Phosphorylation of Eukaryotic Initiation Factor 2 Alpha Regulates Stress in the Human Protozoan Parasite Entamoeba Histolytica

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PHOSPHORYLATION OF EUKARYOTIC INITIATION FACTOR 2 ALPHA REGULATES STRESS IN THE HUMAN PROTOZOAN PARASITE *ENTAMOEBA HISTOLYTICA*

A Thesis
Presented to
the Graduate School of
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In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Biological Sciences

by
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Accepted by:
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ABSTRACT

Entamoeba histolytica is a food- and water-borne intestinal parasite responsible for amoebic dysentery and amoebic liver abscess. The life cycle of E. histolytica alternates between the host-restricted trophozoite form and the highly infective latent cyst stage that is able to persist in the environment. Throughout its life cycle, which may include invasion of tissues in the human host, the parasite is subjected to a variety of stressful conditions. In other systems, stress can trigger the activation of kinases that phosphorylate a serine residue on eukaryotic translation initiation factor-2α (eIF2α). This modification inhibits the activity of eIF2 resulting in a general decline in protein synthesis, and, paradoxically, an up-regulation of the expression of certain genes that permit the cell to counter the stress. Genomic data reveal that E. histolytica possesses eIF2α with a conserved phosphorylatable serine at position 59. Thus, this pathogen may have the machinery for stress-induced translational control. To test this, we exposed E. histolytica trophozoites to six different stress conditions and assessed viability, as well as the level of total and phospho-EhIF2α via Western blot of cell lysates. Long term serum starvation induced an increase in the level of phospho-EhIF2α, but no other stress condition caused a significant change. Long term serum starvation also showed a decrease in polyribosome abundance as observed through sucrose gradient ultracentrifugation; this is consistent with the observation that this condition also induces phosphorylation of EhIF2α. This suggests that the eIF2α-dependent stress response system is operational in E. histolytica and that the system may be activated only by certain stresses. To further examine the role of phosphorylation of EhIF2α during stress,
three transgenic cell lines were created. *EheIF2α*-S59 over-expresses wild type eIF2α protein. *EheIF2α*-S59A expresses eIF2α with the serine-59 residue mutated to an alanine, creating a non-phosphorylatable subunit. *EheIF2α*-S59D expresses eIF2α with the serine-59 residue mutated to an aspartic acid to mimic a phosphorylated residue. *EheIF2α*-S59 exhibited a high level of phosphorylation of the exogenous protein, leading to a decreased growth and polyribosome abundance when compared to the control cell line. *EheIF2α*-S59A had the highest growth rate and retained a high abundance of polyribosome. *EheIF2α*-S59D exhibited the slowest growth rate and had a decrease in polyribosome when compared to control; however, *EheIF2α*-S59D did exhibit the highest survival rate in over half the stress conditions tested. This may indicate the protective nature of phosphorylation of *EheIF2α* during times of stress.
DEDICATION

This body of work would not have been possible without the incredible support of my parents, James and Marcia, my sisters, Hannah and Helena, and my partner in life, Roddey. You have supported me throughout my life, but especially during this most strenuous time. I am always thankful for having you in my life.
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Polyribosome profile for control and mutant eIF2α cell lines after 24 hour induction
CHAPTER ONE

LITERATURE REVIEW

I. Introduction

*Entamoeba histolytica* is the protozoan parasite responsible for amoebic dysentery and liver abscess in humans and non-human primates. The life cycle of *E. histolytica* does not require an intermediate host, as it is transmitted from human to human, often through fecally contaminated food or water (Fig 1). This mode of transmission makes amoebic dysentery a disease of importance to underdeveloped areas with no water filtration or treatment systems. In the late 1990s, the WHO estimated that 500 million people around the world were infected with *Entamoeba*, with 40,000-100,000 deaths annually [1]. Because of the ease of transmission in water, *Entamoeba histolytica* is classified as a Class B bioterrorism agent, further highlighting the need for information about this parasite.

*E. histolytica* cysts are formed in the large intestine and exit the host with the fecal matter. Cysts are typically rounded, quadrinucleated and stable (desiccation-tolerant, acid-tolerant, heat-tolerant; and detergent-resistant) [reviewed in 2]. These latent cysts readily survive the extreme conditions in the external environment. To continue the life cycle, the cyst must be ingested where it can pass unharmed through the acidic condition of the stomach into the small intestine. Once there, unknown triggers cause the cyst to undergo excystation, resulting in eight active trophozoites for every cyst. These amoeboid trophozoites continue down the intestinal tract until reaching the large intestine. This is the location of intraintestinal infections of *E. histolytica*, where cells replicate by binary
Figure 1.1: Life cycle of *Entamobea histolytica*

The infective stage, a latent cyst, is ingested, usually due to fecally contaminated food or water. This cyst can pass through the stomach unharmed and enter the small intestine. Here, unknown factors trigger an excystation event and 8 trophozoites emerge for every one cyst. These active trophozoites migrate through the small intestine and into the large intestine, where *E. histolytica* interintestinal infections occur. A small percentage of these trophozoites will encyst, though the molecular mechanism that triggers this process is unknown. These cysts can survive outside the host and be passed to another individual. Intestinal disease can occur when the trophozoites begin degrading the intestinal cells. If the trophozoites leave the large intestine and enter the bloodstream, extraintestinal complications can occur, often in the liver, lung, and brain [3]. Image modified from the Center of Disease Control [4].
fission and feed on bacteria and intestinal cells while replicating through binary fission. The parasite is able to internalize host cells through the process of phagocytosis or internalizing pieces of living cells through the process of trogocytosis. Trophozoites can also invade through the intestinal wall to cause extraintestinal infections in the liver, lungs, or brain [3].

