescence response to oncogenic RAS: Nicholas Blazanin, Christian John, Alayna Craig-Lucas and Dr. Adam Glick

Effect of Oncogenic Ras on the IRE1Aα Pathway in Human Keratinocytes: Alayna Craig-Lucas

Leadership Positions:

2014-2015: The Penn State IFC/Panhellenic Dance Marathon Hospitality Captain
2013: HOBY (Hough O'Brien Youth Leadership) Associate Facilitator

Volunteer Work:

Fall 2014: Mid State Literacy Center ESL Tutor
2011-2014: Honors College Day of Service, Welcome Crew, Penn State Day of Service

2012-2014: The Penn State IFC/Panhellenic Dance Marathon Morale Committee Member (Position: Weekend Warrior)

Work Experience:

Summer 2012: Medical externship with orthopedic surgeon Dr. John Doherty
2009-Present: Heart to Art-part time staff member.
2007-Present: Laura Craig Galleries- part time staff member