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THE INTERSECTION OF EXTRACELLULAR SIGNALING AND STRESS PATHWAYS

By
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B.S. University of Wyoming, 2014

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THE INTERSECTION OF EXTRACELLULAR SIGNALING AND STRESS PATHWAYS

By Sari Mayhue

Thesis Advisor: Dr. Joshua Kelley


Cell growth is reliant on the flawless orchestration of cellular signaling and is crucial to evade cancer metastasis. It is important to understand key elements of cellular processes like gene regulation and stress signaling and how they contribute to oncogenesis. Cancer cells prove exceptionally adaptive as they effectively evade cellular stress, thus encouraging a tumor hospitable environment and subsequently cancer metastasis. Protein-folding and cellular homeostasis are essential functions of the endoplasmic reticulum (ER). An overabundance of protein accumulation within the ER jeopardizes cellular homeostasis causing stress. Under ER stress, these functions fail to maintain cellular stability resulting in the activation of the unfolded protein response (UPR). The UPR allows the cell the capacity to overcome cellular instability through the IRE1α, ATF6, and PERK sensor-driven pathways. It has been recently shown that the tumor necrosis factor alpha induced protein-8 type 1 (TNFAIP8L1) gene plays a role in tumor proliferation, although its participation within ER stress has not been established. By inducing cellular stress, we aim to characterize the effects of cellular stress and the associated pathways it uses to allow cancer metastasis. Similarly in yeast, cellular mechanism dysfunction affects the cell’s ability to maintain homeostasis and cell cycle activity. In Saccharomyces
cerevisiae, complex molecular networks manage responses to extracellular stimulus and internal stresses simultaneously. Signaling pathways utilize internal switches such as g-proteins, which are conserved from yeast to human. We investigate how stress signaling and internal stress encourages cytokinetic defects. Pathway coordination of molecular machinery is an integral piece of genomic stability. The complete grasp of how these signaling pathways are regulated and their potentially unique contribution to medicine have yet to be discovered.
DEDICATION

I would like to dedicate this thesis work to my children, Dania, Carter, Kyle, Raegynn, and Jerek.

No challenge or journey is too big nor too long to attempt with the company of family.
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This has been quite the journey and I have many people to personally thank for assisting me along the way. First, I would like to specially thank my advisor, Josh, for teaching me a wide variety of lab techniques and skills and all his patience and excellent science and career advice and all the support he has given over the past few years. I would like to thank my committee members for all the advice and guidance given along the way allowing me to become a critical thinking scientist. The graduate students of the Kelley lab, Kat, Cory, and Ahmed have truly been a caring and supportive team and I cannot thank them enough. Most importantly, I would like to give the biggest thanks to my husband, Shawn and our kids, and my sister, Jozi. They all have been my biggest fans and supporters and words cannot express how much I appreciate them.
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CHAPTER 1
CANCER

In past decades, cancer has progressed into a leading health concern worldwide. Because a majority of cancers are chemoresistant and have shown to promote tumor persistence with current interventions, the discovery of new therapeutic targets would considerably impact therapy strategies. More recently, therapies have shifted to a molecular targeted approach [1]. Tumor suppressing/promoting genes and signaling cascades may offer previously unexplored avenues for novel therapeutic approaches. Cancer propagates from six biological events to include cell death evasion, proliferation, bypassing tumor suppressor activity, allowing for replicative immortality, stimulating angiogenesis, and instigating invasion and metastasis [2]. Stress signaling is an important component in the propagation of these events.

1.1. Stress Signaling

Stress signaling greatly contributes to the formation and metastasis of tumors. All cell types undergo stress and must adapt to their stress to ensure homeostasis is a constant component within the cell. Methods to combat such stress are initiated through signaling. Dependent on the type of stress a cell undergoes, it will either strive to activate survival capabilities or it will attempt to commence death of the cell [3]. Cells rely on the activation of signaling pathways in response to homeostatic conditions [4]. When these signals fail to recognize cellular homeostasis and initiated pathways are unable to correct cellular stress, oncogenic cells are capable of tumor formation and metastasis.

1.2. Cellular Checkpoints

Cells are comprised of intricate signaling cascades that work together with checkpoints to maintain cellular homeostasis and cellular function. Mechanisms that actively prevent
advancement in the cell cycle in order to address and amend failure in crucial processes like DNA replication are referred to as a cell cycle checkpoint [7]. Variations within these cell cycle checkpoints and repairing pathways may lead to cancer metastasis [8]. Proper cell regulation is highly controlled by checkpoints within mitosis to ensure faithful cell division. Genes can also play a critical role in cellular checkpoints. The Unfolded Protein Response acts to recognize unrest in the ER and consequently triggers signaling pathways in attempt to restore normal ER function.

1.3. Tumor Necrosis Factor (TNF) and Cancer

Tumor Necrosis Factor (TNF) acts as a cytokine member of the inflammatory response. When first discovered, this small protein proved to facilitate both tumor progression and tumor cell suppression [9]. As research evolved over TNF function, it was found to participate in an array of physiological tasks associated with immunity and development [10]. A typical attribute of cancer, cellular stress, contributes to inflammation triggering TNF function. A notable role for TNF is cell signaling affecting both anticancer and cancer propagation [11]. TNF has proven a vital avenue for cancer therapies given its multifaceted involvement in cell signaling and its influence over cancer progression.

1.4. G Protein Coupled Receptors (GPCRs) and Cancer

G Protein Coupled Receptors (GPCRs) are cell surface receptors that respond to extracellular signals. Signaling through GPCRs affects invasion and proliferation within cancer biology [12]. GPCRs are instrumental to various physiological functions, as well as cancer metastasis [13]. Their role in a magnitude of cellular functions from maintaining cell survival and motility to players of tumor growth and proliferation renders them an integral part of the human physiology. Recently, GPCRs have shown to participate in substantial roles in the most common cancers
including lung, breast, colorectal prostate, and gastric cancers [14]. GPCR signaling cascades have become of interest for potential avenues in cancer therapeutics and autoimmune diseases [15].

1.4.1. GPCRs and TNF in Cancer

Involvement of both GPCRs and TNF signaling cascades are seen in a wide variety of disease onset including tumor growth and metastasis. Interaction between receptors like GPCRs have shown to play a role in neoplastic cells [16]. It is well established that TNF is highly involved in tumor immune surveillance, as well as tumor development and persistence [17]. In addition, crosstalk between GPCRs and TNF indicates the role pro inflammatory mechanisms have in regulating GPCRs [18]. G protein-coupled receptor kinase (GRKs) activation has shown dependency upon TNF-α for activation in HEK293 cells [19].

