View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Clemson University: TigerPrints

Clemson University
TigerPrints

All Theses

Theses

5-2019

Phosphorylation of Eukaryotic Initiation Factor-2 Alpha in Response to Endoplasmic Reticulum Stress and Nitrosative Stress in the Protozoan Parasite, *Entamoeba histolytica*

Heather Andrews Walters
Clemson University, h.m.andrews2013@gmail.com

Follow this and additional works at: https://tigerprints.clemson.edu/all_theses

Recommended Citation

Walters, Heather Andrews, "Phosphorylation of Eukaryotic Initiation Factor-2 Alpha in Response to Endoplasmic Reticulum Stress and Nitrosative Stress in the Protozoan Parasite, *Entamoeba histolytica*" (2019). *All Theses*. 3057.
https://tigerprints.clemson.edu/all_theses/3057

This Thesis is brought to you for free and open access by the Theses at TigerPrints. It has been accepted for inclusion in All Theses by an authorized administrator of TigerPrints. For more information, please contact kokeefe@clemson.edu.

PHOSPHORYLATION OF EUKARYOTIC INITIATION FACTOR-2 ALPHA IN RESPONSE TO ENDOPLASMIC RETICULUM STRESS AND NITROSATIVE STRESS IN THE PROTOZOAN PARASITE, *ENTAMOEBA HISTOLYTICA*

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Biological Sciences

---

by
Heather Andrews Walters
May 2019

Accepted by:
Dr. Lesly Temesvari, Committee Chair
Dr. Lukasz Kozubowski
Dr. Zhicheng Dou

Abstract

Entamoeba histolytica is an intestinal parasite infecting over 50 million people worldwide and is the causative agent of amebic dysentery and amoebic liver abscess. In the human host and nonhuman primates, *E. histolytica* experiences stress brought on by nutrient deprivation and the host immune response. To be a successful parasite, *E. histolytica* must counter the stress; therefore, understanding the stress response may uncover new drug targets. In many systems, the stress response includes down-regulation of general protein translation, which is regulated by phosphorylation of eukaryotic initiation factor (eIF-2α). Previous work in *E. histolytica* has demonstrated that *Eh*eIF-2α phosphorylation increases significantly when exposed to long-term serum starvation, oxidative stress, and long-term heat shock. However, the effects of nitrosative and endoplasmic reticulum (ER) stresses, on the eIF2α protein translation control system have yet to be evaluated. Nitrosative stress is part of the host's immune response and ER stress can be caused by several physiological or pathological factors. We treated *E. histolytica* cells with different reagents to induce nitrosative stress (DPTA-NONOate and SNP) or ER stress (BFA and DTT). We examined the morphology of the ER, tracked phosphorylation of eIF2α, and assessed protein translation in the control and stressed cells. While all four stress-inducing reagents caused a global reduction in protein translation, only DTT was capable of also inducing changes in the morphology of the ER (consistent with ER stress) and phosphorylation of *Eh*eIF-2α. This suggests that DTT authentically induces ER stress in *E. histolytica* and that this stress is managed by the eIF2α-based system. This was supported by the observation that cells expressing a non-phosphorylatable version of eIF2α were also highly sensitive to DTT-stress. Since protein translation decreased in the absence of phosphorylation of eIF2α (after treatment with DPTA-NONOate, SNP or BFA),

the data also indicate that there are alternative protein-translational control pathways in *E. histolytica*. Overall, our study further illuminates the nitrosative and ER stress responses in *E. histolytica*.

ACKNOWLEDGEMENTS

I’d like to thank Dr. Temesvari for being an incredible mentor. Her passion and enthusiasm for research and teaching has been truly inspiring. She is the scientist that I will always inspire to be. I would also like to thank Dr. Lukasz Kozubowski and Dr. Zhicheng Dou for their support and guidance during this project. Their advice has invaluable.

Thank you to Brenda Welter and Alex Villano for your never-ending support and advice, for all the answered phone calls and text messages, and for trying to teach me everything you know!

Finally, I would like to thank my husband, Joshua, who probably knows as much about this thesis as I do. Your support and encouragement during the last two years have been instrumental in my success. Thank you for taking true joy in my work and for picking up the slack when science took up 100 percent of my time. I couldn’t have accomplished this without you.

Financial assistance for this work was provided by grants NIH R21 AI108287 and NIH COBRE GM10904, both awarded to Dr. Lesly Temesvari. The Department of Biological Sciences financially supported me with a teaching assistantship and funded my studies here at Clemson University. The funding agencies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. This content is solely the responsibility of the authors and does not necessarily represent the views of the National Institute of Allergy and Infectious Diseases, the National Institutes of Health.

TABLE OF CONTENTS

Page

TITLE PAGE……………………………………………………………………….i

ABSTRACT…………………………………………………………………………...ii

ACKNOWLEDGEMENTS…………………………………………………………..iv

LIST OF FIGURES………………………………………………………….....…..vii

CHAPTER

1. LITERATURE REVIEW…………………………………………………………...1

   I. Introduction………………………………………………………………......1
   II. Stress Resonse in *Entamoeba Histolytica*…………………………...5
   III. Translation regulation via eIF-2α kinases and how they are found in other eukaryotic pathogens …………………………………………...…..10
   IV. Summary…………………………………………………………………..17
   V. Literature cited……...………………………………………………………19

2. PHOSPHORYATION OF EUKARYOTIC INITIAITON FACTOR 2-ALPHA IN RESPONSE TO ENDOPLASMIC RETICULUM STRESS AND NITROSATIVE STRESS IN ENTAMOEBA HISTOLYTICA.…………....…27

   I. Abstract……………………..…………………………………………..27
   II. Introduction………………………………………………………………..28
   III. Materials and Methods……………………………………………..31
   IV. Results………………………………………………………………….35
   V. Discussion…………………………………………………………...48
   VI. Acknowledgements……………………………………………………55
   VII. Literature Cited …………………………………………………………..56

LIST OF FIGURES

Figure Page

1.1 Life Cycle of *Entamoeba histolytica* ……………………………………2

1.2 eIF-2α Kinases……………………………………………………………..13

2.1 Viability of stressed *E. histolytica* trophozoites in response to various stresses……………………………………………………..36

2.2 Representative immunofluorescence microscopy images of stressed and unstressed trophozoites………………………37

2.3 Percentage of trophozoites with fragmented ERs under various stress conditions……………………………………………………40

2.4 Levels of phosphorylated:total *Eh*eIF-2α in response various stresses……………………………………………………………41

2.5 Trophozoites exposed to nitrosative stress and long-term serum starvation…………………………………………………………43

2.6 Representative images of SUnSET Western Blots for each stress condition…………………………………………………………..46

2.7 Expression of exogenous forms of *Eh*eIF-2α in transgenic cells is tetracycline inducible………………………………………………49

2.8 Viability of transgenic cell lines expressing modified forms of *Eh*eIF-2α when exposed to 10 mM DTT……………………………50

CHAPTER ONE

LITERATURE REVIEW

I. Introduction

Entamoeba histolytica is the causative agent of amebic dysentery and amebic liver abscess. In the late 1990's the World Health Organization (WHO) estimated that 50 million people worldwide were infected with *E. histolytica*, which causes over 100,000 deaths annually (1). Infection occurs when the infectious cyst form of the organism (see Figure 1.1) is ingested from contaminated food and water. According to a 2015 report published by the WHO, approximately 946 million people defecate in the open and 10% of the world's population consumes crops that have been irrigated with contaminated water. Therefore, there is considerable global risk for acquiring infection and it is not surprising that this disease is prevalent in developing countries where sanitation is substandard, such as the middle east and sub-Saharan Africa (2).

This pathogen not only affects indigenous populations, but also poses great risks to American travelers, soldiers, and aid-workers (3,4). In March 2018, USA Today reported that American soldiers were deployed in countries where this parasite is highly prevalent, including Afghanistan, Iraq, Syria, Niger, Yemen, and Somalia (5). *E. histolytica* is classified as a category B biodefense pathogen by the National Institute of Allergy and Infectious Disease for several reasons. First, the organism can be manipulated genetically. Thus, it is conceivable that a hyper-virulent strain of the parasite could be

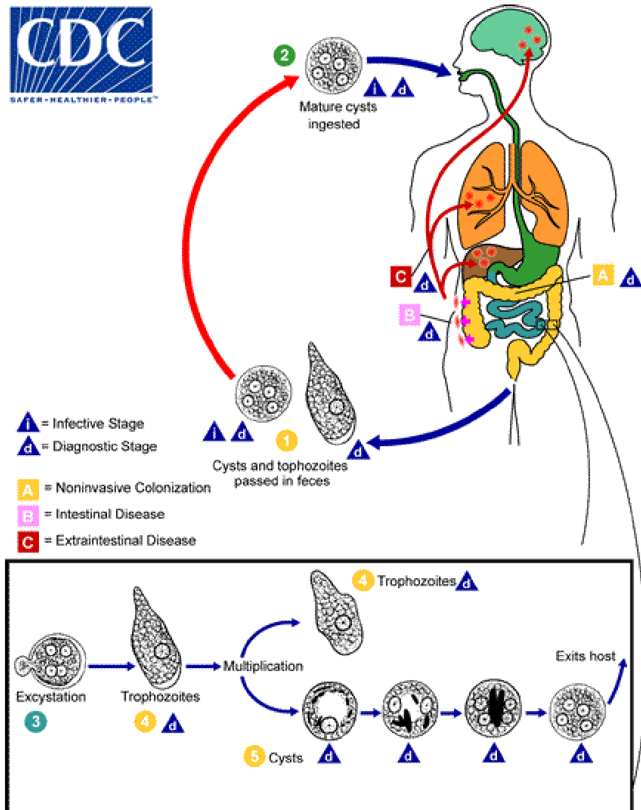

Figure 1.1: Life Cycle of *Entamoeba Histolytica*

The infective stage of the parasite is the latent cyst, which can persist in the environment for extended periods of time. Quadrinucleated cysts are ingested through contaminated food or water. As the cysts pass through the digestive system unharmed, unknown signals trigger excystation in the small intestine, where each cyst can release 8 mature trophozoites. The motile trophozoites travel to the large intestine, where trophozoites feed on the mucosal lining or on natural gut flora. Unknown cues trigger encystation of a small percentage of trophozoites, and cysts and trophozoites are passed back into the environment through feces. Trophozoites are vulnerable outside of their human host, while the cysts can persist and continue the life cycle. Image modified from the Centers of Disease Control, 2019.

developed. Second, the cysts can persist in harsh environments for long periods of time due to their chitinous cell wall. For example, this form of the parasite is resistant to heat, desiccation, low pH, and disinfectants, such as chlorine (4,6). Third, infection can be acquired by ingesting a relatively low dose of cysts (4). Ultimately, *E. histolytica* continues to pose a considerable threat to public health.

Life Cycle

This microaerophilic parasite has a simple two-stage lifecycle (See Figure 1.1), comprised of the infective latent cyst and the pathogenic trophozoite. Cysts can persist in harsh conditions for long periods of time due to the its chitinous cyst wall. Once ingested, quadrinucleated cysts travel through the digestive system, where unknown cues trigger excystation in the small intestine. Each cyst can release 8 mature trophozoites. Motile trophozoites travel to the colon and adhere to the protective mucin layer via a galactose and N-acetyl-D-galactosamine (Gal/GalNAc)–specific lectin (7), In the large intestine, infection can take one of two routes: trophozoites can either feed on the mucosal lining and natural gut flora, establishing an asymptomatic infection or trophozoites can degrade the mucin and invade the underlying intestinal epithelium, establishing an invasive infection.

During symptomatic infection, trophozoites may completely degrade the intestinal epithelium and enter the bloodstream, allowing the parasite to establish extra-intestinal infections in organs such as the liver, lungs, and rarely, the brain (8,9). In both types of infections, trophozoites reproduce by binary fission and continue feeding on host cells and gut-dwelling bacteria until unknown signals trigger trophozoite aggregation and subsequent encystation. Mature trophozoites and cysts are then passed through the feces and back into the environment (1). To date, encystation of *E. histolytica* has not been

observed *in vitro*. Therefore, *Entamoeba invadens,* a parasite that causes amebiasis in reptiles, has been used as a model organism. Synchronous encystation of *E. invadens* can be triggered *in vitro* by a combination of glucose starvation, serum starvation, and osmotic shock (10).

Treatment

Current treatment for amebiasis is metronidazole, an antibiotic from a class of drugs known as nitroimidazoles. These drugs function by passively diffusing into anaerobic cells as an inactive prodrug. Once in the cytoplasm, the drug is reduced into a short-lived nitroso free radical, which damages DNA, ultimately leading to cell death. This drug has been used to successfully treat amebiasis, trichomoniasis, giardiasis, and anaerobic bacterial infections for over 55 years (11). However, high toxicity and severe side effects have been observed when treating amebiasis. Metronidazole is highly absorbed in the small intestine and *E. histolytica* colonizes the large intestine. Therefore, treatment of amebiasis typically requires a high dosage of metranidazole, which in turn, leads to side effects like liver toxicity (12,13). Additionally, there are concerns that the parasite could quickly develop drug resistance. While drug resistance is not prevalent in *E. histolytica*, occasional reports of metronidazole failures, not attributable to patient non-compliance, suggest the possibility for the development of clinical resistance (14–16). Furthermore, metronidazole-resistant strains have been produced in the laboratory (16). One way in which resistance could occur is through re-oxidation of the drug in the presence of molecular oxygen, converting the drug back to its inactive form (11). Finally, metronidazole is thought to be carcinogenic (17). Since metronidazole is presently the only treatment for invasive amebiasis, there is an urgent need to develop a more efficient drug or a vaccine (4).

II. Stress Response in *Entamoeba histolytica*

Beside the environmental stresses experienced by the cyst form of the parasite, the amoeba encounters harsh conditions in the host. These include, low pH (as the parasite passes through the stomach) heat shock (due to fever), and glucose starvation (in the large intestine), and the presence of immune modulators such as reactive oxygen and reactive nitrogen species produced by neutrophils and macrophages (9). It has been hypothesized that nitrosative stress may also induce endoplasmic reticulum (ER) stress in this parasite (18). ER stress is characterized by a buildup of misfolded proteins and disturbances in ER function. Despite facing these various stressors while invading its human host, *E. histolytica* is able to ultimately persist and establish infection (19). Therefore, this amoeba must be able to adapt to its ever-changing environment. Exploring the stress response of this pathogen could be critical in revealing new drug targets.