For *E. histolytica* to establish infection in the colon, adhesion to host mucosal layer and cells is necessary. In the amoebae, one important cell surface adhesion complex is the Gal/GalNAc lectin. This lectin complex is composed of a heavy (Hgl), intermediate, (Igl) and light (Lgl) subunit and is named for its affinity for galactose (Gal) and N-acetyl-D-galactosamine (GalNAc) on host cells and mucin. Along with a large family of transmembrane kinases (TMKs), the Gal/GalNAc lectin plays a role in phagocytosis, contact-dependent cytotoxicity of host cells, and host cell death [reviewed in 5]. Secreted proteases and hydrolases assist the parasite in disruption of host cell layers, and are also involved in the proteolytic inactivation of host antibodies and complement. *E. histolytica* also secretes pore forming proteins, known as amoebapores. These also disrupts host cell membrane integrity which facilitates their uptake by phagocytosis and the invasion of the parasite into internal cell layers [reviewed in 6].

Within the large intestine, some trophozoites will undergo encystation in order to exit the host and continue the life cycle. To date, complete encystation of *E. histolytica* has never been achieved in *in vitro* culture. Therefore, to examine the pathways that regulate encystation, another *Entamoeba* species has been used: *Entamoeba invadens* [2]. *E. invadens* infects reptilian hosts in the same manner as *Entamoeba histolytica* and is often
used as a model organism, as encystation can be induced in vitro by simultaneous application of osmotic stress, glucose starvation, and serum deprivation.

The current treatment for Entamoeba infection is metronidazole, a drug developed in the 1960s to treat bacterial infections as well as protozoan infections [7]. Metronidazole is absorbed very well in the upper gastrointestinal (GI) tract and can be found in most body fluids with few side effects [8]. However, given the site of infection (lower GI), higher doses of metronidazole are required to treat amoebiasis. This can result in systemic toxicity. However, clinical studies have shown less than 50% parasite clearance after treatment with metronidazole and reoccurrence of infection after an initial relief of symptoms [9]. In countries where infections are prevalent, dose compliance can also be problematic if the patient stops taking or cannot access the medication. Given these toxicity, efficacy, and compliance issues, it is clear that novel drugs are necessary to reduce or even eliminate the risk of acquiring Entamoeba histolytica.

II. The Stress Response in Entamoeba histolytica

At different points in the life cycle, E. histolytica must combat stress. Local inflammatory responses can cause temperature increases, as well as oxidative and nitrosative stresses. Nutrients can become scarce during periods of infection, and the overgrowth of enteric bacteria can overcome small populations of the parasite in the intestine. E. histolytica must possess a cellular response to survive and overcome these extreme environmental conditions. Since simultaneous application of osmotic stress, glucose starvation, and serum deprivation induces encystation in E. invadens, stage-
conversion is also presumed to be a response to stress. Considering that the stress response is so important to survival and encystation in *Entamoeba*, it is conceivable that the molecular components of the stress response system may serve as targets for new drugs. Such drugs could cause death of the trophozoites by hindering their ability to counter stress and/or to undergo encystation. The importance of the stress response system is *E. histolytica* has provided the impetus for many *in vitro* studies, which are summarized in this section.

**Glucose Deprivation**

*Entamoeba histolytica* does not possess a functional tricarboxylic acid cycle or a mitochondrial electron transport chain, forcing the parasite to rely on glycolysis and fermentation for energy production [3]. Within the human host, *E. histolytica* will experiences different concentrations of extracellular glucose in a site-specific manner. During the initial invasion in the colon, glucose levels are severely reduced due to efficient glucose absorption in the small intestine. However, within the liver, glucose levels are higher, allowing for glucose uptake by the parasite [reviewed in 10].

*E. histolytica* is routinely cultured *in vitro* in TYI-S-33 media that contains a glucose concentration of 12 mM [11]. Reduction of glucose can induce phenotypic changes. For example, short term glucose starvation (STGS; 12 hours) increases virulence by enhancing hemolytic activity, cytopathic activity, and adhesion to mammalian cells [12]. Although the mechanism by which STGS enhances virulence is unknown, proteomic analysis of these stressed cells by mass spectrometry revealed 49 proteins that exhibited at
least a two-fold change in expression. While decreases in metabolic proteins and increases in protein synthesis enzymes were discovered, an unexpected result also showed a decrease in the proteins associated with virulence, such as amoebapore proteins and a cysteine proteinase, during STGS. This is inconsistent with the observation the STGS increases virulence.

Long term glucose starvation (LTGS; over 1 month) results in an overall reduction in ATP levels. Microarray analysis revealed differential expression of 56 proteins in LTGS, with several virulence factors being upregulated, such as the Gal/GalNAc lectin, cysteine proteinase 4, and pore-forming peptides. Recovery in a high glucose media resulted in further changes in expression, such as the upregulation of cysteine proteinases, tyrosine kinases, cyst-wall specific proteins, and a multitude of other proteins [13]. It is important to note that glucose starvation, both short and long term, does not stimulate the expression of heat shock proteins, known for assisting in overcoming stress and in protein folding.