1.5. Yeast as a Model Organism for Cancer

Although not as common as the murine model for cancer research, yeast has been a used as a model in certain aspects of cancer research, such as anticancer drug responsiveness [108]. Yeast have demonstrated to be quite an advantageous model due to several similarities to mammalian cells like cell cycle control. Mammalian apoptosis can be similarly mimicked by a programmed cell death process that yeast endure [20]. Cellular adaptations produced by molecular pathways give indication of oncogenic cell features. In both mammalian tumor cells and yeast cells, cells use similar tactics to provoke increased anti-apoptosis mechanisms to increase survival [3, 21].
CHAPTER 2

PROLONGED ER STRESS DOWNREGULATES TNFAIP8L1 EXPRESSION IN LUNG CANCER CELLS

2.1. TNFAIP8 Family

The Tumor necrosis factor alpha induced protein-8 type 1 (TNFAIP8) family consist of 4 genes that play a roles tumor suppression and promotion. TNFα is an inflammatory cytokine involved in cell signaling regulating inflammation [22]. Upon activation of the NF-κB pathway, expression of TNFAIP8, also known as TIPE, family proteins are induced [23, 24]. All members of the family, to include TNFAIP8, TNFAIP8L1, TNFAIP8L2, TNFAIP8L2, possess similar homology to one another and have been shown to participate in cell proliferation and cell death [25]. Additionally, the TNFAIP8 family appears to have a vital role in immune homeostasis [26].

2.1.1. Tumor Necrosis Factor Alpha Induced Protein-8 Type 1 (TNFAIP8L1)

TNFAIP8L1 (TIPE1) has been previously characterized to play a role in tumor suppression and is of notable interest as very little is known about its role in tumor suppression under the context of ER stress. TIPE1 is known to interact with small signaling G protein, Rac1 and a negative regulator of mTOR signaling which implies its involvement to enable cell death [23, 27].

TNFAIP8L1 has demonstrated oncogenic involvement in certain cancers. TNFAIP8L1 deficiency has shown to stimulate lung cancer progression, prevent cell proliferation and induce apoptosis indicating an anti-tumor role [26]. Other research revealed TNFAIP8L1 to have a tumor suppressor role within Hepatocellular carcinoma cells (HCC) and furthermore, exhibiting increased expression of TNFAIP8L1 in adjacent non tumor tissues in HCC and cell growth inhibition [28].
2.2. Pleckstrin Homology (PH) Domains and Phosphoinositide (PIPs)

Pleckstrin Homology (PH) domains are significant to intracellular signaling and protein recruitment. They possess high affinity and specificity and is a commonly found domain in humans [5]. Phosphoinositides (PIPs) are phospho lipids in which their primary role is protein interaction. The popularly known PIP, phosphatidylinositol 4,5-biphosphate, or PIP2, is the main substrate to phospholipases C, or PLC, exemplifying protein-lipid interaction [6]. PH domains account for the biggest lipid binding domain family and are critical to cellular signaling. Upregulated phosphoinositide signaling plays an important contributor to certain cancers. The TNFAIP8 family participates as transfer proteins of PIP2 and PIP3 [107]. Upon conversion of PIP2 to PIP3, the recruitment of PH domain proteins can ensue. This process includes AKT proteins through mTOR (mammalian target of rapamycin) signaling, which is an associated pathway of TNF signaling [106].

2.3. MTOR Signaling Pathway

The mTOR signaling pathway is part of the phosphatidylinositol 3-kinase-related kinase family and is involved in cell survival. Dysregulation in the mTOR pathway is associated with among other diseases, some cancers [103]. TNF is a known activator of phosphatidylinositol 3-kinase (PI3-kinase) and Akt serine-threonine kinase, which participates in the initiation of NF-κB signaling [104]. Members of the TNFAIP8 family have presented a distinctive role in down regulating the mTOR-Akt-ULK1 pathway while inducing autophagy in gastric cancer cells [105]. MTOR signaling possess a crucial role in aspects of cellular stability influencing cell fate and potential oncogenesis.
2.4. Non Small Cell Lung Cancer (NSCLC) Cell Lines

Lung cancer is the third highest diagnosed cancer in the United States [29]. In addition, lung cancer is responsible for the greatest fatal outcomes over all other cancers [29]. Lung cancer can be divided into 2 main categories, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), which account for approximately 80-85% and 10-15% of lung cancer diagnosis, respectively [30]. There are somewhere between 300 to 400 various types of lung cancer cell lines [31]. NSCLC cell lines were shown to be an appropriate model in cancer biology [32]. Here, we use two NSCLC lines, A549 cells and H1299 cells. The A549 cell line is derived from lung carcinoma tissue of a 58 year old Caucasian male [33]. The H1299 cell line is derived from a lymph node metastasis of the lung from a 43 year old Caucasian male [34]. Both cell lines are adherent cells and have demonstrated to be suitable transfection hosts.

2.5. Endoplasmic Reticulum (ER) Stress

Cancer cells operate by evading cellular checkpoints which prevent abnormalities to destructively alter the cell and allow for proliferation. The endoplasmic reticulum (ER) is responsible for cellular homeostasis and protein folding. Under normal conditions, calcium concentrations and oxidizing redox potential are greater than that found in the surrounding environment [35]. Protein folding capacity and processes become altered resulting in protein aggregation or accumulation [36]. This is due to disturbances in the calcium concentration or redox state of the ER [36]. Genomic instability and protein mutation promote greater complications for proper protein folding in the endoplasmic reticulum [37]. This is a common occurrence in both solid and hematopoietic tumors [37]. The ER is equipped with mechanisms in response to failed homeostasis and misfolded or unfolded protein accumulation. When such events occur, the unfolding protein response is initiated.
2.6. Unfolded Protein Response

One cellular checkpoint occurs when the ER undergoes stress due to an overabundance or misfolding of proteins and initiates the unfolded protein response (UPR). The UPR attempts to resolve ER stress and allow for homeostasis to return. The UPR has been implicated in the enablement or inhibition within a variety of pathophysiological processes. [38]. Abundant proteins in the ER, like Binding-immunoglobulin protein (BiP), also known as GRP-78, act to chaperone folding proteins in the ER. GRP-78 aids in the prevention of protein aggregation and proper folding conformation [39]. Degradation and autophagy pathways are initiated in the event proper protein conformation has been unsuccessful [40].

The UPR is comprised of three transmembrane sensors; protein kinase RNA-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring protein-1α (IRE1α) that act to assist in maintaining cellular homeostasis. Persistent activation of the PERK pathway precedes to apoptotic events [41]. Upon activation of the UPR, the ATF6 pathway is transported out of the ER to the Golgi apparatus and aids in mechanisms of ER degradation [42, 26, 43]. Cell survival is the primary mission of the IRE-1α pathway. The ER chaperone, BiP, is activated during cellular instability allowing the transmembrane sensors to initiate their own activation to participate in the restoration of cellular homeostasis [38]. However, when cellular stability is unattainable, apoptosis of the cell is promoted.