Nitrosative Stress

As trophozoites degrade the mucus layer of the large intestine, intestinal epithelial cells release proinflammatory factors including interleukin-1, interleukin-8, and tumor necrosis factor-α, which recruit macrophages, natural killer cells, and neutrophils to the site of invasion. The primary response of these immune effector cells is to release reactive nitrogen species (RNS) in micromolar concentrations (9). The effect of RNS on target cells has been termed nitrosative stress. (20). RNS attack cell components, such as proteins, lipids, and nucleic acids, of invading organisms. RNS lead to the S-nitrosylation of proteins within the cell, which is the covalent attachment of an NO group to the thiol side chain of

cysteine residues. This modification leads to aberrant protein activity by inducing conformation changes. Thus, S-nitrosyltion of proteins can also lead to an accumulation of misfolded proteins, which may induce ER stress (20,21). S-nitrosylation of key glycosylation enzymes in this parasite results in inhibition of glycolysis (18) and fragmentation of the ER, triggering cell death (18). Furthermore, nitrosative stress inhibits protein synthesis by inducing cleavage of ribosomal proteins (22). Finally, S-nitrosylation of cysteine proteases results in decreased amoebic virulence because these enzymes are responsible for the parasite's destruction of the mucus layer of the colon (20).

E. histolytica possesses detoxification enzymes and repair systems that cope with nitrosative stress (23). One such mechanism involves a DNA methyltransferase, Dnmt2, which is part of the canonical methyltranserfase family of proteins that include Dnmt1 and Dnmt3. In support of this, the Ankri research group showed that when mutant cells overexpressing Dnmt2 were subjected to nitrosative stress, the transgenic cells exhibited higher viabilities than wildtype control parasites (22). In *E. histolytica*, Dnmt2 catalyzes $tRNA^{Asp}$ methylation, which maintains protein synthesis, by protecting the tRNAs from degradation during protein synthesis. The depletion of specific tRNAs may cause ribosomes to stall or fall off the mRNA during translation, leading to reduced protein synthesis. Additionally, tRNA cleavage that results from unmethylated tRNAs have been proposed to inhibit translation initiation, by displacing eukaryotic initiation factor 4F (eIF4F) from capped mRNAs (24) . Therefore, methylation of tRNAs maintains protein translation, which can aid in countering damage induced during nitrosative stress (22).

Another way in which *E. histolytica* circumvent host defense is by inhibiting macrophages from releasing RNS by producing prostaglandin E2 (PGE), a principle mediator of inflammation. PGE prevents RNS synthesis by triggering the protein kinase C

pathway. The parasite also produces monocyte locomotion inhibitory factor (MLIF), an anti-inflammatory factor, that prevents immune effector cells from producing RNS. *E. histolytica* can also quickly destroy host immune cells by inducing apoptosis or simply by phagocytosis. In one *in vitro* study, one trophozoite was able to kill 3000 neutrophils in 22 hours (9).

There is also evidence to suggest that *E. histolytica* adapts to nanomolar levels of RNS because nitric oxide appears to be a homeostatic regulator of the gastrointestinal muscosa. Studies show that nitric oxide influences microvascular and epithelial permeability and maintains adequate perfusion in the cells of the large intestine. So, there are homeostatic concentrations of nitric oxide, which could prepare invading parasites to withstand a larger RNS released by immune effector cells (25).

While *E. histolytica* possesses mechanisms to counter nitrosative stess, and may also adapt to nanomolar levels of RNS, nitrosative stress still results in high parasite mortality. Therefore, there is much more to learn about how this parasite responds to this stressor. To further evaluate this mechanism, transcriptomic studies have been conducted with parasites that were exposed to nitrosative stress. Santi-Rocca, *et al.* (18) and Vicente, *et al.* (23) found that when this organism was exposed to RNS, several heat shock proteins (Hsp) were upregulated by 2 or 4-fold, respectively, compared to unstressed trophozoites. Hsp are molecular chaperones that aid in protein folding and degradation. Therefore, the protective function of these Hsp may be overwhelmed by the increase in misfolded proteins (18,23). Vicente, *et al.* (23) found that the largest group of genes upregulated were signaling proteins such as protein kinases, phosphatases, and acetyltransferases. Other genes that were upregulated in these studies encoded proteins involved in metabolism, and nucleic acid repair (23). The exact mechanisms of how these upregulated

genes counter nitrosative stress are currently unknown. More studies are needed to further illuminate this stress response pathway.

Endoplasmic Reticulum Stress

In mammalian cells, the endoplasmic reticulum (ER) is a membrane bound organelle that is responsible for calcium storage and the synthesis, modification, and folding of secretory proteins (18,21). *E. histolytica* lacks many organelles that are found in mammalian cells, such as mitochondria and peroxisomes. Additionally, this amoeba has no recognizable Golgi apparatus or rough ER but possesses a simple endomembrane system. In 2008, Teixeira, *et al.* demonstrated the presence of a continuous ER in *E. histolytica* by using a green fluorescent protein (GFP) tagged N-terminal signal sequence, which contained a FLAG epitope and C-terminal ER retention peptide, KDEL. KDEL is a specific sequence of amino acids, lysine, aspartic acid, glutamic acid, and leucine, that retains a protein to the ER. By using immunofluorescence and confocal microscopy, this group showed that the GFP-tagged protein resided within a continuous compartment, that was responsible for N-linked glycosylation of membrane proteins that contained the conserved KDEL signal. These data support the idea that the molecular mechanisms regulating basic vesicle trafficking are conserved in this parasite. Prior to this study, it was thought that these protein modifications occurred in cytoplasmic vesicles (21,26).

ER stress is caused by an accumulation of misfolded proteins in the ER, and is induced by various physiological and pathological stresses, including glucose deprivation, hypoxia, oxidative stress, inflammatory cytokine, and an increase in protein folding demand. In mammalian cells, ER stress can also be induced by mutant protein expression or by using pharmacological reagents, such as Brefeldin A, dithiothreitol, and tunicamycin.

These reagents induce the buildup of proteins within the ER. For example, Brefeldin A inhibits transport of proteins from the ER to the Golgi Apparatus, while simultaneously inducing the retrograde transport of proteins from the Golgi to the ER. Dithiothrietol disrupts the formation of disulfide bonds and tunicamycin inhibits the addition of the dolichol phosphate during N-linked glycosylation of proteins. (21,27). Since *E. histolytica* experiences harsh environments while invading the host, it is likely to encounter stressors that are known to induce ER stress. However, data supporting this hypothesis are limited. Several studies show that stress (e.g., overexpression of mutant proteins, exposure to RNS) induces morphological changes in the ER, such as fragmentation and dispersal into vesicles. (18,28).

In higher eukaryotes, ER stress activates a mechanism known as the unfolded protein response (UPR), an evolutionary conserved adaptive response, which functions to alleviate stress and restore the ER to homeostasis. The UPR is a signaling cascade that consists of three main signaling proteins: Inositol Requiring Enzyme 1 (IRE1), PKR-like ER kinase (PERK), and Activating Transcription Factor 6 (ATF6). Under normal conditions, these ER-resident transmembrane proteins are rendered inactive by ER bound heat shock protein, immunoglobin binding protein (BiP). BiP senses misfolded proteins and subsequently dissociates from each of these proteins. Once free, IRE1, ATF6, and PERK initiate complex signaling cascades that ultimately aim to reduce ER stress by altering gene expression (21).

Microarray analysis of trophozoites exposed to RNS showed that there was a marked increase in Hsp, upregulation of DNA repair and redox gene expression, and an upregulation of glycosylation related gene expression. However, there was no evidence for an unfolded protein response in this organism, as there are no recognizable orthologs

of ATF6 or IRE1 (18). While it has been shown that *E. histolytica* experiences ER stress, the exact mechanism by which this parasite is able to counter this stress, remains largely unknown. Therefore, more studies are needed to elucidate this mechanism.

III. Translation Regulation via eIF-2α Kinases and how they are found in other eukaryotic pathogens.

While many of the countermeasures described above aim to neutralize the stress that a cell is experiencing, eukaryotic cells have also developed methods of countering stress, by decreasing global protein translation. This mechanism involves the eukaryotic initiation factor 2 (eIF-2) pathway. Not only does the eIF-2 mechanism result in decreased protein translation, but it facilitates an increase in the expression of a subset of stress-specific genes (25).

eIF-2 is a multi-subunit protein complex, that initiates protein translation in a GTP-dependent manner by delivering the Met-tRNA$_i$ to the ribosomal initiation complex. eIF-2 is composed of alpha, beta, and gamma subunits. Under normal conditions, eIF-2 is bound to GTP (active), and associates with Met-tRNA$_i$, delivering it to the 40S ribosomal subunit of the initiation complex. To release the Met-tRNA$_i$, eIF-2 must hydrolyze its bound GTP, resulting in an inactive eIF-2-GDP complex. To be reactivated, eIF-2 requires a guanine nucleotide exchange factor (GEF), eIF-2B, to exchange its bound GDP for GTP (29). Under stressful conditions, eIf-2α kinases are activated in a stress-specific manner and interact with eIF-2. This interaction induces a conformational change of eIF-2, exposing serine 51 of the alpha subunit (eIF-2α). Once exposed, eIF-2α is phosphorylated on serine

51 and becomes an inhibitor of its own GEF, eIF2B. Therefore, the eIF-2 complex becomes inactive, ultimately reducing general protein translation.

Simultaneous to a reduction in general protein synthesis, translation of select mRNAs is, paradoxically, initiated. The resulting proteins are needed for the stress response, and include activating transcription factor 4 (ATF4), activating transcription factor 3 (AFT3), and cationic amino acid transporter-1 (CAT-1) (30). During unstressed conditions, the translation of these stress-specific genes is inhibited by the presence of upstream short open reading frames (ORFs), which attract ribosomes to translate short peptides, preventing the flow of scanning ribosomes, to the genuine stress-specific gene sequence. Phosphorylated eIF-2α not only significantly decreases global translation, but also limits the number of ribosomal complexes, which promotes the translation of these genes (31).

Translation initiation is the rate limiting step of protein synthesis and is the central control point (32), and small increases in phosphorylated eIF-2α profoundly inhibits global protein translation (33). This reduction in protein synthesis allows the cell to direct gene expression to counter damage accrued during stress (34). This mechanism of translational control has been demonstrated extensively in yeast (35,36) and mammalian cells (29), and has also been demonstrated human pathogens: *Toxoplasma gondii* (37,38), *Plasmodium* (39), and *Leishmania* (40).

This system is also conserved in *E. histolytica* (19). Genomic data revealed that *E. histolytica* possesses eIF2α (*Eh*eIF2α) with a conserved phosphorylatable serine at position 59 (Ser^{59}). Hendrick *et al*. (19) exposed cells to different stress conditions and measured the level of total and phospho-*Eh*eIF2α. Long-term serum starvation, long-term heat shock, and oxidative stress induced an increase in the level of phospho-*Eh*eIF2α,

while short-term serum starvation, short-term heat shock, or glucose deprivation did not. Long-term serum starvation also caused a decrease in polyribosome abundance, which is in accordance with the role of this protein complex in protein translation. Hendrick *et al*. (19) also generated transgenic cells that overexpress wildtype *Eh*eIF2α, a non-phosphorylatable variant of eIF2α in which Ser^{59} was mutated to alanine (*Eh*eIF2α-S59A), and a phosphomimetic variant of eIF2α in which Ser^{59} was mutated to aspartic acid (*Eh*eIF2α-S59D). Consistent with the known functions of eIF2α, cells expressing wildtype or *Eh*eIF2α-S59D exhibited increased or decreased translation, respectively. Surprisingly, cells expressing *Eh*eIF2α-S59A also exhibited reduced translation. Cells expressing *Eh*eIF2α-S59D were more resistant to long-term serum starvation underscoring the significance of *Eh*eIF2α phosphorylation in managing stress. Finally, phospho-eIF2α accumulated during encystation in *E*. *invadens*, a model encystation system. Together, these data demonstrate that the eIF2α-dependent stress response system is operational in *Entamoeba* species.

Phosphorylation of eIF-2α is facilitated by specific kinases. Four mammalian eIF-2α kinases have been identified (41) (See Figure 1.2).

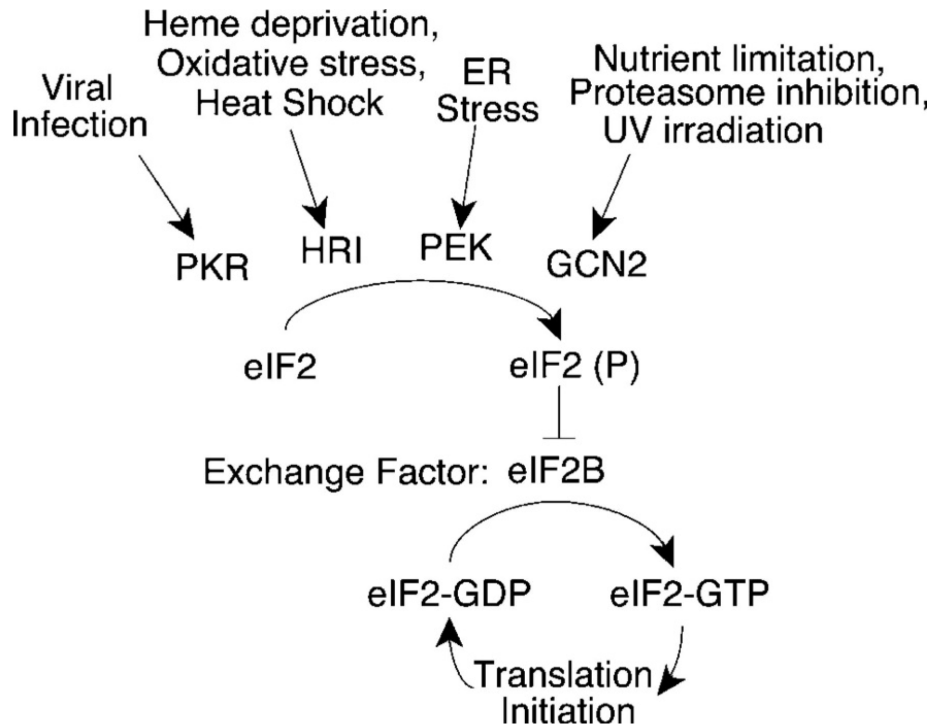

Figure 1.2: eIF-2α Kinases

There are 4 eIf-2α kinases in mammalian cells. PKR, HRI, PEK/PERK, and GCN2. Each kinase is activated by a specific stress, but each kinase functions to phosphorylate the alpha subunit of eIF-2, eukaryotic translation initiation factor. This phosphorylation results in a general decrease in protein translation, which allows the cell time to correct any damage incurred from the stress. Figure taken from Wek, 2006 (36).