Iron Deprivation

Iron is available to in vitro E. histolytica cultures by the addition of ferric ammonium citrate to the medium [11]. An iron chelator, such as 2,2’-dipyridyl, can be used to remove iron from the media [14]. This reduction in iron results in a growth defect that correlates with the amount of iron in the media. Transcriptional analysis of iron-deprived trophozoites revealed an increase in cysteine proteinases, ribosomal proteins, and elongation factor-1 alpha [14].
With no functional mitochondria, the enzymes necessary for glycolysis are essential in parasite survival. One enzyme in the glycolytic pathway is EhADH2, a dual function enzyme responsible for converting acetyl-CoA to an intermediate acetaldehyde, and then to the final product of ethanol. This enzyme is iron-dependent and the activity of both enzymatic steps of EhADH2 can be enhanced with increasing amounts of Fe$^{2+}$. However, the addition of zinc or phenanthroline into the reaction decreases enzyme activity, as these chemicals acts as chelators of iron. Like 2,2'-pyridyl, when zinc and phenanthroline are added into the culture medium, there is a reduction in trophozoite growth [15].

**Serum Deprivation**

In the *E. histolytica* culture media, TYI-S-33, adult bovine serum is used as a lipid source and is usually supplied as a 10-15% v/v additive [11]. Growing trophozoites in reduced serum alters the cell division cycle and synchronizes approximately 95% of the trophozoites to the G0/G1 phase [16]. It has also been demonstrated that serum starvation alters the expression of transmembrane kinases (TMKs). There are 90 putative TMKs in *E. histolytica* that have been grouped into six families, named A through F [17]. Serum starvation can affect the expression of EhTMKBs. For example, during serum starvation (0.5% v/v), the expression of EhTMKB1-9 decreases and the expression of EhTMKB1-18 increases. The predominant EhTMKB1 gene expressed in proliferating cells is EhTMKB1-9. Downregulation of EhTMKB1-9 slows proliferation, and decreases adhesion and cytopathic activity. Specifically, EhTMKB1-9 expression is reduced from 95% to 47% of
the total EhTMKB1 transcripts identified and sequenced after starvation. Inversely, the transcript of EhTMKB1-18 exhibited an increase in expression during serum starvation, from 4% to 80% of the EhTMKB1 transcripts analyzed, even though this gene is predicted to have no protein product [18]. While individual proteins have been analyzed, no “omics” study has been performed on serum-deprived trophozoites, so global protein or mRNA changes have not been reported.

Heat Shock

*Entamoeba histolytica* trophozoites are found in the host’s colon, where temperatures are approximately 37°C. Upon invasion into the intestinal lining and infection of the liver, the host’s inflammatory response may lead to an increase in temperatures, creating a stressful environment for the parasite. The heat shock response is a highly conserved pathway in many systems [19]. This response includes the upregulation of heat shock proteins, which are used to solubilize, unfolded, and ubiquitinated proteins within the cell. A microarray analysis of *E. histolytica* trophozoites exposed to 42°C for 4 hours revealed a massive down regulation of gene expression, with a subset of proteins being up regulated [20]. These upregulated proteins included heat shock proteins and cysteine proteinases.

Additional studies have examined individual proteins and their expression pattern before, during, and after heat shock. One such protein examined was EhHsp100. This protein is expressed during heat shock or the addition of the drugs 5-Azacytidine, an inhibitor of DNA methyltransferase, and Trichostatin A, an inhibitor of histone deacetylase.
Heat shock proteins are not the only differentially expressed genes during heat shock. EhMLBP, *E. histolytica* methyl-binding protein, has heat shock protein-like features, including a heat shock element in its promoter region. After 20 minutes of heat shock, the expression level of EhMLBP is significantly higher than that in control cultures. Heat shock also changes the localization of EhMLBP from the perinuclear area to a uniform distribution in the nucleus [22] and to cytoplasmic granules [23]. Furthermore, EhMLBP interacted with polyubiquitinated proteins within stress granules, an mRNA-containing cytoplasmic granule that assembles during stress (see section III) [23].

The expression or activation of non-heat shock protein-like genes can also be altered during heat shock in *E. histolytica*. Ehssp1, *E. histolytica* stress-sensitive protein, family one, is expressed by multiple genes in a polymorphic manner. During normal growth conditions, a single copy of the Ehssp1 gene is produced; however, during heat shock, multiple polymorphic copies are expressed in as little as 20 minutes [24]. Another protein that is altered during heat shock is EhMAPK, a mitogen-activated protein kinase. While the mRNA levels of this kinase are unaltered during heat shock, an increase in kinase activation and activity, as measured by phosphorylation, was observed. The activation of EhMAPK was measured by Western blot and showed a 2.6 fold increase in phosphorylated kinase when compared to that in control cells [25].

**Oxidative and Nitrosative Stress**

One method a host may utilize to kill a pathogen is the release of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS disrupt the structure
and function of proteins, nucleic acids, and lipids, and can lead to cell death. However, *E. histolytica* has adapted pathways and systems to combat the stress brought about by ROS and RNS.

Proteins can be post-translationally modified during ROS or RNS. In response to oxidative stress, a specific subset of proteins becomes oxidized to protect the majority of the cell from damage. After incubation with hydrogen peroxide, 154 proteins in *E. histolytica* were identified through mass spectrometry to be oxidized. Many different protein classes were represented in these 154 proteins, including transporters, chaperones, oxidoreductases, kinases, and cytoskeletal proteins, to name a few. One protein group that was found to be significantly oxidized involved in translation such as ribosomal proteins and elongation factors. Global protein synthesis was also decreased during oxidative stress, which may have been the results of changes in the expression of protein translation machinery [26]. Nitrosative stress has been used to investigate the role of S-nitrosylated proteins in *E. histolytica*. Treatment with 500 µM S-nitrosocysteine resulted in 142 proteins being S-nitrosylated. These proteins were categorized into protein translation, protein transport, adhesion, and cell metabolism functions [27].