2.7. X-Box Binding Protein 1 (XBP1)

Although a multifunctional protein for a variety of cellular processes, XBP1 is a transcription factor and plays a key role in the UPR. During ER stress and subsequent activation of the UPR, XBP1 is spliced by IRE1, creating the spliced 26 nucleotide XBP1 variant XBP1s containing a C-terminal transactivation domain that is not found in the unspliced XBP1 variant XBP1u [44,
Upon translocating to the nucleus, XPB1s operates by initiating transcriptional mechanism of UPR associated genes [26]. XBP1 has been shown to critical role in for cell stress environments and cell survival [47]. In addition, it has been shown that XBP1s has been an integral part in transcription reprogramming in not only cancers, but also metabolic diseases [26].

2.8. Heat Shock Proteins

Heat shock proteins (Hsp) are expressed during times of cellular stress as they help to protect cellular processes and inhibit apoptotic responses. Overexpression of heat shock proteins are seen in a variety of cancer types [48]. Heat shock protein expression has been linked to metastasis, tumor cell proliferation, and has even been implicated in the prognosis and aggressiveness of certain cancers [48].

2.8.1. Heat Shock Protein 70 (Hsp70)

Hsp70 is specifically involved in protein folding and cell protection during stress. This family of heat shock proteins has been shown to be overexpressed most cancers but notably in breast [49], esophageal [11], colon [50], liver [51], cervical [52], and prostate [53] cancers. More interestingly, Hsp70 is of particular significance as it suppresses the NF-kB pathway, allowing inflammation inhibition, which has been associated to patient prognosis is lung cancer [54] melanomas [55], cholangiocarcinoma [56], and squamous cell carcinoma [57, 58, 59, 60].

2.9. Methods

2.9.1. Cell Culture

A549 human epithelial lung cancer cells (ATCC, CCL-185) and H1299 human epithelial lung cancer cells (ATCC, CRL-5803) were grown at 37°C in a humified atmosphere of 5% CO₂ in Roswell Park Memorial Institute medium 1640 with glutamine (Corning, Corning, NY)
supplemented with either 10% Fetal Select or Fetal Bovine Serum (Atlas Biologicals, fort Collins, CO). Phosphate Buffered Saline (VWR, Radnor, PA) was used as a washing agent. Both cell lines were regularly passaged every 2-3 days to ensure proper health and growth.

2.9.2. ER Stress Inducing Drug Exposure

A549 and H1299 Cells were plated in 12-well plates and exposed to either Brefeldin A (BFA) (Millipore Sigma, Burlington, MA) at a concentration of 0.5 g/ml for 24 hours at 37°C in a humidified atmosphere of 5% CO₂ or Thapsigargin (TG) (Sigma-Aldrich, Burlington, MA) at a concentration of 100nM for 16 hours at 37°C in a humidified atmosphere of 5% CO₂. RNA from cells was collected and qPCR was performed as described in a later section.

2.9.3. Chemical Inhibitors

Cells were plated in 12-well plates and exposed to one of the three inhibitors: PERKi (Millipore Sigma, Burlington, MA), AEBSF (Millipore Sigma, Burlington, MA), and STF083010 (Tocris, Minneapolis, MN) at concentrations of 0.5 μM, 300 μM, 50 μM, respectively, plus BFA at a concentration of 0.5 g/ml for 24 hours at 37°C in a humidified atmosphere of 5% CO₂. RNA from cells was collected 24 hours later.

2.9.4. RNA Isolation

Cells were initially plated at appropriate concentrations in either 12 or 24-well plates. At 24 hrs post drug exposure, cells were washed in Dulbecco’s Phosphate Buffered Saline 1x (PBS) and RNA was collected using the Aurum Total RNA Fatty and Fibrous Tissue Kit (BioRad, Hercules, CA). Cells were then used in qPCR and further stored at -80°C.

2.9.5. Quantitative PCR

Cells were collected at appropriate time points and RNA was collected using the Aurum Total RNA Fatty and Fibrous Tissue Kit (BioRad, Hercules, CA). 20 µl reactions were performed using
the Luna Universal One-Step RT qPCR Kit (New England Biolabs, Ipswich, MA). CT values generated and analyzed with the CFX Manager version 3.1 software (BioRad, Hercules, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. Other primers used in this study include human TNFAIP8L1, human TNFAIP8v1, human TNFAIP8v2, human TNFAIP8L3, XBP-1, HSPA6, HSPA1A, and HSPA1B. QPCR data was graphed using GraphPad Prism version 8 (GraphPad Software, La Jolla, CA).

2.9.6. Transfection with siRNA and plasmid

For siRNA-mediated knockdowns, A549 and H1299 cells were transfected with DharmaFECT1 and DharmaFECT2 (Dharmacon, Lafayette, CO), respectively. SiRNAs were mixed with Opti-MEM and DharmaFECT transfection reagent for 24 hours according to manufacturer’s directions. For plasmid transfections, a cDNA3.1 HA tagged plasmid overexpressing TNFAIP8L1 was obtained from Genscript (Piscataway, NJ). The cDNA3.1 HA tagged plasmid overexpressing TNFAIP8L1 was mixed with Opti-MEM and A549 cells were transfected with ViaFECT (Promega, Madison, WI).

2.9.7. RNAseq and analysis

Human lung cancer cell line A549 were plated in a 12 well plate and exposed to Brefeldin A (BFA) at a concentration of 0.5ug/mL at 37°C in a humidified atmosphere of 5% CO₂ for 24 hours. Total RNA was collected as previously described and prepared in accordance with KAPA Stranded RNA-Seq Library Preparation Kit with poly(A) selection and 201-300bp insert size (KAPA Biosystems, Wilmington, MA). Library construction and RNAseq were performed by Quick Biology (Pasadena, CA). Sequence of 150bp paired end reads was conducted by Illumina HighSeq 4000 (Illumina Inc., San Diego, CA).
2.9.8. Statistical Analysis

All experiments were performed in triplicate. All presented data is shown as the Mean ± SD with P-values calculated with 2-tailed t-test. Statistical analysis for all experiments were performed using GraphPad Prism version 8 (GraphPad Software, La Jolla, CA).

2.10. Results

It has been previously shown that *TNFAIP8L1* participates as a tumor suppressor yet the mechanism by which it is regulated under cellular stress, specifically ER stress is poorly understood. Upon cellular stress induction, we intend to illustrate the effects of cellular stress and the correlated mechanism used to promote cancer metastasis. We hypothesize that *TNFAIP8L1* is regulated by one of the ER sensor-driven pathways that mediates the UPR.