Heme regulated inhibitor (HRI)

HRI, also known as EIF2AK1, is an eIF-2α kinase expressed in erythrocytes, macrophages, and hepatocytes (42). This kinase has two roles during development: to couple the synthesis of globin genes to the amount of heme present and to promote survival of erythroid cells when intracellular iron levels are low. Additionally, HRI has been implicated in the stress response during proteasome inhibition and signaling during erythroid differentiation. When HRI is synthesized, the protein is bound by heme, which triggers autophosphorylation, stabilizing it against aggregation and generating an HRI dimer. The HRI dimer senses heme concentrations. When levels are high, heme binds to the kinase, where it inhibits any further phosphorylation. When low levels of heme are detected, the kinase is activated by multiple autophosphorylations, and then phosphorylates eIF-2α (42).

This decrease in global protein translation in erythrocytes coordinates globin mRNA translation with available iron, preventing accumulation of misfolded globin proteins in the absence of heme. Furthermore, in the liver, HRI activation negatively regulates enzymes involved in the metabolism of L-tryptophan, and in murine macrophages, HRI is required for maturation (42).

dsRNA-dependent Protein Kinase (PKR)

PKR, also known as EIF2AK2, is located within the nucleus and cytosol of various cell types. Its transcription is induced by interferon, an antiviral protein that is secreted in response to viral infections. When bound to dsRNA, PKR dimerizes, autophosphorylates, and becomes active. Once active, PKR is able to phosphorylate eIF-2α. This phosphorylation results in reduced translation of viral mRNAs and can lead to apoptosis if the viral infection can’t be controlled. Moreover, PKR is involved in several signaling

pathways and can be activated independently of dsRNA, by oxidative stress, ER stress, and protein activator (PACT). Upon activation, PKR can phosphorylate p53, facilitate activation of STAT transcription factors and MAPK, and mediate NF-κB activation (42,43).

Pancreatic eIF-2α Kinase/PKR like ER kinase (PEK/PERK)

PERK or EIF2K3 is a transmembrane ER protein and represents one major arm of the unfolded protein response. PERK's regulatory region is located within the ER lumen, and the kinase domains lies in the cytosol. The luminal domain senses misfolded proteins within the ER. During unstressed conditions, the luminal domain of PERK is bound by ER chaperone, BiP/GRP78, which renders the kinase inactive. Within minutes of sensing ER stress, BiP/GRP78 dissociates, and PERK is then free to oligomerize and autophosphorylate. Once active, the cytosolic kinase domain phosphorylates eIF-2α, to reduce global protein translation. This decrease in protein synthesis slows the flow of newly synthesized proteins into the ER, ultimately allowing the cell time to refold proteins or degrade any critically misfolded proteins (29).

PERK can also be activated by calcium fluctuations in the ER, oxidative stress, and hypoxia. In some studies, PERK has been implicated in cancer. It is known that solid tumors tend to grow in hypoxic areas and are invasive and chemoresistant. Since PERK can be activated by hypoxia, it can increase tumor size, vascularization, and cell survival. Additionally, it was found that tumors deficient in PERK were smaller than their wildtype counterparts, and cancer cells that were PERK deficient were stalled in the cell cycle because of ROS induced damaged. Finally, PERK has also been implicated in Wolcott–Rallison syndrome in humans, which is a disease characterized by lifelong diabetes, as well as skeletal and pancreatic defects (42).

General Control non-inducible-2 (GCN2)

Also known as EIF2K4, GCN2 is an eIF-2α kinase that is activated by amino acid starvation, UV irradiation, and viral infections (29). In yeast and mammals, GCN2 is also activated by glucose starvation. Expressed at high levels in the brain, GCN2 is kept inactive by several auto-inhibitory molecular interactions. During amino acid starvation, uncharged tRNAs accumulate and bind to the histidyl-tRNA synthase-like domain on GCN2, which results in allosteric rearrangements and dimerization. Subsequent autophosphorylation allows GCN2 to phosphorylate eIF-2α. While phosphorylated eIF-2α results in decreased protein translation, it also prompts translation of specific mRNAs, such as ATF4, which is critical in the stress response, as it induces the expression of amino acid biosynthetic enzymes and amino acid transporters (31,44).

This kinase is present in most eukaryotes and is involved in major biological processes. In mammals, this kinase is crucial for long-term memory formation, feeding behavior and immune system regulation. Evidence for this comes from studies that showed that the accumulation of uncharged tRNAs lead mice to reject diets low in amino acids, while mutant mice lacking functional GCN2, did not discriminate between amino acid-rich and amino acid-deficient foods. It was also found that when wildtype mice were fed diets deficient in amino acids, lipid metabolism was affected, leading to decreased liver mass and adipose tissue (31,42,44).

Conservation of eIF-2α kinases in lower eukaryotes

Translation regulation via eIF-2α phosphorylation is necessary to counter various stresses and is an evolutionary conserved mechanism in eukaryotes. In *Plasmodium falciparum*, the causative agent of malaria in humans, *Pf*PK4 resembles mammalian HRI eIF-2α kinase and is also inhibited by heme. Zhang, *et al*. (39) showed that *Pf*PK4 is

required for development of *Plasmodium* blood stage development and regulates protein translation by phosphorylating eIF-2α in trophozoites, schizonts, and gametocytes. Another parasite, *Toxoplasma gondii* has four putative eIF-2α kinases, two of which most closely resemble GCN2. *Tg*IF2K-A, the most characterized, appears to be a transmembrane ER protein, like PERK, and phosphorylates eIF-2α when exposed to ER stress. *Tg*IF2K-B has no orthologs, but is a true eIF-2α kinase (37). Furthermore, *Trypanosoma brucei,* the causative agent of African sleeping sickness, has three putative kinases. *Tb*eIF2K2 is a confirmed eIF-2α kinase, *Tb*eIF2K1 is a GCN2 ortholog, while no clear homology has been determined for *Tb*eIF2k3 (34). Genome data demonstrate that there are 2 putative eIF-2α kinases in *E. histolytica* (EHI_035950 and EHI_109700) and 2 putative eIF-2α kinases in *E. invadens* (accession numbers). However, these have not been authenticated.

IV. Summary

E. histolytica faces numerous stressors as it travels through the digestive system and host's tissues. These include changes in pH, glucose deprivation, osmotic shock, heat shock, and immune pressure, which could impart oxidative and nitrosative stresses (19,45). In *E. histolytica*, nitrosative stress may also lead to ER stress (18). To be a successful pathogen, *E. histolytica* must employ mechanisms that counter these stresses. In many species, response to these types of stressors involves translational control by phosphorylating eIF-2α. Hendrick *et al.* (16) demonstrated that long-term serum deprivation, long-term heat shock, and oxidative stress induce phosphorylation of eIF2-α. However, phosphorylation *Eh*eIF-2α has not been measured in response to nitrosative

stress or ER stress. Further investigation of this mechanism in *E. histolytica* may reveal novel pathways for drug development.

Therefore, the aims of this study are as follows:

Aim 1: To investigate the phosphorylation of *Eh*eIF-2α in response to nitrosative stress and ER stress.

In Aim 1, we exposed *E. histolytica* trophozoites to nitrosative stress and ER stress and tracked the levels of phosphorylated *Eh*eIF-2α by western blotting. Our data demonstrate that *Eh*eIF-2α is phosphorylated in response to ER stress but is not phosphorylated in response to nitrosative stress.

Aim 2: To determine if phosphorylation of *Eh*eIF-2α is necessary to counter ER Stress.

In Aim 2, we exposed transgenic cells that express variant forms of *Eh*eIF-2α to ER stress and assessed viability using Trypan Blue exclusion. Our data demonstrate that our phosphomimmetic and overexpressing cell lines are not better capable of handling stress as we predicted. However, our nonphosphorylatable cell line had significantly lower viability when exposed to ER stress.

V. Literature Cited

1. Bercu TE, Petri W a, Behm JW. Amebic colitis: new insights into pathogenesis and treatment. Curr Gastroenterol Rep [Internet]. 2007;9(5):429–33. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17991346

2. Grebmer K, Bernstein J, de Waal A, Prasai N, Yin S, Yohannes Y. UNICEF and WHO, 2015 Update and MDG Assessment - Progress on Sanitation and Drinking Water. 2015;

3. Kasper MR, Lescano AG, Lucas C, Gilles D, Biese BJ, Stolovitz G, et al. Diarrhea outbreak during U.S. military training in El Salvador. PLoS One. 2012;7(7):1–8.

4. Shirley DAT, Farr L, Watanabe K, Moonah S. A review of the global burden, new diagnostics, and current Therapeutics for amebiasis. Open Forum Infect Dis. 2018;5(7):1–9.

5. Michaels J. Fighting Terrorism: These are the most dangerous places U.S. troops are deployed. USA Today [Internet]. 2018 Mar 5; Available from: https://www.usatoday.com/story/news/world/2018/03/05/look-most-dangerous-places-u-s-troops-deployed/396737002/

6. Eichinger D. Encystation in parasitic protozoa. Curr Opin Microbiol. 2001;4(4):421–6.

7. Petri W a, Singh U. Diagnosis and Management of Amebiasis. Clin Infect Dis. 1990;29:1117–25.

8. Tanyuksel, M. Petri W. Laboratory Diagnosis of Amebiasis. Clin Microbiolody Rev. 2003;16(4):713–29.

9. Begum S, Quach J, Chadee K. Immune evasion mechanisms of Entamoeba histolytica: Progression to disease. Front Microbiol. 2015;6(DEC):1–8.

10. Wang Z, Samuelson J, Clark CG, Eichinger D, Paul J, Van Dellen K, et al. Gene discovery in the Entamoeba invadens genome. Mol Biochem Parasitol. 2003;129(1):23–31.

11. Löfmark S, Edlund C, Nord CE. Metronidazole Is Still the Drug of Choice for Treatment of Anaerobic Infections. Clin Infect Dis [Internet]. 2010;50(s1):S16–23. Available from: https://academic.oup.com/cid/article-lookup/doi/10.1086/647939

12. Ralston K, Petri WA. Tissue Destruction and invasion by Entamoeba histolytica. Trends Parasitol. 2011;27(6):254–63.

13. Cherian PT, Wu X, Yang L, Scarborough JS, Singh AP, Alam ZA, et al. Gastrointestinal localization of metronidazole by a lactobacilli-inspired tetramic acid motif improves treatment outcomes in the hamster model of Clostridium difficile infection. J Antimicrob Chemother. 2015;70(11):3061–9.

14. Seifert K, Duchêne M, Wernsdorfer WH, Kollaritsch H, Scheiner O, Wiedermann G, et al. A New Approach for Chemotherapy Against Entamoeba histolytica. 2000;31:6–7.

15. Knight R. The chemotherapy of amoebiasis. 1980;577–93.

16. Samarawickrema NA, Brown DM, Upcroft JA, Thammapalerd N, Upcroft P. Involvement of superoxide dismutase and pyruvate:ferredoxin oxidoreductase in mechanisms of metronidazole resistance in Entamoeba histolytica. 1997;833–40.

17. Bendesky A, Menéndez D, Ostrosky-wegman P. Is metronidazole carcinogenic ?

2002;511:133–44.

18. Santi-Rocca J, Smith S, Weber C, Pineda E, Hon CC, Saavedra E, et al. Endoplasmic reticulum stress-sensing mechanism is activated in entamoeba histolytica upon treatment with nitric oxide. PLoS One. 2012;7(2):1–14.

19. Hendrick HM, Welter BH, Hapstack MA, Sykes SE, Sullivan WJ, Temesvari LA. Phosphorylation of Eukaryotic Initiation Factor-2α during Stress and Encystation in Entamoeba Species. PLoS Pathog. 2016;12(12):1–14.

20. Nagaraja S, Ankri S. Utilization of Different Omic Approaches to Unravel Stress Response Mechanisms in the Parasite Entamoeba histolytica. Front Cell Infect Microbiol [Internet]. 2018;8(February):1–12. Available from: http://journal.frontiersin.org/article/10.3389/fcimb.2018.00019/full

21. Oslowski CM, Urano F. Measuring ER stress and the unfolded protein response using mammalian tissue culture system. Methods Enzymol [Internet]. 2013;490(508):71–92. Available from: http://www.sciencedirect.com/science/article/pii/B9780123851147000040

22. Hertz R, Tovy A, Kirschenbaum M, Geffen M, Nozaki T, Adir N, et al. The Entamoeba histolytica Dnmt2 homolog (Ehmeth) confers resistance to nitrosative stress. Eukaryot Cell. 2014;13(4):494–503.

23. Vicente JB, Ehrenkaufer GM, Saraiva LM, Teixeira M, Singh U. Entamoeba histolytica modulates a complex repertoire of novel genes in response to oxidative and nitrosative stresses: Implications for amebic pathogenesis. Cell Microbiol. 2009;11(1):51–69.

24. Tuorto F, Liebers R, Musch T, Schaefer M, Hofmann S, Kellner S, et al. RNA cytosine methylation by Dnmt2 and NSun2 promotes tRNA stability and protein synthesis. Nat Publ Gr [Internet]. 2012;19(9):900–5. Available from: http://dx.doi.org/10.1038/nsmb.2357

25. Kolios G, Valatas V, Ward SG. Nitric oxide in inflammatory bowel disease: A universal messenger in an unsolved puzzle. Immunology. 2004;113(4):427–37.

26. Teixeira JE, Huston CD. Evidence of a continuous endoplasmic reticulum in the protozoan parasite Entamoeba histolytica. Eukaryot Cell. 2008;7(7):1222–6.

27. Galluzzi L, Diotallevi A, Magnani M. Endoplasmic reticulum stress and unfolded protein response in infection by intracellular parasites. Futur Sci Open Access [Internet]. 2017;00(3):FSO198. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28883998%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5583660

28. Welter BH, Temesvari LA. Overexpression of a mutant form of EhRabA, a unique rab GTPase of entamoeba histolytica, alters endoplasmic reticulum morphology and localization of the Gal/GalNAc adherence lectin. Eukaryot Cell. 2009;8(7):1014–26.