As during heat shock, EhMAPK’s mRNA levels remained unchanged during oxidative stress. However, different concentrations of hydrogen peroxide resulted in phosphorylation and activation of EhMAPK. A low concentration of H\(_2\)O\(_2\) (0.5 mM) resulted in a 1.8-fold increase in phosphorylated EhMAPK. Conversely, treatment with a higher concentration of H\(_2\)O\(_2\) (2 mM) resulted in a 4-fold decrease in phosphorylated
EhMAPK. The viability of *E. histolytica* grown in 2 mM H$_2$O$_2$ was severely reduced [25]. This suggests that there is a threshold of radical oxygen that results in a stress response; however, beyond that threshold, *E. histolytica* cannot overcome the stress and dies.

Cells can also change gene expression in response to ROS or RNS stress. As discussed earlier, Ehssp1 is a protein that exhibits polymorphic expression during heat shock. A similar expression pattern was observed during exposure to excess oxygen [24]. Microarray analysis of *E. histolytica* cells exposed to 1mM H$_2$O$_2$ for one hour displayed a total of 284 differentially expressed genes: 185 upregulated and 102 downregulated [28]. In the same study, the authors used 200 µM DPTA-NONOate to induce RNS formation. This resulted in a greater number of genes (1,036 total) differentially expressed: 443 upregulated and 593 downregulated. Of the known proteins upregulated in both stress conditions, most were categorized as DNA, protein and lipid repair proteins as well as those involved in signaling and regulatory pathways [28].

An additional microarray analysis of adherent *E. histolytica* cells incubated with the nitric oxide donor sodium nitroprusside (SNP) showed that 365 genes were upregulated and 103 genes were downregulated. Proteins involved in macromolecule binding, oxidoreduction, and glycolytic and hydrolytic reactions were the most differentially expressed genes. During incubation with SNP, the unfolded protein response was not noted, but the endoplasmic reticulum became fragmented and appeared as a vesicle-like structure [29].
Several specific proteins have been analyzed further for their role in virulence during ROS and RNS exposure. EhSIAF (*E. histolytica* stress-induced adhesion factor) and EhPTPA (*E. histolytica* phospholipid transporting P-type ATPase/flippase) were both upregulated during ROS and RNS stress conditions, as expression of these proteins was absent during normal growth conditions. Overexpression of either of these two proteins results in an increased resistance to oxidative stress and an increase in adherence to Chinese hamster ovarian (CHO) cells [30].

Oxidative stress, in combination with the addition of trace amounts of cations to the medium, induces the formation of cyst-like structures (CLS) *in vitro*. These CLSs are phenotypically similar to clinical cysts. For example, CLSs are resistance to detergents such as SDS, Sarkosyl and Triton. They exhibit a rounded morphology and 55% of these structures display an increase in the number of nuclei compared to 10% of control cells not exposed to oxidative and cation stress [31]. This lends support to the notion that encystation is a stress response.

III. Overview of translational control of stress

During stress, it is important for cells to prioritize protein synthesis, so as to not waste energy needed to overcome the stress. One response that leads to an overall decrease in protein synthesis is the phosphorylation of the alpha subunit of the eukaryotic translation initiation factor-2 (eIF2). eIF2 is a multimeric protein composed of three subunits (alpha, beta, and gamma) and is responsible for delivering initiator methionyl-tRNA (Met-tRNAi).
to the 40S ribosomal complex for translation initiation. In its inactive form, eIF2 is bound to GDP. To activate eIF2, this GDP must be exchanged for GTP, a reaction catalyzed by eIF2B. Once bound to GTP, eIF2 is able to bind Met-tRNA\textsubscript{i} and associate with the 40S ribosomal subunit. This association stimulates eIF5 to bind to the complex. eIF5 stimulates an unknown GTPase which hydrosizes the eIF2-bound GTP [32]. This inactive eIF2 disassociates from the ribosomal complex due to a reduction in affinity, and is ready to begin the process again (Fig 2) [33].