2.10.1. BFA and TG Initiates ER Stress and Subsequently UPR in Lung Cancer Cells

We began to test this hypothesis by looking at the effects of ER stress on *TNFAIP8L1* transcript expression. Previous studies investigating ER stress in cancer cells used the drugs, Brefeldin A (BFA) and Thapsigargin (TG). BFA works to inhibit the transportation of proteins, causing a buildup of proteins in the ER. BFA was used as an ER stress inducer to activate the UPR in hepatic stellate cells (HSC) [38]. Thapsigargin has also been used as a pharmaceutical ER stress inducer as seen in a study with both LNCaP prostate cells and HCT116 colon cancer cells [61]. Thapsigargin functions as an ER Ca$^{2+}$ ATPase inhibitor, depleting ER calcium stimulating an ER stress response. We exposed 2 different lung cancer cells, A549 and H1299 NSCLC lines, to BFA and TG at a concentration of 0.5 µg/ml for 24 hours and 100 nM for 16 hours, respectively. Optimal concentrations of ER stress inducing agents we established by previously conducted ER stress research (Osłowski, 2013, Linder, 2020) Quantitative PCR revealed a downregulation in transcript expression of tumor suppressor gene, *TNFAIP8L1*, indicating a trigger of ER stress
(Figure 1). Although both the A549 and H1299 cell lines both showed a downregulation in relative expression of 26.5-fold and 3-fold, respectively, the A549 line revealed much more apparently transcript expression reduction. Furthermore, a more robust response was witnessed with the use of BFA over TG and therefore moving forward, BFA was the primary pharmacologically ER stress inducer utilized in subsequent experiments.
Figure 1. BFA and TG Initiates ER Stress and Subsequently UPR in Lung Cancer Cells. **Top.** A549 (right) and H1299 (right) cells were exposed to BFA at a dose of 0.5 µg/ml for 24 hrs. Methanol was used for the control. RNA was isolated and transcript expression was measured through qPCR methods. The H1299 and A549 cells showed a 3-fold and 26.5-fold reduction respectively in TNFAIP8L1. ****p < 0.0001. **Bottom.** Downregulation of TNFAIP8L1 by Thapsigargin. A549 (left) and H1299 (right) cells were exposed to TG at a concentration of 100 nM for 16 hrs. Methanol and DMSO were used as controls. RNA was isolated and transcript expression was measured through qPCR methods. The H1299 and A549 cells showed a 1.5-fold and 3-fold reduction respectively in TNFAIP8L1. **p < 0.01; ***p < 0.001.
2.10.2. ER Stress Demonstrates Regulation of *TNFAIP8* Gene Family

Other members of the *TNFAIP8* gene family were subsequently exposed to BFA and transcript expression was measured for both variants of *TNFAIP8* (*TNFAIP8v1* and *TNFAIP8v2*) and *TNFAIP8L3*. The family member *TNFAIP8L2* was not measured for this experiment as it is not highly expressed in either cell lines. After exposing the A549 and H1299 cells to BFA for 24 hrs at a dose of 0.5 µg/ml, we found that transcript expression of *TNFAIP8v1* was not significantly downregulated (Figure 2). Interestingly, *TNFAIP8v2* transcript expression exhibited a significant downregulated upon ER stress in both the A549 and H1299 cells (Figure 2). It was also discovered that *TNFAIP8L3* showed a significant down regulation in transcript expression in both the A549 and H1299 cells (Figure 2).
Figure 2. ER Stress Demonstrates Regulation of **TNFAIP8** Gene Family. A549 (top) and H1299 (bottom) cells were exposed to BFA at a dose of 0.5 µg/ml for 24 hrs. Methanol was used for the control. RNA was isolated and transcript expression was measured through qPCR methods. Both cell lines show a significant downregulation of **TNFAIP8v2** and **TNFAIPL3**. ****p < 0.0001. ***p < 0.001.
2.10.3. Splicing of XBP-1 Indicates IRE-1alpha activation (activation of UPR).

Upon ER stress, the UPR becomes activated leading to the subsequent initiation of one of the three sensors, ATF6, PERK, or IRE-1α. The IRE-1α is the only pathway that entails the splicing of XBP1. ER Stress inducing drugs have been shown to produce a significant amount of cellular stress and therefore are quickly able to initiate all three UPR sensors [99]. To test whether *TNFAIP8L1* is regulated through the IRE-1α, we initially assessed transcript expression of both variants of XBP1, XBP1v1 (unspliced) and XBP1v2 (spliced) after undergoing ER stress. A549 cells were exposed to BFA, and quantitative PCR showed a 15-fold transcript expression in the cells that underwent ER stress (Figure 3). This data provided us encouraging evidence the IRE-1α pathway of the UPR was the probable mechanism employed in the regulation of *TNFAIP8L1* while under ER stress.
Figure 3. Splicing of XBP-1 Indicates IRE-1alpha activation (activation of UPR) Top. A549 cells were exposed to BFA at a dose of 0.5 µg/ml for 24 hrs. Methanol was used for the control. RNA was isolated and transcript expression was measured through qPCR methods. Exposure to BFA reveals a 15-fold transcript expression in XBP1v2 (spliced). Bottom. Analysis from RNA sequencing verification of spliced region of XBP1 upon BFA exposure (top) in comparison to unspliced region of XBP1 not exposed to BFA. Top data from my undergrad mentee Sam Mildrum. Bottom data generated from RNAseq project in collaboration with Ben King.
2.10.4. TNFAIP8L1 Expression is Partially Downregulated Through an IRE-1α Dependent and not the PERK or ATF Pathways

We did consider the other two UPR sensor pathways may have some regulation over TNFAIP8L1. To rule both the ATF6 and PERK mechanisms out, we exposed H1299 cells to BFA with the addition of the corresponding pharmacological inhibitor and measured the relative expression of TNFAIP8L1. The PERK and ATF6 pathways were examined using the inhibitors PERKi and AEBSF, respectively. It was noted that after subjecting the cells to BFA and subsequently the pharmacological inhibitor, there was no significant rescue of TNFAIP8L1 transcript expression leading us to believe both PERK and ATF6 molecular mechanism lack regulation over TNFAIP8L1 during ER stress (Figure 4A). The evidence led to the possibility of the UPR sensor IRE-1α maintaining some form of regulation over TNFAIP8L1 while under ER stress. Both A549 and H1299 cells were exposed to ER stress and preceded by the IRE-1α pharmacological inhibitor, STF083010, and transcript levels of TNFAIP8L1 were measured. It was revealed that BFA exposure induced a downregulation in TNFAIP8L1 expression as expected, but interestingly, after the addition of the IRE-1α inhibitor, partial rescue was observed, as TNFAIP8L1 transcript expression was partly upregulated in comparison to BFA exposure without the pharmacological inhibitor, STF083010 (Figure 4B). To verify these findings, we repeated the experiment with the A549 cells using an IRE-1α siRNA to knockdown IRE-1α expression and indeed discovered similar results implicating the IRE-1a pathway as a partial regulator of TNFAIP8L1 (Figure 4B).
Figure 4A. TNFAIP8L1 Expression is Partially Downregulated Through an IRE-1α Dependent and not the PERK or ATF Pathways