29. Wek RC, Cavener DR. Translational Control and the Unfolded Protein Response. Antioxid Redox Signal [Internet]. 2007;9(12):2357–72. Available from: http://www.liebertonline.com/doi/abs/10.1089/ars.2007.1764

30. Cao SS, Kaufman RJ. Unfolded protein response. Curr Biol [Internet]. 2012;22(16):R622–6. Available from: http://dx.doi.org/10.1016/j.cub.2012.07.004

31. Castilho BA, Shanmugam R, Silva RC, Ramesh R, Himme BM, Sattlegger E. Keeping the eIF2 alpha kinase Gcn2 in check. Biochim Biophys Acta - Mol Cell Res [Internet]. 2014;1843(9):1948–68. Available from: http://dx.doi.org/10.1016/j.bbamcr.2014.04.006

32. Ventoso I, Kochetov A, Montaner D, Dopazo J, Santoyo J. Extensive translatome remodeling during ER stress response in mammalian cells. PLoS One. 2012;7(5):1–12.

33. McEwen E, Kedersha N, Song B, Scheuner D, Gilks N, Han A, et al. Heme-regulated inhibitor kinase-mediated phosphorylation of eukaryotic translation initiation factor 2 inhibits translation, induces stress granule formation, and mediates survival upon arsenite exposure. J Biol Chem. 2005;280(17):16925–33.

34. Vonlaufen N, Kanzok SM, Wek RC, Sullivan WJ. Stress response pathways in protozoan parasites. Cell Microbiol. 2008;10(12):2387–99.

35. Wek RC, Cannon JF, Dever TE, Hinnebusch a G. Truncated protein phosphatase GLC7 restores translational activation of GCN4 expression in yeast mutants defective for the eIF-2 alpha kinase GCN2. Mol Cell Biol. 1992;12(12):5700–10.

36. Zhan K, Vattem KM, Bauer BN, Thomas E, Chen J, Wek RC, et al. Phosphorylation of Eukaryotic Initiation Factor 2 by Heme-Regulated Inhibitor Kinase-Related Protein Kinases in Schizosaccharomyces pombe Is Important for Resistance to Environmental Stresses Phosphorylation of Eukaryotic Initiation Factor 2 by Heme-Regul. 2002;22(20):7134–46.

37. Sullivan WJ, Narasimhan J, Bhatti MM, Wek RC. Parasite-specific eIF2 (eukaryotic

initiation factor-2) kinase required for stress-induced translation control. Biochem J [Internet]. 2004;380(Pt 2):523–31. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1224182&tool=pmcentrez&rendertype=abstract

38. Joyce BR, Tampaki Z, Kim K, Wek RC, Sullivan WJ. The unfolded protein response in the protozoan parasite Toxoplasma gondii features translational and transcriptional control. Eukaryot Cell. 2013;12(7):979–89.

39. Zhang M, Mishra S, Sakthivel R, Rojas M, Ranjan R, Sullivan WJ. PK4, a eukaryotic initiation factor 2a(eIF2a) kinase, is essential for the development of the erythrocytic cycle of Plasmodium. Proc Natl Acad Sci. 2012;109(10):3956–61.

40. Cloutier S, Laverdière M, Chou MN, Boilard N, Chow C, Papadopoulou B. Translational control through eIF2alpha phosphorylation during the Leishmania differentiation process. PLoS One. 2012;7(5).

41. Wek RC, Jiang H-Y, Anthony TG. Coping with stress: eIF2 kinases and translational control. Biochem Soc Trans [Internet]. 2006;34(1):7–11. Available from: http://biochemsoctrans.org/lookup/doi/10.1042/BST0340007

42. Donnelly N, Gorman AM, Gupta S, Samali A. The eIF2α kinases: Their structures and functions. Cell Mol Life Sci. 2013;70(19):3493–511.

43. Garcia MA, Gil J, Ventoso I, Guerra S, Domingo E, Rivas C, et al. Impact of Protein Kinase PKR in Cell Biology: from Antiviral to Antiproliferative Action. Microbiol Mol Biol Rev [Internet]. 2006;70(4):1032–60. Available from: http://mmbr.asm.org/cgi/doi/10.1128/MMBR.00027-06

44. Anda S, Zach R, Grallert B. Activation of Gcn2 in response to different stresses. PLoS One. 2017;12(8):1–13.

45. Pineda E, Perdomo D. Entamoeba histolytica under Oxidative Stress: What Countermeasure Mechanisms Are in Place? Cells [Internet]. 2017;6(4):44. Available from: http://www.mdpi.com/2073-4409/6/4/44

CHAPTER TWO

PHOSPHORYATION OF EUKARYOTIC INITIAITON FACTOR 2-ALPHA IN RESPONSE TO ENDOPLASMIC RETICULUM STRESS AND NITROSATIVE STRESS IN ENTAMOEBA HISTOLYTICA

Heather A. Walters[1,2], Lesly A. Temesvari[1,2]

[1] Department of Biological Sciences

[2]Eukaryotic Pathogens Innovations Center

Clemson University

Clemson, South Carolina United States of America 29634

*Corresponding author

Email: ltemesv@clemson.edu (LAT)

I. Abstract

Entamoeba histolytica is an intestinal parasite infecting over 50 million people worldwide and is the causative agent of amebic dysentery and amoebic liver abscess. In the human host, *E. histolytica* experiences stress brought on by nutrient deprivation and the host immune response. To be a successful parasite, *E. histolytica* must counter the stress; therefore, understanding the stress response may uncover new drug targets. In many systems, the stress response includes down-regulation of general protein translation, which is regulated by phosphorylation of eukaryotic initiation factor (eIF-2α). Previous work in *E. histolytica* has demonstrated that *Eh*eIF-2α phosphorylation increases significantly when exposed to long-term serum starvation, oxidative stress, and long-term heat shock. However, the effects of nitrosative and endoplasmic reticulum (ER) stresses, on the eIF2α protein translation control system have yet to be evaluated. Nitrosative stress is part of the host's immune response and ER stress can be caused by several physiological or pathological factors. We treated *E. histolytica* cells with various reagents to induce nitrosative stress (DPTA-NONOate and SNP) and or ER stress (BFA and DTT). We examined the morphology of the ER, tracked phosphorylation of eIF2α, and assessed protein translation in the control and stressed cells. While all four stress-inducing reagents caused a global reduction in protein translation, only DTT was capable of also inducing changes in the morphology of the ER (consistent with ER stress) and phosphorylation of *Eh*eIF-2α. This suggests that DTT authentically induces ER stress in *E. histolytica* and that this stress is managed by the eIF2α-based system. This was supported by the observation that cells expressing a non-phosphorylatable version of eIF2α were also highly sensitive to DTT-stress. Since protein translation decreased in the absence of

phosphorylation of eIF2α (after treatment with DPTA-NONOate, SNP or BFA), the data also indicate that there are alternative protein-translational control pathways in *E. histolytica*. Overall, our study further illuminates the stress response to nitrosative stress and ER stress in *E. histolytica*.

II. Introduction

Entamoeba histolytica is an enteric parasite which causes amoebic dysentery and amoebic liver abscess in humans and nonhuman primates. This parasite has a two-stage life cycle, consisting of the infective, environmentally-stable cyst form and the pathogenic trophozoite. *E. histolytica* cysts are transmitted via fecal-contaminated food and water, making this disease prevalent in sub-Saharan Africa and southern Asia where sanitation is substandard. As of 2015, 663 million people worldwide utilized unprotected drinking water sources, including wells, springs, and surface water. Additionally, 946 million people continue to practice open defecation, furthering the spread of this disease (1). Globally, more than 50 million people become infected with the parasite, with over 100,000 deaths annually (2).

E. histolytica is ingested as a latent cyst and travels through the digestive system until unknown cues trigger the excystation of 8 trophozoites in the small intestine. The trophozoites travel to the large intestine where they feed on the natural gut flora and mucosal cells that compose the endothelial lining. In some cases, the trophozoites can degrade the mucosal layer and enter the blood stream where they cause extra-intestinal infections in the liver, lungs, or, rarely, the brain. The parasite faces numerous stressors

as it traverses the digestive system and host tissues. Environmental stressors could include glucose deprivation, osmotic shock, and heat shock, while immune pressure could impart oxidative and nitrosative stresses (3). In addition, increased protein demand caused by physiological and pathological stressors could induce endoplasmic reticulum (ER) stress (4). To be a successful parasite, *E. histolytica* must be able to counter all of these various stressful conditions. Understanding the stress response of *E. histolytica* may uncover a new target for drug therapy.

The phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF-2), has been implicated in managing the stress response in *E. histolytica* (3) and other systems such as *Toxoplasma gondii* (5), *Plasmodium* (6), and *Leishmania* (7). eIF-2 is a heterotrimeric protein composed of three subunits: alpha (α), beta (β), and gamma (γ). Under normal conditions, the eIF-2 complex, when bound to GTP, delivers the Met-tRNA$_i$ to the initiation complex to initiate translation. Once the GTP is hydrolyzed, eIF-2B, a guanine nucleotide exchange factor, reactivates eIF-2 by exchanging the bound GDP for GTP. However, certain stresses have been shown to phosphorylate serine 51 of the eIF-2α subunit, which causes the protein complex to become an inhibitor of eIF-2B, rather than a substrate. This inhibits the function of eIF-2, ultimately leading to a decrease in general protein translation (3,8). In *E. histolytica*, these stresses include long-term serum starvation, long-term heat shock, and oxidative stress (3). However, the response to ER stress and nitrosative stress in *E. histolytica* has yet to be investigated.

Reactive nitrogen species (RNS) and reactive oxygen species (ROS) are released by neutrophils and macrophages as an immune defense response (9). Both RNS and ROS attack cell components, such as proteins, lipids, and nucleic acids, inducing ntirosative and oxidative stress in invading organisms. *E. histolytica* has detoxification

enzymes and repair systems that cope with both reactive species in a similar fashion (10). Hendrick, *et al.* showed that oxidative stress induces the phosphorylation of *EheIF-2α* (3), but the phosphorylation of *Eh*eIF-2α in response to nitrosative stress has yet to be evaluated.

It is likely that *E. histolytica* will experience nitrosative and ER stress as it invades hosts tissues; therefore, elucidating the associated stress response may reveal novel drug targets, which could produce novel therapies to eliminate the infectious disease caused by this parasite. In this study we investigated the molecular mechanisms that regulate nitrosative and ER stress in this pathogen. Specifically, we exposed *E. histolytica* trophozoites to rapid nitric oxide (NO) donors, dipropylenetriamine NONOate (DPTA-NONOate) and sodium nitroprusside (SNP) or to ER stress-inducing reagents, dithiothreitol (DTT) and Brefeldin A (BFA). DTT is a reducing agent that blocks disulfide bond formation and BFA disrupts the transport of proteins from the ER to the Golgi Apparatus, while simultaneously inducing transport of proteins from the Golgi Apparatus to the ER. We examined the morphology of the ER, tracked phosphorylation of *Eh*eIF-2α, and assessed protein translation in the control and stressed cells. While all four stress-inducing reagents caused a global reduction in protein translation, only DTT was capable of also inducing changes in the morphology of the ER (consistent with ER stress) and phosphorylation of *Eh*eIF-2α. This suggests that DTT authentically induces ER stress in *E. histolytica* and that this stress is managed by the eIF2α-based system. This was supported by the observation that cells expressing a non-phosphorylatable version of eIF2α were also highly sensitive to DTT-stress. Since protein translation decreased in the *absence* of phosphorylation of eIF2α (after treatment with DPTA-NONOate, SNP or BFA), the data also indicate that there are alternative protein-translational control pathways in *E.*

histolytica. Overall, our study further illuminates the nitrosative and ER stress responses in *E. histolytica.*

III. Material and Methods

Cell Culture

E. histolytica trophozoites (strain HM-1:1MSS) were cultured axenically in TYI-S33 medium at 37˚C. Cells were passaged into fresh media every 72 to 96 hrs and were grown in 15 mL glass screw cap culture tubes (11). The generation of transgenic cell lines overexpressing exogenous wildtype or mutant forms of *Eh*eIF-2α was previously described by Hendrick, *et al.* (3). Transgenic cells were cultured at 37˚C in TYI-S33 medium supplemented with 6 μg mL^{-1} G418 (Fisher Scientific; Fair Lawn, NJ, USA) and 15 μg mL^{-1} hygromycin (Fisher Scientific). Twenty-four hrs prior to all experiments, expression of exogenous *Eh*eIF-2α was induced by adding 5 μg mL^{-1} tetracycline to the culture medium (3).

Stress Induction

To induce ER stress, log-phase trophozoites were incubated with either Dithiothreitol (DTT; Sigma-Aldrich St. Louis, MO, USA) or Brefeldin A (BFA; Thermo Scientific; Hercules, CA, USA) (4,12,13) in 13 mL TYI-S-33 culture medium for 1 hr at 37˚C. Controls were incubated with diluents, sterile water or dimethyl sulfoxide (DMSO; MP Biomedicals, Solon, OH, USA), respectively (4). To induce nitrosative stress, log-phase trophozoites were incubated with either sodium nitroprusside (SNP; Sigma-Aldrich) (14) or dipropylenetriamine NONOate (DPTA NONOate; Enzo Life Sciences, Farmingdale, NY, USA) (10) in 13 mL TYI-S-33 culture medium for 1 hr at 37˚C. Finally, wild type trophozoites were exposed to a combination of nitrosative stress and long-term serum starvation. Trophozoites were incubated with 1 mM SNP or 300 μM DPTA-

NONOate for 1 hr at 37˚C in 13 mL TYI-S-33 culture medium. Cells were then incubated on ice for 8 minutes to detach cells from the glass, transferred to 15 mL plastic centrifuge tubes, and pelleted at 500 x *g* for 5 minutes. The supernatant was removed and discarded, and cells were resuspended in serum free culture medium. Cells were then incubated at 37˚C for 24 hrs to induce long-term serum starvation.

After each treatment, trophozoites were incubated on ice for 8 minutes after stress induction to detach the cells from the glass culture tube. Viability was immediately assessed using microscopy and Trypan Blue exclusion (VWR, Radnor, PA, USA).