To conserve energy during suboptimal growth conditions, the activity of eIF2\textalpha{} is downregulated by phosphorylation. Stress activates eIF2\textalpha{} kinases, which phosphorylate a serine (Ser\textsuperscript{51} in mammalian systems) on eIF2\textalpha{}. This phosphorylation changes eIF2 from a substrate for eIF2B to a competitive inhibitor, keeping eIF2-eIF2B in an inactive complex. This decreases the active eIF2-GTP complexes in the cell, slowing down the initiation of protein translation [32]. The level of phospho-eIF2\textalpha{} required to halt translation can vary from as low as 15% to as high as 60% [34]. The ratio of eIF2\textalpha{}:eIF2B can also alter the overall effect of this phosphorylation of eIF2\textalpha{}, depending on the type of cell, with some cells expressing a 10:1 ratio of eIF2 to eIF2B, but some cells can express much higher levels of eIF2, resulting in a 2:1 ratio [34]. By balancing these ratios, cell can maintain a balance of protein translation and energy conservation. While phosphorylation of eIF2\textalpha{} halts protein translation, it does not directly alter transcription. Newly transcribed mRNAs can become abundant in the cell. If the mRNA is targeted for degradation, it can accumulate in a cytoplasmic granule, known as a P body. Here, the 5’ cap structure on the
In normal conditions, the trimer eIF2 has two forms: GDP bound (inactive) or GTP bound (active). When bound to GTP, eIF2 binds to Met- tRNAᵢ and delivers it to the 43S ribosomal complex for translation initiation. Once Met- tRNAᵢ is unbound to eIF2, the GTP is hydrolyzed by an unknown GTPase; this activity is stimulated by the binding of eIF5 to the complex. To become active again, an exchange factor, eIF2B, must facilitate the exchange of GDP for GTP, beginning the cycle again. During stress, eIF2α kinases become activation, phosphorylating a key serine residue on the eIF2α subunit. This changes the binding properties of the eIF2-GDP complex and becomes a competitive inhibitor for eIF2B. This sequesters the exchange factor, keeping active eIF2-GTP complex levels low. In turn, overall protein synthesis slows as translation cannot be initiated [Reviewed in 32].
mRNA can be removed and the mRNA denatures. Stress granules can also appear during times of low translation rates. These cytoplasmic granules are made of untranslated mRNAs, as well as subset of translation initiation factors, including eIF2. The appearance of stress granules is promoted by eIF2 phosphorylation, indicating their role in sequestering mRNAs during times of low translation rates [35].

To date, 4 families of eIF2α kinases (EIF2AK1-4) have been described: HRI (heme-regulated inhibitor), PKR (protein kinase double-stranded RNA-dependent), PERK/PEK (PKR-like ER kinase), and GCN2 (general control non-derepressible-2). Each family is activated by different stress conditions (Fig 3). While mammalian systems encode for all four families of kinases, other systems may only express a subset of these kinases.

**Heme-regulated Inhibitor (HRI)**

HRI is also known as EIF2AK1 and is expressed within erythrocyte precursors, liver cells, and macrophages. Within the erythrocyte precursors, the kinase works to balance the production of α and β globin to iron levels and inhibit toxic levels of these components. HRI is used to sense intracellular levels of heme through its heme-binding domain; when heme levels are high, this bound heme keeps HRI from forming a catalytically active dimer. During low iron levels, HRI can dimerize and become active, halting protein translation through the phosphorylation of eIF2α [36]. Within the liver, HRI is most notably involved
In mammalian systems, there are four eIF2α kinases: HRI (heme-regulated inhibitor), PKR (protein kinase double-stranded RNA-dependent), PERK/PEK (PKR-like ER kinase), and GCN2 (general control non-derepressible-2). While some activation conditions overlap, some are specific to individual kinases. Above, several of these activation conditions are highlighted [33].
in regulating liver cell translation during heme deficient periods. Within macrophages, HRI assists in forming an inflammatory response, as well as macrophage maturation [33].

Protein Kinase Double-stranded RNA-Dependent (PKR)

PKR is also known as EIF2AK2, and was found to be activated during viral infection. By phosphorylating eIF2α, the cell inhibits the translation of viral mRNA, slowing viral infection and replication. This is due to a double-stranded RNA binding domain found on the N-terminus of the kinase, which can bind to the nucleic acid of invading viruses. PKR expression is induced by interferon, which is usually released by the host in response to a viral invasion. PKR can also become activated during oxidative and ER stress independently of viral infection [33].

Found in the cytosol and the nucleus, PKR is activated after dimerization and autophosphorylation. eIF2α is not the only target for PKR; it can also phosphorylate p53 and activate STAT and NF-Kβ. These additional substrates can change the final fate of the cell, due to their ability to promote apoptosis. The loss or knockdown of PKR within mice does not result in a detectable phenotype during reproduction and normal growth conditions. However, due to the impact viral infections can cause on a system, it’s important to note the possible redundancies in the anti-viral reaction [33].
PKR-like ER Kinase (PERK/PEK)

PERK is also known as PEK or EIF2AK3 and is a transmembrane endoplasmic reticulum protein. The N-terminus is localized to the ER lumen and the C-terminus is found in the cytoplasmic space. During normal growth conditions, immunoglobulin binding protein (BiP) binds to PERK, keeping it in a monomer form within the ER lumen. However, when misfolded proteins begin accumulating within the ER, BiP disassociates from PERK. PERK can then form a homodimer during this unfolded protein response (UPR) and become autophosphorylated to become an active kinase. To halt the synthesis of new proteins during this buildup of non-functional proteins, PERK phosphorylates eIF2α to inhibit translation initiation. While PERK activation allows time for the cell to battle ER stress, constitutive activity by PERK is a pro-death signal, suggesting that long-term ER stress is fatal to a cell. The loss of PERK in humans results in Wolcott-Rallison syndrome (WRS). WRS is an extremely rare autosomal recessive disorder bought on by a mutation the catylatic domain of the PERK gene. Though there are different types of mutations, all resulting PERK proteins are non-functional kinases. General characteristiss of WRS is the development of insulin-dependent diabetes before birth or during infancy, bone dysplasia, and hepatic dysfunction [37]. The molecular mechanisms resulting in the anomalies are unknown [33].
General Control Non-derepressible-2 (GCN2)

GCN2 (or EIF2AK4) acts as a sensor for amino acid and glucose levels. Activation is carried out through the binding of uncharged tRNA to a histidyl tRNA synthetase (HisRs)-related domain within the kinase. Like previously described kinases, dimerization is also required for activation, and autophosphorylation helps stabilize and optimize the kinase’s activity. Currently, the only known substrate of GCN2 is eIF2α. GCN2 can also be activated during viral infection and can bind viral genomic RNA directly through its HisRs domain [33]. In humans, this kinase is predominantly expressed within the brain, where it is hypothesized to protect neural tissue during periods of amino acid starvation. Through unknown feedback mechanisms, GNC2 activation can alter the diet of tested mice, resulting in a selective feeding behavior to alleviate the stress by ingesting more essential amino-acid rich foods [38].