Top. H1299 cells were exposed to BFA + PERKi at a concentration of 0.5 µM for 24 hours. Methanol and DMSO were used as controls. RNA was isolated and transcript expression was measured through qPCR methods and showed no significant downregulation of TNFAIP8L1 in H1299 cells. Bottom. H1299 cells were exposed to BFA + AEBSF at a concentration of 300 µM for 24 hours. Methanol and sterile water were used as controls. RNA was isolated and transcript expression was measured through qPCR methods and showed no significant downregulation of TNFAIP8L1 in H1299 cells. n.s. = not significant: **p < 0.01; ***p < 0.001.
Figure 4B. TNFAIP8L1 Expression is Partially Downregulated Through an IRE-1α Dependent and not the PERK or ATF Pathways. (Pharmacological inhibition of IRE1α pathway rescues TNFAIP8L1 expression following ER stress). H1299 and A549 cells were exposed to BFA + STF083010 at a concentration of 50 µM for 24 hours. Methanol and DMSO were used as controls. RNA was isolated and transcript expression was measured through qPCR methods. **p < 0.01: ***p < 0.001:

Top Left. H1299 cells with BFA showed a 2-fold reduction and BFA + STF showed a 1.5-fold reduction in TNFAIP8L1. Top Right. A549 cells with BFA showed a 23-fold reduction and BFA + STF showed an 8-fold reduction in TNFAIP8L1. **p < 0.01: ***p < 0.001: ****p < 0.0001. Bottom. SiRNA-mediated knockdown of IRE1α rescues TNFAIP8L1 expression following ER stress. TNFAIP8L1 expression in A549 cells was knocked down by siRNA. At 24 hrs post transfection, cells were exposed to BFA at a dose of 0.5 µg/ml. Negative controls for siRNA and BFA exposure were included. RNA was isolated and TNFAIP8L1 transcript expression was measured through qPCR methods. n.s. = not significant; **p < 0.01; ***p < 0.001.
2.10.5. Rescue of *TNFAIP8L1* During ER Stress Affects HSP70 Family Signaling

In addition to examining *TNFAIP8L1* regulation during ER stress, we investigated outcomes of regulation. Heat shock proteins, especially the Hsp70 family, are notable components in cellular stress responses. As cells become stressed or exhibit inflammation resulting in protein misfolding, Hsp70 can respond to such stress by attaching to exposed hydrophobic surfaces inhibiting other interactions from occurring thus preventing aggregation to ensue [100]. To investigate whether the tumor suppressor, *TNFAIP8L1*, and its regulation has any correlation to other cellular stress response mechanisms. A549 cells were overexpressed with L1 and then subjected to BFA to induce ER stress. RNA was collected and then sequenced. We found that among many genes that displayed altered expression, the Hsp70 family was one of them. The MA plot (a graphical method to display plotted values of the mean average (A) against the log ratio(M) of gene expression) revealed a multitude of genes indicating expression when exposed to ER stress (Figure 5). From the MA plot, genes that showed highest expression are noted in the line circle figure (Figure 5). The heatmap shows gene expression and measures similarities within the gene expression when overexpressed with L1 and exposed to BFA in comparison to non-overexpressed L1 cells lacking ER stress (Figure 5). Members of the HSP70 family are significantly altered when overexpressed with L1 and exposed to BFA. To further probe these conclusions, QPCR methods were performed. Transcript expression of Hsp70 paralogs, HSPA1A, HSPA1B, and HSPA6 revealed Hsp expression increased in the presence of BFA and decreased in the presence of overexpressed *TNFAIP8L1* (Figure 5). These results proposed the idea that when the cell is under stress, Hsp functions to assist the cell back to homeostasis and when the tumor suppressor *TNFAIP8L1* is present in abundance, Hsp expression is less robust as the cell is not under unmanageable stress.
Figure 5. Rescue of TNFAIP8L1 During ER Stress Affects HSP70 Family Signaling (RNAseq reveals Upregulated genes when Exposed to BFA).
Figure 5 (cont’). Rescue of *TNFAIP8L1* During ER Stress Affects HSP70 Family Signaling (RNAseq reveals Upregulated genes when Exposed to BFA).
Figure 5 (cont’). Rescue of *TNFAIP8L1* During ER Stress Affects HSP70 Family Signaling (RNAseq reveals Upregulated genes when Exposed to BFA). A549 cells were overexpressed with L1 and subjected to BFA at a dose of 0.5 µg/ml. RNA was collected and sequenced. **Top Left.** The heat map displays gene expression and measures similarities within the gene expression. Members of the HSP70 family are significantly altered when overexpressed with L1 and exposed to BFA. **Top Right.** QPCR data of transcript expression of Hsp70 paralogs, HSPA1A, HSPA1B, and HSPA6. HSP expression increases in the presence of BFA and decreases in the presence of overexpressed *TNFAIP8L1*. **Top (second page).** Differential gene expression upon prolonged ER stress. MA plot displaying differential gene expression when exposed to ER stress. A549 human lung cancer cells were exposed to Brefeldin A at a concentration of 0.5ug/mL for 24 hours. RNA from cells were collected and used for RNAseq analysis. **Bottom (second page).** Gene networking map displaying data from RNAseq analysis indicates upregulated genes (green) and downregulated genes (red). QPCR figure design made in collaboration with Sari Mayhue and Con Sullivan. Heat map, MA plot, and gene networking map provided by Ben King.
2.11. Discussion and Future Directions