Immunofluorescence Microscopy

To examine the morphology of the ER during stress, we carried out immunofluorescence (IF) microscopy, using anti-KDEL (ER marker) antibodies (Abcam, Cambrige, UK). Cells were treated with 10 mM DTT, 300 µM DPTA-NONOate, 1 mM SNP, or 350 µM BFA for 1 hr at 37˚C and were fixed with 4% (vol/vol) paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes at room (RT). After permeabilization with 0.2% (vol/vol) Triton X-100 in PBS for 5 minutes at RT, nonspecific binding sites were blocked by incubation with 3% bovine serum albumin–10% goat serum–PBS for 30 minutes at RT. The trophozoites were then incubated with a 1:140 dilution of primary anti-KDEL (anti-mouse) antibody overnight at 4˚C. Following primary antibody incubation, fixed cells were incubated with secondary antibody, Alexa Fluor 488 (green) (Life Technologies, Carlsbad, CA, USA) conjugated to goat anti-mouse. To stain nuclei, cells were then incubated with 1:1000 DAPI [5mg mL^{-1}] (Invitrogen, Carlsbad, CA, USA) for 12 minutes at RT. Stained cells were then mounted onto glass slides in 1:1 PBS:glycerol and observed using a Leica SPE laser scanning confocal microscope (Wetzlar, Germany).

Western Blotting

To determine the levels of total and phosphorylated eIF-2α, SDS-PAGE and Western Blotting were performed. Control and stressed *E. histolytica* trophozoites ($3x10^5$) were pelleted by centrifugation at 500 *x g* for 5 minutes. Cells were resuspended in NuPAGE LDS sample buffer (Life Technologies), heated for 5 minutes at 100˚C, and loaded on a precast NuPAGE 12% Bis-Tris Gel (Life Technologies). The gels were electrophoresed at 200 V for 45 minutes and separated proteins were transferred using a blotter apparatus to polyvinylidene difluoride membranes (PVDF; Life technologies) at 12 V for 1.5 hrs in Towbin Transfer Buffer. The membranes were blocked with 5% w/v Blotting Grade powdered milk blocker (Bio-Rad Laboratories, Hercules, CA) and 0.5% w/v bovine gelatin (Sigma-Aldrich) in TBST (50 mM Tris, 150 mM NaCl, 0.5% (v/v) Tween 20) for 35 minutes at 37°C. Membranes were incubated overnight at 4˚C in either anti-total eIF-2α (diluted 1:1000 in TBST) or anti-phosphorylated eIF-2α (diluted 1:1333 in TBST) antibodies, which were developed and tested as previously described (3). The membranes were then washed in TBST for 45 minutes with 6 buffer changes, incubated for 1 hr at 22°C with commercially available horseradish peroxidase-conjugated goat anti-rabbit (dilution factor 1:5000 in TBST) (Fisher Scientific), and washed again for 45 minutes in TST (50 mM Tris, 328 mM NaCl, 0.05% v/v Tween20) with 6 buffer changes. The blots were developed using a commercially available Enhanced ChemiLuminescence Western Blotting detection system (ThermoScientific) according to the manufacturer's instructions. Proteins were quantified by scanning densitometry using ImageJ software (version 1.51, National Institute of Health, USA). Gels were stained with Bio-Safe G250 Coomassie Stain (Bio-Rad Laboratories) to measure load.

SUrface SEnsing of Translation (SUnSET)

SUnSET was previously used to measure levels of general protein translation in *E. histolytica* (3). Wildtype trophozoites were exposed to vehicle control, 10 mM DTT, 1 mM SNP, 300 µM DPTA-NONOate, or 350 µM BFA for 1 hr at 37˚C. Then, to assess levels of general protein levels in stressed and unstressed cells, we incubated cells ($2x10^5$) with 10 µg mL^{-1} puromycin (Sigma-Aldrich) for 15 min before or after incubation with 100 µg mL^{-1} cycloheximide for 10 minutes. All incubations were held at 37°C. Next, cells were pelleted, and proteins were precipitated using 20% (v/v) TCA and incubating on ice for 10 minutes. Proteins were separate by centrifugation at 2200 *x g* for 5 minutes (4˚C) and washed with 5% (v/v) TCA. The protein pellet was resuspended in 2X SDS running buffer and incubated at 95˚C for 10 minutes. The lysates were immediately analyzed using Western Blotting as described above. Primary mouse anti-puromycin monoclonal antibodies (Sigma-Aldrich) were used at a 1:100 dilution and secondary horseradish peroxidase-conjugated goat anti-rabbit antibodies (Fisher Scientific) were used at a dilution of 1:2500. As a loading control, PVDF membranes were stained with Bio-Safe Coomassie G-250 Stain (Bio-Rad Laboratories) or Ponceau Stain (Sigma-Aldrich).

Statistical Analysis

All values are given as means ± standard error of at least 3 trials. Means of treated groups were compared against the appropriate control and statistical analyses were performed using GraphPad Prism v.6.05 software with a one-way analysis of variance (ANOVA) and a Tukey-Kramer multiple-comparison test. In all cases, p-values of less than 0.05 were considered significant.

IV. Results

Immunofluorescence microscopy confirms ER Stress in *E. histolytica* when exposed to DTT

While DTT and BFA have been shown to induce ER stress in mammalian cell culture (4), these reagents have not previously been used to induce ER stress in this parasite. Additionally, another study shows that exposure to the NO donor, SNP, causes ER stress in *E. histolytica* (14). Therefore, we wanted to determine if these reagents could induce ER stress by examining the morphology of the ER after treatment. First, viability of trophozoites exposed to DTT, BFA, SNP, or DPTA-NONOate was assessed. (Figure 2.1). Our tested concentrations of DTT, BFA, and SNP did not induce significant cell death. DPTA-NONOate produced significant cell death at 500 µM; therefore, we did not use that concentration in further experiments.

We then carried out immunofluorescence microscopy with an anti-KDEL antibody on stressed and unstressed cells. KDEL is a peptide signal sequence at the C-terminus of proteins that are destined to remain in the ER, and is commonly used as an ER marker (14,15). An unstressed ER should appear as a continuous intracellular compartment, that extends throughout the cytoplasm, while a stressed ER should appear fragmented with many vesicle-like structures (14). We captured confocal images of trophozoites exposed to DTT, BFA, SNP, DPTA-NONOate, or the appropriate vehicle control (See Figure 2.2). To avoid bias, images were blindly scored, and the cells were classified as “stressed” or “unstressed” based on the fragmentation of the ER within each condition. Figure 2.2 shows representative images of cells exposed to each stress condition.

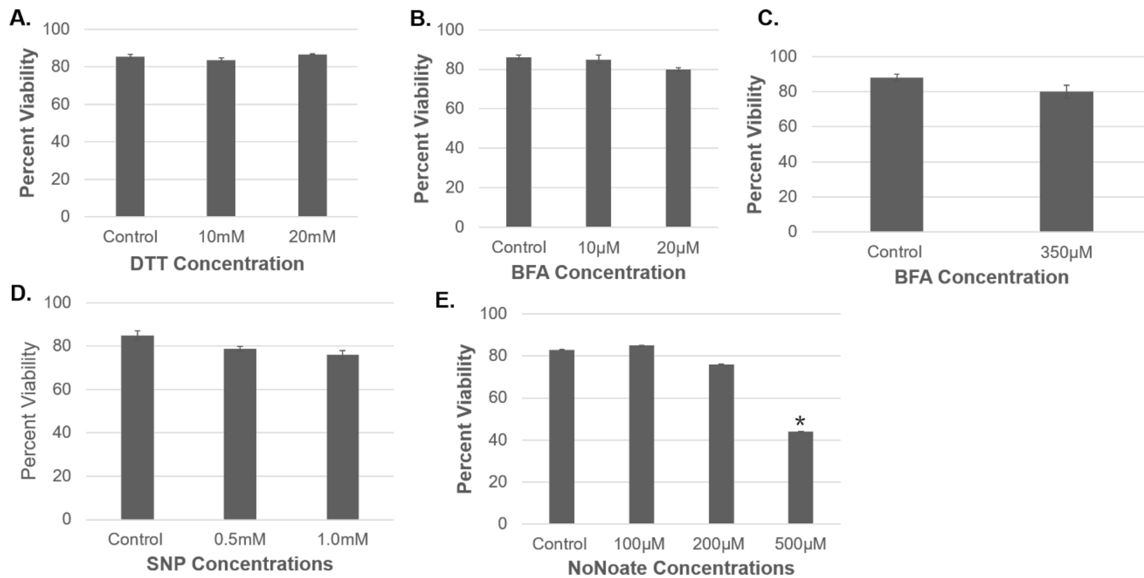

Figure 2.1: Viability of Stressed *E. histolytica* trophozoites

E. histolytica trophozoites were stressed as described in the text. Sterile deionized water was used as the vehicle control for experiments using DTT, SNP, and DPTA-NONOate, while DMSO was used as the vehicle control for BFA. A) Viability of trophozoites when exposed to 10 mM and 20 mM concentrations of DTT. B.) Viability of trophozoites exposed to 10 µm and 20 µM BFA. C.) Viability of trophozoites exposed to 350 µM BFA. D) Viability of trophozoites exposed to 0.5 mM and 1 mM SNP. E.) Viability of trophozoites exposed to 100 µM, 200 µM, and 500 µM DPTA-NONOate. * represents a significant result (p value < 0.05). Viability of trophozoites was quantified for each condition using Trypan Blue Exclusion. Results represent the mean (+/- standard error of at least 3 separate trials).

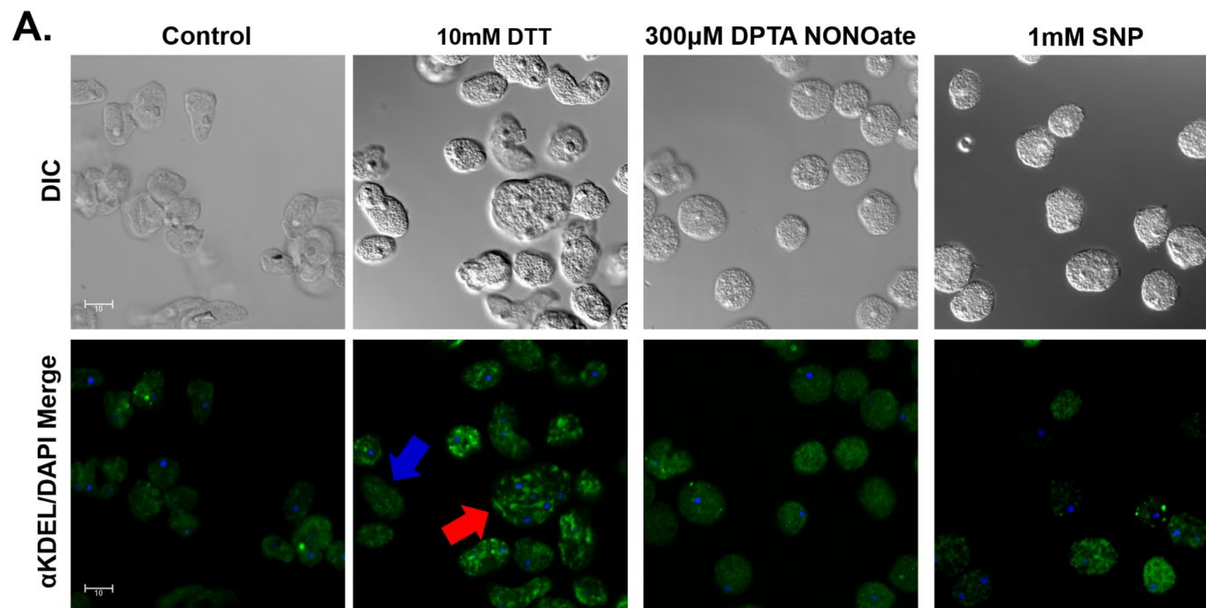

Figure 2.2A: Representative immunofluorescence microscopy images of stressed and unstressed trophozoites

Trophozoites were incubated with appropriate vehicle control (dH_2O), 10 mM DTT, 300 μM DPTA-NONOate, or 1 mM SNP. Cells were stained with anti-KDEL antibody (green) to visualize the ER and DAPI (blue) to visualize the nucleus. **Top Row:** DIC images. **Bottom Row:** Corresponding anti-KDEL/DAPI merged images. The red arrow indicates a representative cell with a stressed ER, while the blue arrow indicates a representative cell with an unstressed ER. DTT-treated cells showed a higher incidence of fragmented ERs than other conditions. Scale bars represent 10 μm.

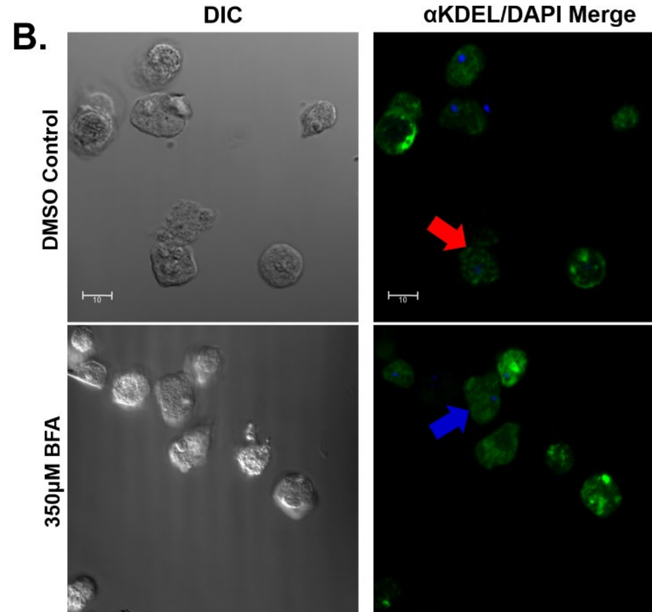

Figure 2.2B: Representative immunofluorescence microscopy images of stressed and unstressed trophozoites

Trophozoites were incubated with appropriate vehicle control, DMSO (top row) or 350 μM BFA (bottom row). Cells were stained with anti-KDEL antibody (green) to visualize the ER and DAPI (blue) to visualize the nucleus.

First Column: DIC images. **Second Column:** Corresponding anti-KDEL/DAPI merged images. The red arrow indicates a representative cell with a stressed ER, while the blue arrow indicates a representative cell with an unstressed ER. BFA-treated cells did not show a higher incidence of fragmented ERs than the control cells. Scale bars represent 10 μM.