IV. Stress-induced control of protein translation in eukaryotes

*Plasmodium spp.*:

*Plasmodium* is the apicomplexian responsible for the malaria. As of 2015, there are an estimated 214 million cases of malaria worldwide, with the majority of cases occurring in Africa and Southeast Asia. The annual death toll associated with malaria is currently estimated to be 438,000. It is estimated the global cost of controlling malaria infections is roughly $2.5 billion, highlighting the economic burden of this parasite. There are currently
5 species of *Plasmodium* that causes disease in humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*. The onset of symptoms, including chills and fevers, is usually associated with the lysis of host blood cell. The interval of symptoms, as well as molecular diagnostic tests, can determine the species responsible for individual infections; however, co-infections with multiple species is possible. In the past, chloroquine was used as a treatment for malaria infections; contemporary treatments include drugs from the artemisinin class, often in combination with other medications [39].

*Plasmodium* utilizes two hosts during its complex life cycle in order to reproduce and spread: the definitive female *Anopheles* mosquito host and the vertebrate intermediate host. Within the human host, *Plasmodium* cells have two different cycles: the exo-erythrocytic cycle within liver cells and the erythrocytic cycle within red blood cells (Fig 4). In these two cycles, sporozoites will invade liver cells, or schizonts will invade erythroid cells. These stages grow as immature and mature trophozoites, then develop into mature schizonts, that can continue the erythrocytic cycle of cellular invasion. However, in the mosquito host, the dormant stage of the sporozoites is also important in elimination of this parasite, as this stage is what initially enters the blood stream of the human host [40].

*Plasmodium* possesses three putative eIF2α kinases. Several different species of *Plasmodium* were used to assess the functions of these kinases. PfIK1 is a GCN2-like kinase found in the *Plasmodium* asexual human blood stage that becomes activated during amino acid starvation. *Plasmodium* cell lines lacking PfIK1 showed no increase in phospho-eIF2α after amino acid starvation. However, deletion of this kinase did not alter
Within the human host, the cells have two different cycles: the exo-erythrocytic cycle within liver cells and the erythrocytic cycle within red blood cells. During this blood stage, the cell can undergo asexual reproduction or develop into gametocytes for sexual reproduction. These gametocytes are taken up by an *Anopheles* mosquito during a blood meal. Sexual reproduction occurs within the gut of the mosquito to begin the sporogonic cycle. This ends with the sporozoite stage entering the salivary glands. Here, the parasite remains dormant until the mosquito takes a blood meal. The sporozoites are injected into the human host and must survive in the blood stream until they can invade liver cells and continue the life cycle. The dormant stage of the sporozoites allows the parasite to halt protein translation and store energy, as well as mRNA transcripts. Once protein translation begins again within the schizonts in the liver cell, these stored mRNAs will be transcribed to assist with cell development and survival [40]. The eIF2α kinases, highlighted in green, are shown with the life cycle where they are most prominently expressed. Figure is modified from the Center of Disease Control [42]
any developmental stages [41]. The second *Plasmodium* kinase, PK4, is required for completion of the erythrocytic cycle of the parasite, *PK4* cell lines were less able to infect mouse models [40].

PfIK2, the final eIF2 kinase, is expressed predominantly in sporozoites found in the mosquito’s salivary gland and is thought to be life cycle dependent, rather than stress dependent. In PfIK2 knockout strains, eIF2α was not phosphorylated in the sporozoites and the infectivity of this life stage was significantly decreased. Phosphorylation is thought to be necessary to keep transcribed mRNAs from being translated at the wrong life stage. By keeping these mRNAs in stress granules and decreasing global protein translation, *Plasmodium* is prepared for the next life stage while in the salivary gland of the mosquito [43]. This control of latency is key to *Plasmodium*’s initial infection of the human host, as it keeps energy stores high should the parasite have to remain in this life stage for longer periods of time.

Taken together, these studies highlight the importance of eIF2α phosphorylation in *Plasmodium* during its multiple life stages and the unique conditions required for eIF2 kinase activation.

*Toxoplasma gondii*

*Toxoplasma gondii* is the protozoan parasite responsible for toxoplasmosis in humans. Estimates of infected individuals have been as high as 25-30% of the world
population. Most infections occur after the consumption of infected meat, but can also occur after the ingestion of any fecal oocysts (Fig 5). Occasionally, blood transfusions or organ transplantation can introduce *T. gondii* into the human host. The highest disease burden comes from transmission from a mother to a developing fetus. Outcomes can be as severe as miscarriages and abnormalities occurring in fetuses infected during early developmental stages [44].

*T. gondii* has three major life stages that have been studied: the active tachyzoites and the latent tissue bradyzoites, and the latent fecal oocysts (Fig 5). It is the cycling of tachyzoites to bradyzoites in the human host that is of particular interest. *T. gondii* possesses four authentic eIF kinases, named TgIF2K-A-D. TgIF2K-A localizes to the parasite’s endoplasmic reticulum around the nucleus. It is known that inducers of ER stress, such as sodium arsenite and calcium ionophore induce phosphorylation of eIF2α. It is highly likely the kinase responsible for this phosphorylation is TgIF2K-A [47] because of the kinase’s location in the ER. TgIF2K-B is expressed in the cytosol of the parasite; however, the conditions necessary to activate this kinase remains unknown [47].