This project encompassed a broad investigation to determine regulation of tumor suppressor, \textit{TNFAIP8L1}, while under internal stress. It was determined that when lung cancer cell lines are exposed to ER stress, \textit{TNFAIP8L1} reveals a downregulation in transcript expression. Upon further investigation, we concluded regulation of \textit{TNFAIP8L1} is partially mediated by the IRE-1\(\alpha\) pathway of the UPR. The involvement of IRE-1\(\alpha\) in cellular stress leads to not only cancers, but an array of other diseases including central nervous system and autoimmune diseases [62, 63]. The mechanism of IRE-1\(\alpha\) pathway functions initially by activation and dimerization upon detachment of GRP78 and followed by splicing the coding region of the transcription factor XBP1 [63, 45]. Both the PERK and ATF6 UPR sensor pathways did not appear to retain any regulation over \textit{TNFAIP8L1}. An in-depth RNA sequencing analysis led us to the observation of Hsp expression while cells were overexpressed with L1 and undergoing ER stress. Heat shock proteins are well known for their involvement in inflammation and cellular stress. It was to no surprise that during cellular stress, and upregulation of Hsp genes are highly expressed and lose expression in the company of overexpression of tumor suppressor, \textit{TNFAIP8L1}. Heat shock proteins, while possessing diverse functions in response to cell stress to include involvement in apoptotic pathways, can occasionally promote anti-apoptotic mechanisms. Hsp 70 has been shown to play a role in anti-apoptotic pathways, ensuring cytoprotective measures for the cell leading to tumor persistence and chemoresistance [64]. Along with heat shock 70, the heatmap revealed another heat shock protein, DNAJB1, part of the HSP40 family, to possess increased expression when overexpressed with \textit{TNFAIP8L1} and exposed to ER stress. Heat shock protein 40 is linked to essential protein functions such as folding and degradation, as well as interacting with HSP70 members. Moving forward, investigating Hsp expression through apoptosis assays
in overexpressed L1 cells may lead to a firmer grasp on the interaction between heat shock proteins and tumor suppressor gene, TNFAIP8L1. Another avenue to explore is to consider alternate ER stress inducing approaches. Investigating TNFAIP8L1 regulation while ER stress via nutrient deprivation and oxidative stress. Alternative methods of stress induction may impact cell response to stress and pathways utilized to help control homeostasis and the regulation of tumor suppressor gene, TNFAIP8L1. Understanding how tumor suppressors operate while under internal stress such as ER stress may provide useful information for future gene targeted therapies.
CHAPTER 3

STRESS SIGNALING AND MOLECULAR COORDINATION IN SACCHAROMYCES CEREVISIAE

3.1. Yeast as a Model Organism

GPCR signaling cascades and mechanisms of its machinery are highly conserved between humans and yeast. G-proteins have proven exceptional models to research cell signaling [65]. Unlike other model organisms, the simple eukaryotic organism, *Saccharomyces cerevisiae*, contains a critical mitotic checkpoint that serves an identical function in humans [66]. Yeast models allows us to easily investigate the coordination of machinery in simultaneous applications of extracellular signaling and internal stress responses. With the ease of propagation and gene manipulation, yeast prove a practical model to study cell cycle functions and signaling cascades [67]. Furthermore, yeast is well known for versatility and genomic manipulation as it became the first eukaryotic organism to have its genome sequenced [68]. Universal signaling pathways in mammalian cells are also present in yeast cells [69].

3.1.1. Gradient Tracking in Yeast

Similarly to chemotaxis mechanisms, yeast use gradient tracking for mating purposes. Pheromone is detected by the GPCR and allows for directed growth to the potential mating partner. *Saccharomyces cerevisiae* consists of two haploid mating partners, MATα and MATα [70]. Haploid yeast cells of one mating type will mate with a haploid cell of the other mating type. GPCRs will bind to the peptide pheromones that are secreted by the mating partner [71]. Once peptide-receptor interaction has occurred, the receptor acts as a guanine-nucleotide exchange factor (GEF) on Gα, allowing GDP to be replaced with GTP triggering dissociation of
Gβγ, via conformational change with GPCR [72]. This directs the initiation of mating pathway signaling. Upon mating with the corresponding mating partner, a diploid is produced as MATα/MATα (figure 6) [70]. Yeast mating by external stimulus is a basic cellular property that heavily depends on cellular and cytoskeletal reorganization to respond [73]. Once successful cell division has occurred, a yeast cell will organize its molecular machinery to orchestrate a shmooo projection when an external stimulus is available. The mating pathway relies on protein-protein interaction to efficiently construct scaffolds for the shmoo projection to reach its mating partner. This aspect of a yeast cell’s life may become disrupted if the cell has not achieved fidelity in cell division. These similar mechanisms are used in other physiological settings such as wound healing, inflammation, and tumor metastasis [74].

3.2. Regulator of G protein Coupled Receptor Signaling (RGS)

Regulators of GPCR signaling (RGS) are proteins that act to inactivate G protein via GTP hydrolysis. An important aspect of the RGS mechanism is that it allows GPCR signaling to ensue. In *Saccharomyces cerevisiae*, the RGS, referred to as Sst2, is a negative regulator of the pheromone pathway yeast use to detect a mating partner. Sst2 exposed its ability to foster pheromone desensitization in vivo [75]. Sst2 belongs to the RGS family, which is identified as the GTPase activating protein (GAP) in mammalian cells [75]. Interestingly, Sst2 shares similar sequence in the N-terminal domain to the human neurofibromatosis tumor suppressor gene [76].

3.3. Cytokinesis

Cytokinesis is the final step of mitosis and is reliant on tightly coordinated signaling before the cell can respond to vegetative growth or mating stimulus. Defects arising from the cytokinetic phase affect the cell’s ploidy and typically results in the generation of aneuploid cells [77]. Aneuploid cells account for almost 80% of all solid tumors [77]. This is regarded as a classic
trait in the production of tumors and the progression in cancer proliferation. During successful completion of the mitotic cycle, vegetative growth and sexual conjugation of yeast are preceded by cellular polarization that involve spatiotemporal regulation [78].

3.4. NoCut Pathway

The final stages of cytokinesis undergo checkpoints that are activated in a DNA damaging event. In *Saccharomyces cerevisiae*, early spindle damage and chromosomal segregation leads to a delay in abscission, thus prompting activation of the NoCut pathway [79]. The NoCut pathway serves as a checkpoint in the cytokinetic phase. The checkpoint pathway uses aurora kinase, Ipl1, and anillin-related protein, Boi2, to inhibit abscission of the cell [80]. Once activated, an aurora kinase signaling cascade is activated to correct the internal stress that is delaying the completion of cytokinesis. Further signaling extends to other proteins that are involved in GPCR signaling. Budding yeast formin, Bnr1, is responsible for assembly of filamentous structures and actin cables during polarized growth [101] Bnr1 and another formin, Bni1, localize to the bud neck and bud tip, as well as cable polymerization [102].

3.5. Methods

3.5.1. Yeast Strains

*Saccharomyces cerevisiae* strains were grown in filtered liquid synthetic complete media with 2 % dextrose (SCD) at 30 °C and cultured to an OD600 of 0.6 – 0.8. Cells were treated with 30 μM α-factor for 90 minutes. Cells were then fixed in 10 % paraformaldehyde, 2 % glucose, and 30 μM α-factor for 20 minutes. Upon fixation, cells were washed with 1x phosphate buffered saline (PBS) 3 consecutive times. Fixed cells were stained for 30 minutes with 5 μM of Calcofluor White in conjunction with 50 μg/mL of Concanavalin A. A second 3-consecutive
wash with 1x PBS was performed and then imaged using fluorescent microscopy. Randomly chosen fields were imaged and cells scored for cytokinetic failure.