Treatment with DTT, DPTA-NONOate, or SNP resulted in a higher percentage of cells with fragmented ERs (Fig. 2.2 and Figure 2.3). Although, these increases were not statistically significant, DTT produced the highest percentage of trophozoites showing ER morphology consistent with ER-stress (approaching significance, p-value=0.0660). Surprisingly, treatment with BFA resulted in a lower percentage of cells with fragmented ERs when compared with its DMSO control. While BFA causes ER stress in mammalian cells, it does not seem to cause ER stress in this parasite based on microscopic observations of ER morphology.

DTT-mediated ER stress induces phosphorylation of *Eh*eIF-2α, while nitrosative stress does not

The levels of total and phosphorylated *Eh*eIF-2α were measured in control and stressed trophozoites by Western Blotting using antibodies that specifically recognize total or phosphorylated *Eh*eIF-2α (3) (Figure 2.4). We observed a basal level of phosphorylated *Eh*eIF-2α in control unstressed trophozoites, which was consistent with the findings of Hendrick, *et al*. (3). Treatment with DTT resulted in significant phosphorylation of *Eh*eIF-2α (Figure 2.4A). Treatment with lower concentrations of BFA did not induce the phosphorylation of *Eh*eIF-2α; however, treatment with a higher concentration of BFA induced phosphorylation of *Eh*eIF-2α, albeit the increase was not statistically significant (Figure 2.4B). Overall, this is consistent with our conclusion that BFA does not seem to cause ER stress in this parasite. Surprisingly, nitrosative stress did not induce the phosphorylation of *Eh*eIF-2α. In fact, the levels of phosphorylated *Eh*eIF-2α decreased when trophozoites were exposed to reagents that cause nitrosative stress. Reactive nitrogen species are known to damage proteins, lipids, and other cellular

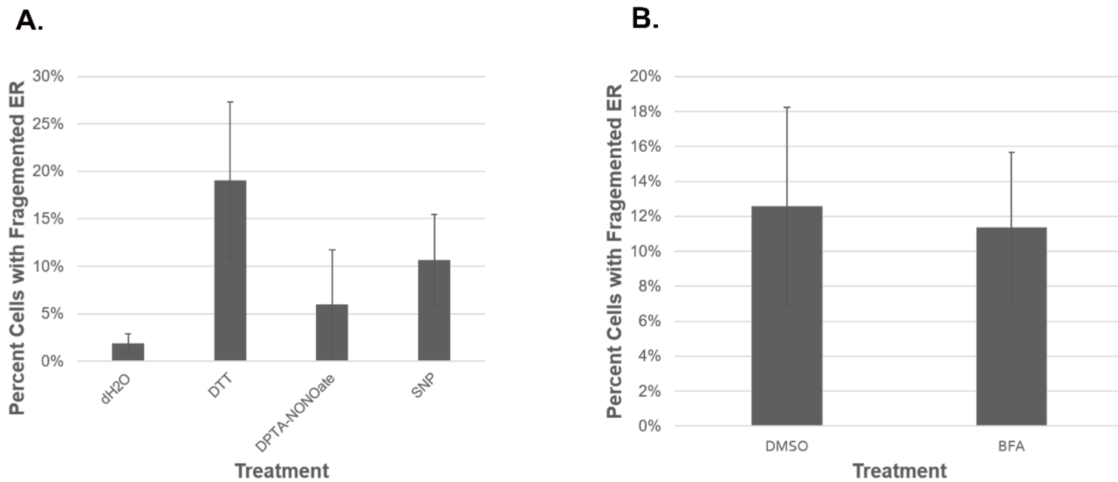

Figure 2.3: Percentage of trophozoites with fragmented ER under various stress conditions

Trophozoites were incubated with appropriate vehicle control (dH2O), 10 mM DTT, 300 µM DPTA-NONOate, or 1 mM SNP. Cells were stained with anti-KDEL antibody to identify the ER and DAPI to visualize the nucleus. Cells where then mounted on glass slides and visualized using a Leica SPE confocal microscope. Cells with fragmented ERs were identified as “stressed”, while cells with continuous ERs were identified as “unstressed”. The percentage of fragmented ERs was then calculated. No condition produced a significant increase in percentage of fragmented ERs compared to the appropriate control (p-value>0.05), but the increase in fragmented ERs when incubated with DTT is approaching significance (p-value=0.0660). Results represent the mean (+/- standard error of at least 3 separate trials).

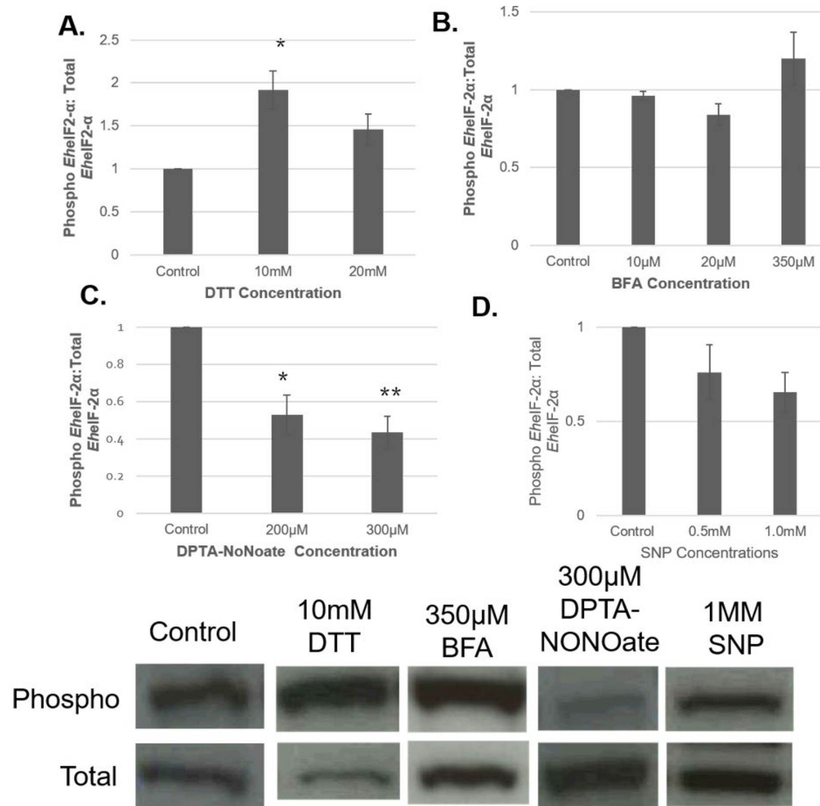

Figure 2.4: Levels of phosphorylated and total *Eh*eIF-2α when exposed to various stresses

Levels of total and phosphorylated *Eh*eIF-2α were measured using Western blotting and the ratios of total *Eh*eIF-2α: phosphorylated *Eh*eIF-2α were calculated using scanning densitometry after the bands were corrected for load variation as described in the text. The ratio for the control was arbitrarily set to 1.0 and the ratios for the treated cells were compared to the control. The trophozoites that were exposed to 10 mM DTT had significantly increased phosphorylation of *Eh*eIF-2α compared to the control (* represents p-value<0.05) (**A**.), while trophozoites exposed to 200μM and 300μM DPTA-NONOate decreased significantly (* represents p-value <0.05, ** represents p-value<0.01). Furthermore, trophozoites exposed to SNP and BFA did not have significant changes in levels of phosphorylated *Eh*eIF-2α compared to the total levels (p-value >0.05). All other comparisons were not statistically significant. Results represent the mean (+/- standard error) of at least 3 separate trials. Moreover, representative total and phosphorylated *Eh*eIF-2α Western Blot bands are shown for each stress condition. There phosphorylated band is visibly larger than the total for the DTT and BFA treated samples, and the phosphorylated band is much smaller than the total band for the DPTA-NONOate treated sample.

components (9). It is possible that exposure to the NO donors damaged the molecular machinery responsible for phosphorylation of *Eh*eIF-2α. Long-term serum starvation has been shown to significantly induce the phosphorylation of EheIF-2α (3). Therefore, if nitrosative stress damages the machinery needed to phosphorylate *Eh*eIF-2α, pre-treating trophozoites with NO donors prior to starving trophozoites of serum, should prevent phosphorylation of eIF2α. We exposed wildtype trophozoites to 300 μM DPTA-NONOate, 1 mM SNP, or vehicle control for 1 hr at 37˚C and then induced long-term serum starvation by incubating those same trophozoites in serum free culture medium for 24 hrs. Western blotting was performed using anti-total or anti-phosphorylated *Eh*eIF-2α antibodies to track the phosphorylation status of *Eh*eIF-2α.

Figure 2.5A shows the viability of trophozoites when exposed to DPTA-NONOate, SNP, or vehicle control for 1 hr, and then incubated with or without serum for 24 hrs and Figure 2.5B shows ratios of phosphorylated *Eh*eIF-2α: total *Eh*eIF-2α of wildtype parasites after exposure to vehicle control or nitrosative stress and long-term serum starvation. We found that parasites exposed to long-term serum starvation were not only severely less viable than the control that did not experience long-term serum starvation, but also had significantly decreased levels of phosphorylated *Eh*eIF-2α. Furthermore, parasites exposed to both nitrosative stress and long-term serum starvation were significantly less viable and had dramatically less phosphorylation of *Eh*eIF-2α compared to cells in the serum starved control condition. We were surprised to see that phosphorylation of *Eh*eIF-2α was reduced, rather than increased, during long-term serum starvation. But, since our serum starved control also showed a significant decrease in phosphorylated *Eh*eIF-2α

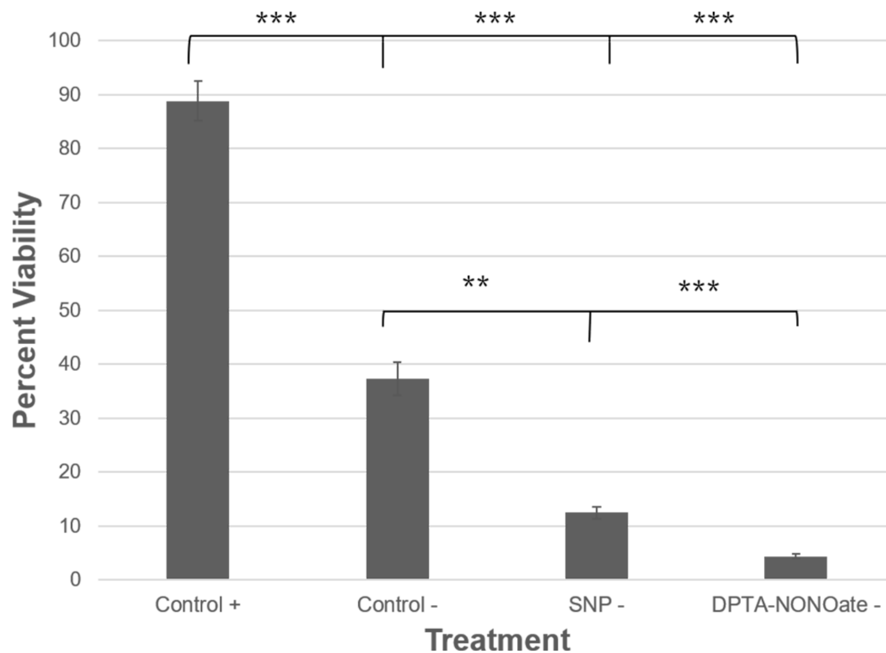

Figure 2.5A: Percent viability of trophozoites exposed to nitrosative stress and long-term serum starvation.

Wildtype trophozoites were incubated with vehicle control or nitrosative stress for 1 hr at 37˚C and then incubated in normal culture medium with or without serum for 24 hrs at 37˚C. After stress induction, viability was assessed using trypan blue exclusion. “Control +” represents cells treated with vehicle control that were not exposed to long-term serum starvation, whereas “control –“ represents cells that were treated with vehicle control and exposed to long-term serum starvation. Also, “SNP-“ and “DPTA-NONOate –“ represents groups of cells that were treated with SNP or DPTA-NONOate, and then exposed to long-term serum starvation. Long-term serum starvation resulted in a significant decrease in viability in all conditions when compared to the non-serum starved control (p-value < 0.001). Additionally, cells treated with SNP or DPTA-NONOate prior to serum starvation display significantly decreased viabilities compared to the serum starved control. (p values < 0.01 and <0.001, respectively). Data represent the mean (+/- standard error) of 3 separate trials.

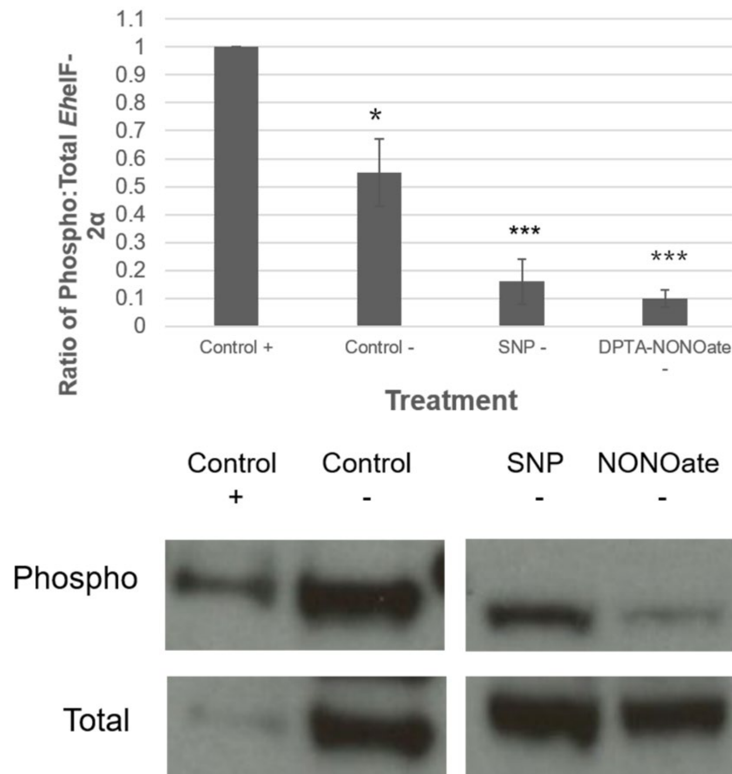

Figure 2.5B: Ratios of phosphorylated:total *Eh*eIF-2α when exposed to nitrosative stress and long-term serum starvation

Wildtype trophozoites were incubated with vehicle control or nitrosative stress for 1 hr at 37˚C and then exposed to long-term serum starvation for 24 hrs at 37˚C. Subsequent Western Blotting was conducted using anti-total or anti-phosphorylated *Eh*eIF-2α antibodies. The "control +" represents cells treated with vehicle control and that were not exposed to long-term serum starvation, where was "control –" represents cells that were treated with vehicle control and exposed to long-term serum starvation. Also, "SNP-" and "DPTA-NONOate –" represents groups of cells that were treated with SNP or DPTA-NONOate, and then exposed to long-term serum starvation. Long-term serum starvation resulted in a significant decrease in phosphorylated *Eh*eIF-2α in all conditions when compared to the non-serum starved control (* represents a p-value < 0.05, *** represents p-value <0.001). Furthermore, representative total and phosphorylated *Eh*eIF-2α Western Blot bands are shown below the figure. One can observe that the phosphorylated *Eh*eIF-2α are smaller and less intense than their corresponding total bands in samples treated with SNP and DPTA-NONOate. Data represent the mean (+/- standard error) of 3 separate trials.

compared to the non-serum starved control, we cannot make a conclusion about whether nitrosative stress damages the machinery necessary for phosphorylation of *Eh*eIF-2α.