Two of the *T. gondii* kinases are characterized as being GCN2-like. TgIF2K-C is expressed in the cytosol of tachyzoites. TgIF2K-C knockout lines were not deficient in progressing through the lytic cycle and had similar virulence as wildtype cells. However, if grown in glutamine-free media, knockout lines had decreased levels of phospho-eIF2α [48]. This is consistent with the known function of GCN2 kinases in amino acid sensing.
While the normal life cycle of *T. gondii* includes the definitive feline host and an intermediate rodent or bird host, humans can become infected when the latent oocyst is ingested. These oocyst further develop into tachyzoites inside the host, and can transition into a latent tissue cyst form, called bradyzoites, during stress. Bradyzoites can excyst after the stress has subsided. Tachyzoites can invade macrophages and subsequently lyse the cell, forming the lytic cycle of the parasite. This constant cycling can continue until the death of the host, or the clearance of the parasite by the immune system [45]. eIF2 kinases, in green, are shown with the life cycle where they are most prominently expressed; although, it is currently unknown if TgIF2K-A and TgIF2K-B have a life stage-specific expression pattern. Figure is modified from the Center of Disease Control [46]
TgIF2-K-D, also expressed in the cytosol, is involved in tachyzoite survival outside of host cells. Once outside the macrophage, the cell must keep translation low to save energy until another macrophage can be invaded. By phosphorylating eIF2α, translation decreases by 90%. TgIF2K-D knockout lines could not compete against wildtype lines and were outgrown after extracellular co-incubation in vitro, as determined by PCR analysis using cell-line specific PCR primers [49].

During the cycling between active tachyzoites and latent bradyzoites in the human host, protein synthesis must be altered to save any available energy. Previous studies have shown that phosphorylation of eIF2α is important for controlling latency. For example, latent bradyzoite formation is initiated when dephosphorylation of eIF2α is inhibited with the phosphatase inhibitor, salubrinol [47]. While the eIF2α kinase responsible for this phosphorylation of eIF2α during stage conversion is unknown, these findings indicate the importance of the eIF2α phosphorylation pathway in overall parasite development.

*Leishmania* *spp.*

*Leishmania* is the causative agent of leishmaniasis, which can occur in three forms: visceral, cutaneous, and mucocutaneous. Roughly 1.3 million cases of leishmaniasis occur annually, with up to 50,000 deaths each year attributed to the visceral form. There are 21 morphologically identical species of *Leishmania* that infect mammals. While the promastigote stage is initially injected into the human host by the sandfly vector, within the macrophages, promastigotes develop into amastigotes, where the only known
*Leishmania* eIF2α is activated. *Leishmania major* and *Leishmania infantum* both have a PERK homologue with approximately 30% identity to the human PERK. This kinase is expressed in both the promastigote and amastigote stages of the *Leishmania* life cycle, but is thought to be responsible for the increased phospho-eIF2α levels in the amastigote stage. Localized to the ER, *Leishmania* PERK has been shown to become activated during the unfolded protein response. To study PERK further, a truncated form of PERK was expressed in cells cultured *in vitro*, acting as a dominant negative. The lack of phosphorylation by PERK caused a significant decrease in differentiation to amastigotes. Amastigote-specific genes had a marked delay in expression, indicating a delay in stage transition. These findings were the same in both axenic cultures and within human and mouse macrophage models. While most eIF2α sequences have a key serine residue that becomes phosphorylated under stress, the eIF2α sequence of *Leishmania* has a threonine at the key phosphorylation residue at position 166. This indicates the conservation of the eIF2α pathway and the kinases associated with it [51].

**Non-pathogenic eukaryotes**

The eIF2-based stress pathway is found in other lower eukaryotic organisms. In yeast, the only known eIF2 kinase is Gcn2. In *Saccharomyces cerevisiae*, Gcn2 is used for sensing and responding to amino acid starvation, specifically tryptophan and arginine. Activation of Gcn2 occurs when uncharged tRNA binds to the histidyl-tRNA synthetase-like domain on Gcn2 [52]. Due to the single eIF2 kinase, *S. cerevisiae* is often used to authenticate eIF2 kinase activity in proteins from other organisms. For example, the *Gcn2p*
Transmission of *Leishmania* occurs after the bite of a female sandfly, when promastigotes are injected into the blood stream. This free-living stage is phagocytized by the host’s macrophages and allows for differentiation into amastigotes. These amastigotes divide within the cell and can infect other cells. During a blood meal, the intermediate host, a sandfly, takes up macrophages infected with amastigotes. Within the sandfly’s gut, amastigotes transition back to a promastigote stage that multiply and migrate to the salivary gland of the sandfly. Here, the parasite is ready to be injected into a mammalian host to continue the cycle (Fig 6). The only known eIF2 kinase, PERK is activated during amastigote development and is indicated in green. Figure is adapted from the Center of Disease Control [50].
gene can be disrupted and replaced with cDNAs that encode entire eIF2α kinases [53] or just active sites [40]. If the exogenous kinase is authentic, expression will induce phenotypic changes, such as reduced growth [53].