3.5.2. Phospho-blotting of Sst2

Phospho-blotting of Sst2 was assessed by Western blotting. Yeast cultures were grown overnight in 30°C. Cells were lysed with TCA buffer and protein concentrations were determined using DC protein assay kit (BioRad). Protein separation was performed with a 7.5% SDS-PAGE and transferred to nitrocellulose at 100 V for 90 mins. Primary antibody (1:1,000) and non-phosphopeptide (1:10,000) were incubated in 1% PBST blocking solution overnight followed by secondary antibody incubation (1:10,000) in 1% PBST blocking solution for 1 hr. Band intensity was detected via Odyssey CLx imaging system (LI-COR) and then quantified using ImageJ.

3.5.3. Spontaneous Cytokinesis Experiments

Yeast cultures were grown overnight in filtered liquid synthetic complete media with 2% dextrose (SCD) at 30 °C and cultured to an OD600 of 0.6 – 0.8 and then treated with 100mM of hydroxyurea for 4 hours at 30C. Cultures were then fixed with ethanol and yeast cells were mounted to slides and imaged on the IX83 epifluorescent microscope (Olympus).

3.5.4. Fluorescent Microscopy Imaging Techniques

Cells were imaged on an Olympus IX83 with a 60X-TIRF 1.49 NA objective, a Photometrics Prime95b camera, Xcite LED 120 Boost fluorescence light source (Excelitas), and filters for DAPI and GFP (Semrock). Cells were grown to mid-log phase (OD600 = 0.1 to 0.8) at 30°C in filtered Synthetic Complete Media with 2% dextrose (SCD). Images on pads were made of 2% agarose in SCD. Imaging was performed with an objective heater (Bioptechs) set to 30°C. Cells were pelleted and then resuspended in SC with 3uM α-factor and placed on an agarose pad.
Images were deconvolved using Huygens (SVI) with the CMLE. Images were quantified using FIJI (Schindelin et al., 2012) and MATLAB (Mathworks)

3.6. Results

External stimulus, such as a pheromone response, has been well studied in yeast. The molecular machinery is comprised of a tightly regulated pathway that orchestrates gradient tracking and shmoo projections towards a mate. Signaling pathways and their components have been identified as targets of checkpoints during normal cell cycle [35]. GPCRs have been shown to possess complex mechanisms in response to a range of extracellular stimuli, including hormones and neurotransmitters [36]. How the molecular machinery responds to internal DNA damage while reacting to extracellular stimulus is not well understood. We hypothesize the pheromone signaling cascade is a shared mechanism when cellular stress arises thus halting mating signaling in order to address complications of cell cycle disarray. Our approach will investigate the ability for yeast to respond to pheromone and how the mating pathway is altered when internal stress arises.

3.6.1. Localization of the RGS is Dependent on Phosphorylation State

In Saccharomyces cerevisiae, GPCRs possess regulators to aid in pheromone response. There are four known regulator proteins of GPCRs, however, GTPase activating protein, Sst2, functions to negatively regulate g-protein [76]. Upon external stimulation, g-protein will dissociate into βγ subunit and α subunit, Gpa1, prompting a downstream cascade of events. Gα-GDP has been shown to negatively regulate mating pathway signaling [83]. Sst2 holds a distinct role aside from the other regulators in that it binds selectively to Gpa1 when in the transitional state conformation [20]. MAP kinase, Fus3, facilitates the phosphorylation of Sst2 [84]. Within the pheromone signaling cascade, pheromone is detected by GPCR, which activates g-protein which
precedes to a cascade of events including to recruit scaffold proteins [71]. MAPK Fus3 is critical to mechanisms of mating and cell cycle arrest [85, 86, 72]. The RGS becomes phosphorylated by MAPK on serine 539 when responding to pheromone [87]. The Hypothesis that phosphorylation of the RGS regulates spatial distribution was assessed using RGS mutants, Sst2 S539D (phosphomimetic) and Sst2 S539A (dephosphorylated) tagged with EGFP and Bem1-Ruby (polar cap localization). Over a 12 hour time course, cells exposed to pheromone at a concentration of 300nM in a microfluidic chamber [88]. Cells were then imaged by epifluorescence microscopy. It was revealed that RGS phosphorylation decreases its localization to the polar cap (Figure 6). This suggests that the WT RGS is in the unphosphorylated form. RGS distribution was also measured employing an averaged 3D-kymograph (Figure 6). The kymographs indicate a broadening of intensity in both the WT and RGS mutant (S539A) around the polar cap at 400mins. However, the RGS mutant (S539D) shows low polar cap association over 400mins. These data indicate a lack of interactions with binding partners at the center of the polar cap under the phosho-form of the RGS.
Figure 6. Localization of the RGS is Dependent on Phosphorylation State. 

A. Confocal images of WT, unphosphorylatable RGS (S539A), phosphomimetic RGS (S539D) fused with GFP in saturating pheromone. 

B. Quantification of the average RGS spatial distribution normalized to the polar cap marker (Bem-1Ruby) in saturating pheromone over a 12hr time course in a microfluidic gradient chamber, imaged by epifluorescent microscopy. Lines are derived from averaging of 180min onward. Bottom graphs display statistical analysis using one-way ANOVA followed by Tukey’s HSD, with statistically (p < 0.05) significant differences in localization noted by bars. Data is derived from n=89 cells (WT), n=88 cells (RGS mutant S539A), n=139 cells (RGS mutant S539D), with 29 time points per cell. 

C. 3-D kymographs of the spatial distribution of the RGS over 12hrs with 37 time points. Data provided by William Simke [89].
3.6.2. RGS Phosphorylation Peaks Early in the Pheromone Response and Diminishes at Later Time Points

Phosphorylation mutants of Sst2 occur at position 539. Upon replacement of serine-539 with alanine, RGS, while still able to negatively regulate Gα, is unable to be phosphorylated, restricting its function to regulate other polarity proteins [87]. To assess phosphorylation, a polyclonal antibody able to detect Sst2 phosphorylation on serine 539 was made and used for Western blotting. The addition of an unphosphorylated peptide was used to block non-specific interactions. We compared Sst2-GFP against bar1Δ and treated both strains with pheromone. Samples were collected every hour over a 240 minute time course. Phosphorylation of Sst2 peaks around 60-90 mins following pheromone exposure which is consistent with previous research [87] and then steadily decreases over a period of 150 mins (Figure 7) suggesting phosphorylation is likely required for mating responses. This may indicate mating cascade signaling disruption. At 90 minutes, yeast are able to complete mitosis and arrest in G1. We can further conclude that phosphorylation of RGS at S539 likely contributes to ensuring mitosis completion.
Figure 7. RGS Phosphorylation Peaks Early in the Pheromone Response and Diminishes at Later Time Points

A. Yeast cells were treated alpha factor for 4 hours and then collected at 1-hour intervals. Western blotting techniques show peak phosphorylation at 60-90 mins and then steadily decreasing thereafter. G6PDH was used as a loading control.

B. Quantification of Western blotting shown in A, normalized to G6PDH levels. Standard error of mean (n=3) is represented by error bars.