Reduced protein translation is observed after treatment with DTT, BFA, SNP and DPTA-NONOate.

A common cellular stress response is to reduce global translation, giving the cell time to reconfigure gene expression to correct any damage caused by stress. Phosphorylation of *Eh*eIF-2α is one mechanism by which cells achieve the decrease in protein translation during stress (3).

To determine if nitrosative stress or ER stress results in reduced protein translation, we carried out SUrface SEnsing of Translation (SUnSET), as this method has been used successfully in *E. histolytica* to measure levels of protein translation (3,16). Briefly, after incubation with DTT, BFA, DPTA-NONOate, SNP, or vehicle control, trophozoites were incubated with puromycin, a tyrosyl-tRNA analog, which becomes incorporated into growing polypeptides, ultimately halting translation. Subsequent Western Blotting with an anti-puromycin antibody allows one to assess the level of puromycin incorporation and thus, the level of active protein translation within the cell.

To ensure that SUnSET accurately assessed protein translation, and to ensure that the anti-puromycin antibody was specific, we artificially halted translation by incubating control cells with cycloheximide (cyclo), prior to incubation with puromycin (puro). Cyclohexamide inhibits translation by blocking translocation of the tRNA molecules during protein translation (17) . The absence of protein bands in the sample treated with cycloheximide and puromycin (Figure 2.6A, Control+Puro+Cyclo) indicates that our

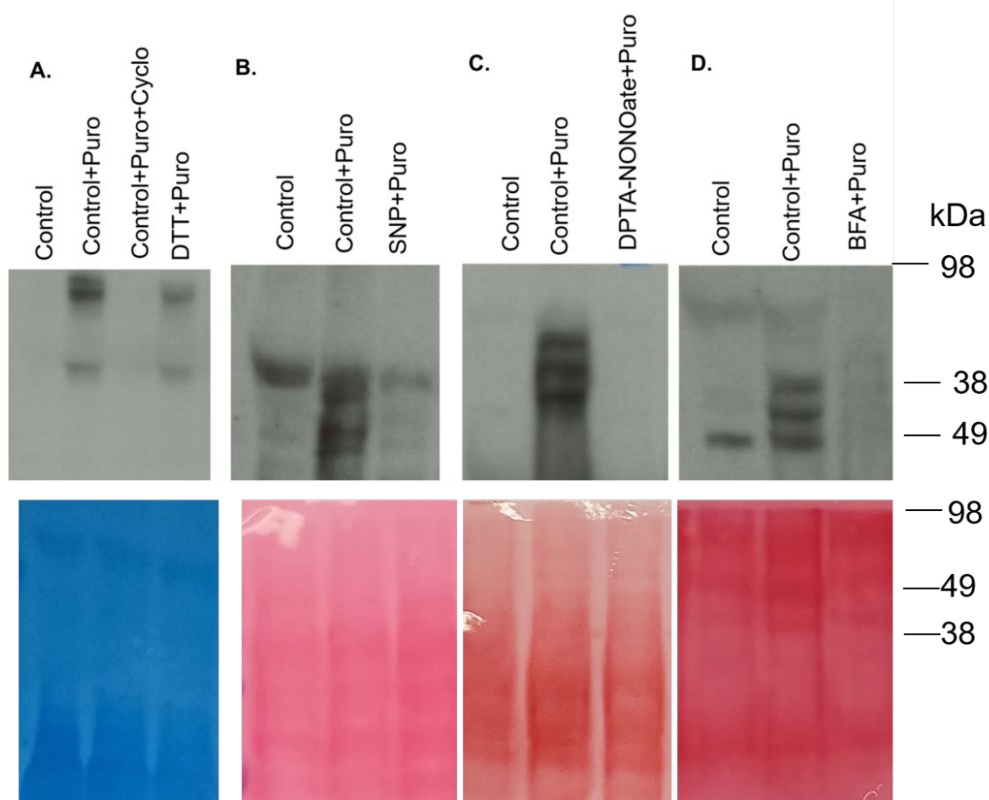

Figure 2.6: Representative images of SUnSET Western blots for each stress condition.

A) Wildtype trophozoites were incubated with vehicle control (1 hr), vehicle control (1 hr) and 10µg mL^{-1} puromycin (15 mins), vehicle control (1 hr), cycloheximide (10 mins), and 10µg mL^{-1} puromycin (15 mins), and 100µg mL^{-1}, or 10mM DTT (1 hr) and 10µg mL^{-1} puromycin (15 mins). Subsequent Western Blotting was performed using an anti-puromycin antibody. The lack of bands seen in the cycloheximide treated sample shows that we can inhibit translation and that the anti-puromycin antibody is specific. Also, the decrease in band intensity between the control+puro and the DTT+puro samples indicates that translation is decreasing upon treatment with DTT. B,C,D) Wildtype trophozoites were again, incubated with vehicle control, vehicle control and 10 µg mL^{-1} puromycin, or stress reagent and 10 µg mL^{-1} . Subsequent Western Blotting was performed using an anti-puromycin antibody. In each stress condition, there is a noticeable decrease in band intensity between the control+puro and stress+puro samples, indicating a decrease in protein translation with treatment with 1 mM SNP, 300 µM DPTA-NONOate, and 350 µM BFA. Below each Western Blot image, is the corresponding PDVF membrane that has either been stained with A) Coomassie or B,C,D) ponceau reagent to confirm loading of equal amounts of cell lysate.

antibody is specific for puromycin incorporation and that this method of measuring translation is accurate.

For all 4 stress treatments, the decrease in protein band density indicates that protein translation is decreased. We expected to see a decrease in protein translation upon treatment with DTT since we saw a significant increase in phosphorylation of *Eh*eIF-2α under the same conditions. However, we were surprised to see a decrease in protein translation upon treatment with BFA, SNP, or DPTA-NONOate since these conditions did not induce an increase in the phosphorylation of *Eh*eIF-2α. These data suggest that there may be an alternate stress response pathway in *E. histolytica* that acts independently of the *Eh*eIF-2α pathway.

Phosphorylation of *Eh*eIF-2α may be required to counter ER stress

Previously our laboratory generated 3 transgenic cell lines that overexpress wildtype or modified versions of *Eh*eIF-2α in a tetracycline-inducible fashion (3). We have a cell line that expresses a phosphomimetic version eIF-2α (D59), in which the serine at position 59 was mutated to aspartic acid, a cell line that expresses a non-phosphorylatable version of eIF-2α (A59), in which the serine at position 59 was mutated to alanine, and finally a cell line that overexpresses wildtype *Eh*eIF-2α (S59). Each of these exogenous proteins have a FLAG tag epitope peptide sequence added to N-terminal end to distinguish the endogendous *Eh*eIF-2α from the exogenous *Eh*eIF-2α. A cell line that expresses luciferase (Luc), an irrelevant protein, in a tetracycline-inducible fashion serves as a control. To confirm expression of the exogenous proteins the transgenic cell lines were incubated with (+) or without (-) 5 μg mL^{-1} tetracycline for 24 hrs and the lysates were subjected to Western Blotting using anti-FLAG tag antibody or an anti-total *Eh*eIF-2α

antibody. The presence of a correctly sized FLAG-tag protein band between 28 and 38 kDa in the lysates from cells incubated with tetracycline confirm that expression of the exogenous is inducible by tetracycline (Figure 2.7).

To determine if phosphorylation of *Eh*eIF-2α was necessary to counter stress, the transgenic cells lines were exposed to 10 mM DTT for 1 hr at 37˚C and viability was measured using trypan blue exclusion (See Figure 2.7). We predicted that our D59 and S59 transgenic cell lines would have an increased viability when stressed and that our A59 cell line would have decreased viability when exposed to stress. As expected the nonphosphorylatable cell line exhibited a significant lower viability (p-value < 0.05) than that of control cells (Figure 2.8). However, unexpectedly, the the phosphomimetic cell line (D59) and the overexpressing cell line (S59) also exhibited slightly lower viabilities compared to that of the control cell line, although these decreases were not statistically significant.

V. Discussion

This study is the first to investigate the phosphorylation of eIF-2α during ER stress and nitrosative stress in *E. histolytica*. Our immunofluorescence microscopy images (Figure 2.2) revealed distinct fragmentation of the ER when parasites are treated with dithiothreitol, indicating ER stress, while the fragmentation did not occur when parasites are treated with Brefeldin A, both of which are known inducers of ER stress (4). Additionally, Western blotting showed significant phosphorylation of *Eh*eIF-2α upon treatment with DTT, but only a slight increase in phosphorylation with BFA (Figure 2.4). These data suggest that *E. histolytica* experiences ER stress when treated with DTT and

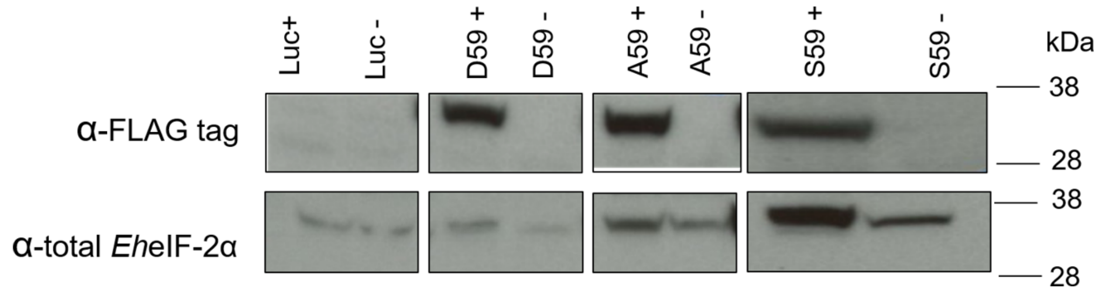

Figure 2.7: Expression of exogenous forms of *Eh*eIF-2α in transgenic cells is tetracycline inducible.

Three *E. histolytica* transgenic cells lines were produced that express exogenous forms of *Eh*eIF-2α in a tetracycline inducible fashion: a phosphomimetic *Eh*eIF-2α (D59), a non-phosphorylatable *Eh*eIF-2α (A59), and a cell line that overexpresses wildtype *Eh*eIF-2α (S59). Each exogenous protein has a FLAG tag epitope peptide sequence added to the N-terminus to distinguish endogenous *Eh*eIF-2α from exogenous *Eh*eIF-2α. Finally, as a control we use a cell line that expresses luciferase (Luc). We incubated all 4 cell lines with (+) or without (-) 5 µg mL^{-1} tetracycline and conducted subsequent Western blotting with an anti-FLAG tag antibody or an anti-total *Eh*eIF-2α antibody. In each cell line expressing an exogenous form of *Eh*eIF-2α**,** we see a clear FLAG-tag protein band after induction with tetracycline, while we see no FLAG-tag band in samples incubated without tetracycline. Additionally, levels of total *Eh*eIF-2**α** are higher than that in cells incubated without tetracycline. These data confirm that our transgenic cell lines are tetracycline inducible.

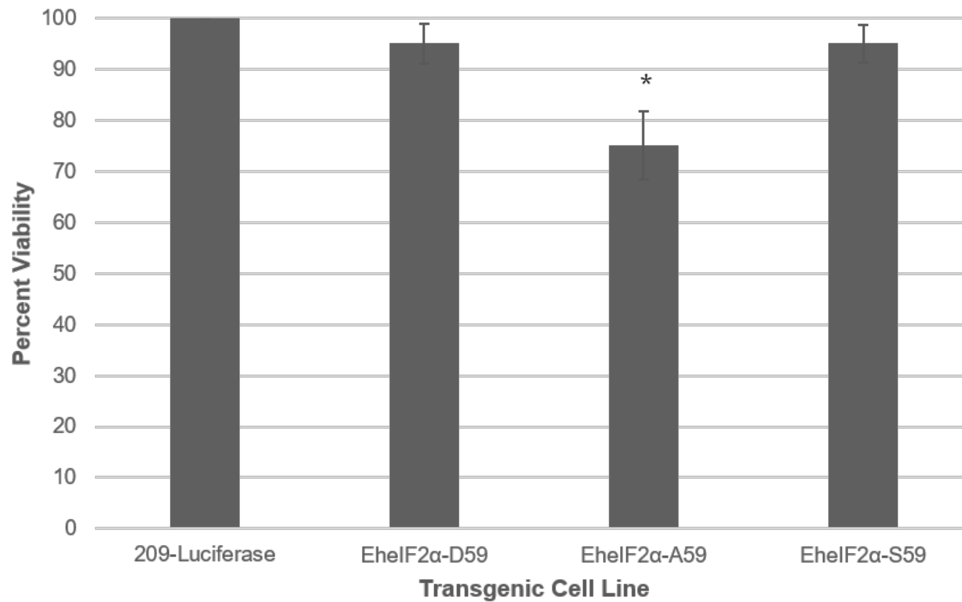

Figure 2.8: Viability of transgenic cell lines expressing modified forms of *Eh*eIF-2α when exposed to 10 mM DTT

Transgenic cell lines were grown for 24 hrs in normal culture medium supplemented with 5 μg mL^{-1} tetracycline. Then, trophozoites were incubated with 10 mM DTT for 1 hr at 37˚C. Viability was quantified using trypan blue exclusion and a hemocytometer. D59 and S59 cell lines did not have significantly different viabilities when compared to the Luciferase line. However, the A59 cell line had a significantly lower viability (p-value <0.05). These data represent the mean (+/- standard error) of at least 3 separate trials.

responds by phosphorylating *Eh*eIF-2α. While a higher concentration of BFA elicited slightly higher levels of phosphorylated *Eh*eIF-2α, ER fragmentation did not occur in this parasite.

BFA disrupts vesicle trafficking by inhibiting the interaction of ADP-ribosylation factor (ARF) with the Golgi membrane. ARF is a small GTP-binding protein involved in vesicle coating and uncoating. Interaction of ARF with Golgi membrane facilitates the guanine nucleotide exchange, which is required for vesicle trafficking. By blocking this interaction, BFA effectively inhibits vesicle trafficking, leading to an accumulation of proteins in the ER (18). In mammalian cells, this ER stress induces the phosphorylation of eIF-2α (4).

At present, it cannot be discerned if BFA causes ER stress in *E. histolytica*. Although we observed augmented levels of phosphorylated *Eh*eIF-2α after treatment with BFA, the increase was not statistically significant and we did not observe fragmentation of the ER after staining with anti-KDEL antibodies. A search of AmoebaDB (amoebadb.org) demonstrates that *E. histolytica* possesses multiple genes encoding conserved ARFs. Thus, in theory, *E. histolytica* has the target for BFA. In a previous study, Welter *et al*. (19) demonstrated that treatment with 350 uM BFA resulted in the accumulation of several secretory proteins in a large tubular compartment that was reminiscent of a swollen and stressed ER. Furthermore, the aberrantly localized secretory proteins co-localized with calreticulin, an ER-resident protein. However, Welter *et al*., (19) did not attempt to assess the morphology of the ER using immunostaining and anti-KDEL antibodies. Therefore, it is possible that BFA causes re-organization of the ER in a way that excludes the co-localization of KDEL-containing proteins and calreticulin. Future studies that attempt to

co-localize KDEL-containing proteins and calreticulin before and after BFA treatment will provide insight into this question.

Furthermore, our data show that nitrosative stress may induce ER stress. We observed some fragmentation of the ER upon treatment with DPTA-NONOate or SNP for 1 hr; however, it was not statistically significant (Figures 2.2 and 2.3). While these data agree with those published by Santi-Rocca, *et al.* (14), further investigation into this mechanism is needed. Based on data showing a similar stress response in *E. histolytica* when exposed to oxidative and nitrosative stresses (10), and the data published by Hendrick *et al.*(3) finding that oxidative stress results in significantly increased levels of phosphorylated *Eh*eIF-2α, we predicted that nitrosative stress would also result in the significant phosphorylation of *Eh*eIF-2α. Surprisingly, our data revealed significantly decreased levels of phosphorylated *Eh*eIF-2α when trophozoites were exposed to DPTA-NONOate or SNP, both strong NO donors (Figure 2.4).

Previously, Hendrick *et al*. showed that long-term serum starvation strongly induced the phosphorylation of *Eh*eIF-2α (3); therefore, to determine if the machinery necessary to phosphorylate *Eh*eIF-2α was damaged by RNS, we exposed trophozoites to SNP or DPTA-NONOate for 1 hr and then immediately exposed to them to serum starvation for 24 hrs. We found that once treated with nitrosative stress, parasites were no longer able to cope with long-term serum starvation by phosphorylating *Eh*eIF-2α and suffered significantly reduced viabilities compared to trophozoites that were only exposed to long-term serum starvation (Figure 2.5). Additionally, we found that control cells had significantly reduced phosphorylation of *Eh*eIF-2α, rather than increased. This is not consistent with the work of Hendrick *et al*. (3); however, for unknown reasons, we

observed significantly higher cell death than in the previous study. Therefore, we currently cannot determine if the translation control machinery is being damaged in the presence of RNS. In the future, it will be necessary to expose cells to a different stress (e.g., long-term heat shock or oxidative stress) known to induce phosphorylation of eIF2a to determine if the NO donors damage the eIF2a phosphorylation machinery.

The eIF-2 mechanism is known to counter stress by reducing global protein translation. This reduction in protein synthesis allows the cell time to repair any damage caused by stress (8,20). To further illuminate the eIF-2 mechanism in this parasite, we measured levels of protein translation in control and stressed trophozoites using SUnSET. Out of our four stress conditions (DTT, BFA, DPTA-NONOate, and SNP) we only detected significantly increased levels of phosphorylated *Eh*eIF-2α in DTT-treated samples. Therefore, we expected to see decreased protein translation in trophozoites exposed to DTT. Unexpectedly, we found that trophozoites displayed reduced translation in all four stress conditions (See figure 2.6). Reduction of protein translation in the absence of phosphorylated *Eh*eIF-2α suggests that there may be another stress-specific response pathway, independent of the eIF-2 mechanism.

Reactive nitrogen species have been found to attack many cell components, damaging proteins, lipids, and DNA. In addition, RNS have been found to decrease protein synthesis by NO-mediated cleavage of ribosomal proteins. So, one possible explanation for decreased phosphorylation of *Eh*eIF-2α and globally reduced translation is RNS-damaged kinases and ribosomal proteins (9). However, Kim *et al.* found that nitrosative stress induces the phosphorylation of eIF-2α in murine macrophages and pancreatic islet

cells (20). That *E. histolytica* does not seem to phosphorylate eIF-2α in the presence of NO donors represents a difference from the conserved mechanism in mammalian cells.

Finally, to determine if phosphorylated eIF-2α is required to counter ER stress in this parasite, our lab previously produced 3 transgenic *E. histolytica* cell lines that express control or modified forms of eIF-2α in a tetracycline-inducible fashion (3). We then exposed these transgenic cell lines to 10mM DTT and measured viability (see Figures 2.7 and 2.8). In agreement with our prediction, the nonphosphorylatable (A59) transgenic cell line suffered significant cell death when exposed to stress. This observation illustrates the need to reduce protein translation by the phosphorylation of *Eh*eIF-2α. However, contrary to our predictions, the phosphomimetic (D59) and overexpressing (S59) cell lines had lower viabilities when exposed to stress than the luciferase expressing control cell line, therefore, they are not better suited to handle stress. This observation suggests that levels of phosphorylated *Eh*eIF-2α above a certain threshold do not aid in the stress response.

Ultimately, these data show that *E. histolytica* experiences ER stress and copes with this stress by phosphorylating *Eh*eIF-2α, and subsequently reducing general protein translation. This parasite may encounter physiological stressors that are known to induce ER stress; such as glucose deprivation, high protein demand, and inflammatory cytokines (4,5). Therefore, the eIF-2 mechanism may be crucial to parasite survival within the human host. Viability was assessed (Figure 2.1) to ensure that the concentrations of DTT were not lethal to the trophozoites. Since the viability of the stressed and the control trophozoites was not statistically significant, we are confident that the viability did not affect the phosphorylation of *Eh*eIF-2α.

However, it is possible that the phosphorylation *Eh*eIF-2α seen with DTT may be due to off-target effects. It is therefore important to confirm ER stress is occurring using other stressors. Possible options include treatment with Tunicamycin, and calcium ionophores, or physiological stressors, such as glucose deprivation, which have been shown to induce ER stress as well. (4). Furthermore, while phosphorylated eIF-2α is notably known for reducing global protein synthesis, this mechanism simultaneously promotes the translation of stress-specific mRNAs (21).

A current goal of our lab is to isolate and identify these mRNAs that are escaping this translational control, as we predict they are critical to the stress response of this parasite. Obtaining these data may reveal novel stress response pathways or may identify novel targets for drug and vaccine development. Moreover, investigation of the *E. histolytica* genome data suggests that *E. histolytica* possesses two putative eIF-2α kinases (EHI_109700, EHI_035950) (22). Authentication of these kinases is crucial to fully understanding the eIF-2 mechanism in *E. histolytica* and may help explain the differences between this conserved mechanism among different species. Nonetheless, these data from this study agree with findings that show phosphorylation of *Eh*eIF-2α is a stress-specific response and further suggests that *E. histolytica* has a sophisticated way of countering stress to survive in various environments (3).

VI. Acknowledgements

The authors would like to thank the Temesvari lab for their support in completing this project. A special thanks to Dr. Zhicheng Dou at Clemson University for the reagent,

Brefeldin A, and his helpful discussion on experimental design. All immunofluorescent images were taken in the Clemson Life Imaging Facility. The authors thank Ms. Rhonda Powell for her assistance and for donating DAPI, a nuclear stain.

Financial assistance for this work was provided by grants NIH R21 AI108287and NIH COBRE GM10904, both awarded to Dr. Lesly Temesvari. The Department of Biological Sciences financially supported me with a teaching assistantship and funded my studies here at Clemson University. The funding agencies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. This content is solely the responsibility of the authors and does not necessarily represent the views of the National Institute of Allergy and Infectious Diseases, the National Institutes of Health.

VII. References

1. Grebmer K, Bernstein J, de Waal A, Prasai N, Yin S, Yohannes Y. UNICEF and WHO, 2015 Update and MDG Assessment - Progress on Sanitation and Drinking Water. 2015;

2. Gunther J, Shafir S, Bristow B, Sorvillo F. Short report: Amebiasis-related mortality among United States residents, 1990-2007. Am J Trop Med Hyg. 2011;85(6):1038–40.

3. Hendrick HM, Welter BH, Hapstack MA, Sykes SE, Sullivan WJ, Temesvari LA. Phosphorylation of Eukaryotic Initiation Factor-2α during Stress and Encystation in Entamoeba Species. PLoS Pathog. 2016;12(12):1–14.

4. Oslowski CM, Urano F. Measuring ER stress and the unfolded protein response using mammalian tissue culture system. Methods Enzymol [Internet]. 2013;490(508):71–92. Available from: http://www.sciencedirect.com/science/article/pii/B9780123851147000040

5. Joyce BR, Tampaki Z, Kim K, Wek RC, Sullivan WJ. The unfolded protein response in the protozoan parasite Toxoplasma gondii features translational and transcriptional control. Eukaryot Cell. 2013;12(7):979–89.

6. Zhang M, Mishra S, Sakthivel R, Rojas M, Ranjan R, Sullivan WJ. PK4, a eukaryotic initiation factor 2a(eIF2a) kinase, is essential for the development of the erythrocytic cycle of Plasmodium. Proc Natl Acad Sci. 2012;109(10):3956–61.

7. Cloutier S, Laverdière M, Chou MN, Boilard N, Chow C, Papadopoulou B. Translational control through eIF2alpha phosphorylation during the Leishmania differentiation process. PLoS One. 2012;7(5).

8. Wek RC, Cavener DR. Translational Control and the Unfolded Protein Response. Antioxid Redox Signal [Internet]. 2007;9(12):2357–72. Available from: http://www.liebertonline.com/doi/abs/10.1089/ars.2007.1764

9. Nagaraja S, Ankri S. Utilization of Different Omic Approaches to Unravel Stress Response Mechanisms in the Parasite Entamoeba histolytica. Front Cell Infect Microbiol [Internet]. 2018;8(February):1–12. Available from: http://journal.frontiersin.org/article/10.3389/fcimb.2018.00019/full

10. Vicente JB, Ehrenkaufer GM, Saraiva LM, Teixeira M, Singh U. Entamoeba histolytica modulates a complex repertoire of novel genes in response to oxidative and nitrosative stresses: Implications for amebic pathogenesis. Cell Microbiol. 2009;11(1):51–69.

11. Diamond, Louis; Harlow, DR; Cunnick C. A new medium for the axenic culture of Entamoeba histolytica and other Entamoeba. Trans R Soc Trop Med Hyg. 1978;72(4):431–2.

12. Delgado-Corona P, Mart??nez-Cadena G, Alvarez AH, Torres-Calzada HE, Avila EE. An extracellular monoADP-ribosyl transferase activity in Entamoeba histolytica trophozoites. J Eukaryot Microbiol. 2002;49(6):454–9.

13. Manning-Cela R, Marquez C, Franco E, Talamas-Rohana P, Meza I. BFA-sensitive and insensitive exocytic pathways in Entamoeba histolytica trophozoites: Their relationship to pathogenesis. Cell Microbiol. 2003;5(12):921–32.

14. Santi-Rocca J, Smith S, Weber C, Pineda E, Hon CC, Saavedra E, et al. Endoplasmic reticulum stress-sensing mechanism is activated in entamoeba histolytica upon treatment with nitric oxide. PLoS One. 2012;7(2):1–14.

15. Teixeira JE, Huston CD. Evidence of a continuous endoplasmic reticulum in the protozoan parasite Entamoeba histolytica. Eukaryot Cell. 2008;7(7):1222–6.

16. Hertz R, Tovy A, Kirschenbaum M, Geffen M, Nozaki T, Adir N, et al. The Entamoeba histolytica Dnmt2 homolog (Ehmeth) confers resistance to nitrosative stress. Eukaryot Cell. 2014;13(4):494–503.

17. Schmidt EK, Clavarino G, Ceppi M, Pierre P. SUnSET, a nonradioactive method to monitor protein synthesis. Nat Methods. 2009;6(4):275–7.

18. Donaldson JG. Mode of action of brefeldin A. Nature. 1992;360:350–2.

19. Welter BH, Temesvari LA. Overexpression of a mutant form of EhRabA, a unique rab GTPase of entamoeba histolytica, alters endoplasmic reticulum morphology and localization of the Gal/GalNAc adherence lectin. Eukaryot Cell. 2009;8(7):1014–26.

20. Kim YM, Son K, Hong SJ, Green A, Chen JJ, Tzeng E, et al. Inhibition of protein synthesis by nitric oxide correlates with cytostatic activity: Nitric oxide induces phosphorylation of initiation factor eIF-2 alpha. Mol Med. 1998;4(3):179–90.

21. Castilho BA, Shanmugam R, Silva RC, Ramesh R, Himme BM, Sattlegger E. Keeping the eIF2 alpha kinase Gcn2 in check. Biochim Biophys Acta - Mol Cell Res [Internet]. 2014;1843(9):1948–68. Available from: http://dx.doi.org/10.1016/j.bbamcr.2014.04.006

22. Vonlaufen N, Kanzok SM, Wek RC, Sullivan WJ. Stress response pathways in protozoan parasites. Cell Microbiol. 2008;10(12):2387–99.