3.6.3. Phosphorylated Sst2 and Kelch-repeat protein, Kel1 Promotes Completion of Cytokinesis Prior to Pheromone Induced Polarization

The concluding step of mitosis, cytokinesis relies on strict signal coordination as the cell prepares to mate or enter vegetative growth. We observed mother-daughter cells in the RGS mutant S539A strains formed mating projections prior to completing cytokinesis (Figure 8A). Previous research concluded interactions between Bnr1 and kel1, both proteins involved in the cytokinetic process, and the RGS [90, 91]. It has also been demonstrated that negative regulation of formin, Bnr1, is mediated by Kel1[92]. We then investigated whether formin, Bnr1, and formin regulator, Kel1 were mediating cytokinetic defects. Kel1Δ and Bnr1Δ cells were exposed to saturating pheromone, stained with both Concanavalin A and Calcofluor White, and imaged with fluorescence microscopy. We discovered Kel1Δ cells failed to complete cytokinesis prior to responding to pheromone in a small portion of cells (Figure 8A, negative data for Bnr1Δ not shown). We found that this frequency occurred 3-4 percent of the time (Figure 8B). These data suggest that the RGS mutant S539A and Kel1 may both be a necessary component in the mechanism that ensures cytokinesis to complete before responding to pheromone. Additionally, we used hydroxyurea (HU), which works to inhibit deoxynucleotide production damaging DNA and pausing cytokinesis [93], to examine RGS phosphorylation ability to foster cytokinesis. Cellular destruction produced by HU exposure stimulates the NoCut pathway. Delay in polarity machinery from mating signaling may provoke defects found in the RGS mutant S539A and Kel1Δ mutant. Encouraging paused cytokinesis in the cells allow us to better characterize mechanisms with modified Sst2 and Kel1 signaling. Cells were pretreated with 100uM of HU for 2 hours and subsequently treated with pheromone and HU together for 4 hours. Cells were then stained with Calcofluor White and imaged with fluorescent microscopy. We determined that WT
cells revealed paused cytokinesis showing round cells joined at the bud neck. Alternate phenotypes were also discovered and scored based on successful cytokinesis before attempting polarized growth or arresting and lacking polarized growth (Figure 8D). Interestingly, the RGS mutant S539A and the Kel1Δ strains both resembled similar phenotypes of hyper-polarized growth, or asymmetric hyperpolarized growth (Figure 8F). The phosphomimetic RGS mutant (S539D) strain revealed repressed polarized growth when exposed to HU. These data implicate that dephosphorylated RGS shares some phenotypic similarity to strain inhabiting a loss of Kel1 function. We can further conclude RGS phosphorylation works to support a Kel1-dependent mechanism inhibiting mating response mechanisms from repurposing polarity processes before successful cytokinesis.
Figure 8. Phosphorylated Sst2 and Kelch-repeat protein, Kel1 promotes completion of cytokinesis prior to pheromone induced polarization. A. Microscopy images of RGS mutant (S539A) and Kel1Δ show failed cytokinesis prior to polarized growth. Cells were stained with both Concanavalin A and Calcofluor White prior to verify open bud neck. B. WT, RGS mutant (S539A), and Kel1Δ were exposed to pheromone in culture for 90 mins then fixed, stained with both Concanavalin A and Calcofluor White, and screened for failed cytokinetic events (n=1412 WT, n=1350 RGS mutant S539A, n=1396 Kel1Δ). C. Experimental design proposing the induction of cytokinetic arrest followed by a delayed pheromone exposure to investigate the role of RGS mutant (S539A) and Kel1Δ. D. Images of WT phenotypes in response to HU + pheromone. First column is DIC images, second column is DIC stacked images, and the third column us cell wall staining with Calcofluor white. Normal response to HU + pheromone encompasses the following: 1. Completion of cytokinesis, but arrest as a circular cell, in the event stress signaling is suppressing the pheromone response (minimal percentage of cells), 2. Lone cell responding to pheromone, 3. Completion of cytokinesis (if cells had resolved their DNA damage), followed by pheromone induced morphogenesis, 4. Arrest of cytokinesis yielding a mother daughter pair not exhibiting polarized growth. E. Frequency plots of normal response to HU and pheromone in the indicated strains. Error bars represent 95% confidence intervals. For each strain, n>640 cells for 3 experiments. F. Asymmetric hyperpolarized growth phenotype of RGS mutant (S539A) and Kel1Δ. G. Frequency plots of asymmetric hyperpolarized growth in response to HU and pheromone in the indicated strains. Figure was created in collaboration with Josh Kelley, Cory Johnson, Sari Mayhue, and Lucas Craig.
3.7. Discussion and Future Direction

This research project aimed to investigate how yeast coordinate extracellular signaling with internal stresses. The induction of cytokinetic defects allowed us to conclude that RGS in a phosphorylated state allows the cell to coordinate internal machinery by pausing cytokinesis without inducing mating polarity thus allowing the cell to prioritize the rectification of internal stress in order to continue signaling responses to external stimuli (figure 9). It was determined that Kel1 and Bnr1 are used to facilitate such a tightly regulated mechanism. Furthermore, we concluded the dephosphorylated RGS mutant, S539A, while critical for spatial regulation of Gα signaling, poses an obstacle for successful cytokinesis allowing internal stress to persist thus inducing cytokinetic defects. We suspect both Kel1 and phosphorylation of RGS are required to mediate the mating signaling cascade, propelled by an external stimulus. Additionally, upon internal stress, the lack of Kel1 and dephosphorylation of the RGS drives the cell into further stress provoking cytokinetic defect to arise. The formin regulator, Kel1, along with its role in mating and involvement with the polar cap, is necessary for function of the mitotic exit network (MEN) [92, 94, 95, 96]. Kel1 participates in the MEN by interacting with Lte1, a Ras regulator, during mitosis [97, 98]. Aside from the interaction between Kel1 and Lte1 in the MEN, they have also been shown to the aid in the inhibition of polarization. [97]. Moreover, deletion of Lte1, causing a lack of suppression of polarized growth, led to asymmetric hyperpolarized growth, much like deletion of Kel1 (Figure 8) [97]. This suggests RGS in the phosphorylated state stimulates the Lte1 polarity suppression pathway, and the dephosphorylation of RGS impedes it.

Characterizing how yeast utilize extracellular signaling such as mating mechanisms in conjunction with competing internal stress may reveal important aspects affecting how signaling
cascades direct important proteins in order to overcome stress and proceed to normal cellular function. Understanding these cellular mechanisms may offer more insight to more effective targeted therapies.
Figure 9. Shared Molecular Machinery in *Saccharomyces cerevisiae*. Diagram of yeast displaying proposed molecular mechanism of the NoCut pathway during the pheromone response. Blue path represents polarized growth. Yellow path represents NoCut checkpoint.
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