*Dictyostelium discoideum* is a free living amoeba that feeds on bacteria in the soil but can be grown axenically in the laboratory. During times of starvation, these amoebae will aggregate and form mounds of approximately 100,000 cells. The mounds eventually differentiate to form fruiting bodies consisting of dormant spores supported by stalk cells. *Dictyostelium* has two eIF2 kinases: IfkA and IfkB. During normal development, the level of phospho-eIF2α increase from a slight basal level slight for several hours, and then return back to the initial basal level. IfkA null lines did not exhibit increased phospho-eIF2α during any stage of development. IfkA knockout lines also had a slight growth defect in axenic conditions, but were able to complete the entire life cycle. This suggests that this kinase, and phosphorylation of eIF2α are not essential to development in this system. Interestingly, IfkA knockout lines formed mounds earlier than the parental strain, with mound size much larger than the parental strain due to the misregulation of countin. Countin is a polypeptide secreted by *Dictyostelium* to regulate the number of cells within a particular mound. All attempts to disrupt the IfkB gene were not successfully, indicating that IfkB, a gene found in all life stages, is essential to development and growth. While these two kinases have domains similar to the mammalian GCN2 kinase, neither were found to be responsible for detecting amino acid starvation in the amoeba [54].
V. Summary

The ability to phosphorylation of eIF2α is utilized by many organisms during times of stress or development. This phosphorylation is catalyzed by the eFI2 kinases in each organism. To date, there has not been a study into the role of eIF2α in *Entamoeba histolytica*, despite the many studies on the effects of stress on the parasite (reviewed above). *E. histolytica* and *E. invadens* each possess eIF2α (EHI_005100 and EIN_242170, respectively) and two presumptive eIF2α kinases (eIF2K) (*Entamoeba histolytica*-EHI_109700, EHI_035950; *Entamoeba invadens*- EIN_059080, EIN_0333330) [55, 56]. The phosphorylated serine of eIF2α (see chapter 2) and critical active site lysines of the kinases (data not shown) are conserved. In *E. invadens* expression of one of the kinases is developmentally regulated (EIN_0333330) [55]. Thus, we hypothesize that this genus uses eIF2α-based machinery to control translation during stage conversion. This hypothesis is supported by the pioneering work of Dr. Gordon Bailey showing that encystation was accompanied by the aggregation of ribosomes into structures known as a chromatoid bodies and by a decrease in the incorporation of exogenous amino acids [57, 58]. Chromatoid bodies are an RNA- and ribosome-containing cytoplasmic structure that assembles during encystation. They are reminiscent of stress granules or P bodies, but the fate of RNAs within chromatoid bodies is unknown. Currently, these *Entamoeba* eIF2α kinases have not been authenticated, nor have the conditions that lead to their activation been discerned.
The percent identity of the catalytic domains of the two *E. histolytica* kinases to the human host orthologs is considerably low at approximately 32% for each [56]. Because of this low identity, it may be possible to use these kinases as potential drug targets. This would prove to be especially useful if either kinase was shown to be necessary for parasite growth or cyst development. If cyst production could be halted, spread of infection could be halted, leading to the decline in *E. histolytica* infections. Toward this end, a better understanding of the eIF2-based stress response system in *E. histolytica* is necessary. Therefore, the aims of this study were to:

1. To define the role of the phosphorylation of the alpha subunit of the eukaryotic initiation factor-2 (eIF2α) in *Entamoeba histolytica*

2. To determine if phosphorylation of eIF2α is necessary to counter stress in the *E. histolytica* system
VI. Literature Cited


CHAPTER TWO

PHOSPHORYLATION OF EUKARYOTIC INITIATION FACTOR-2 α REGULATES STRESS IN THE HUMAN PROTOZOA PARASITE

ENTAMOEBA HISTOLYTICA

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I.  Abstract

*Entamoeba histolytica* is a food- and water-borne intestinal parasite responsible for amoebic dysentery and amoebic liver abscess. The life cycle of *E. histolytica* alternates between the host-restricted trophozoite form and the highly infective latent cyst stage that is able to persist in the environment. Throughout its life cycle, which may include invasion of tissues in the human host, the parasite is subjected to a variety of stressful conditions. In other systems, stress can trigger the activation of kinases that phosphorylate a serine residue on eukaryotic translation initiation factor-2α (eIF2α). This modification inhibits the activity of eIF2 resulting in a general decline in protein synthesis, and, paradoxically, an up-regulation of the expression of certain genes that permit the cell to counter the stress. Genomic data reveal that *E. histolytica* possesses eIF2α with a conserved phosphorylable serine at position 59. Thus, this pathogen may have the machinery for stress-induced translational control. To test this, we exposed *E. histolytica* trophozoites to six different stress conditions and assessed viability, as well as the level of total and phospho-*EheIF2α* via Western blot of cell lysates. Long term serum starvation induced an increase in the level of phospho-*EheIF2α*, but no other stress condition caused a significant change. Long term serum starvation also showed a decrease in polyribosome abundance as observed through sucrose gradient ultracentrifugation; this is consistent with the observation that this condition also induces phosphorylation of *EheIF2α*. This suggests that the eIF2α-dependent stress response system is operational in *E. histolytica* and that the system may be activated only by certain stresses. To further examine the role of phosphorylation of *EheIF2α* during stress, three transgenic cell lines were created. *EheIF2α*-S59 over-
expresses wild type eIF2α protein. *Eh*IF2α-S59A expresses eIF2α with the serine-59 residue mutated to an alanine, creating a non-phosphorylatable subunit. *Eh*IF2α-S59D expresses eIF2α with the serine-59 residue mutated to an aspartic acid to mimic a phosphorylated residue. *Eh*IF2α-S59 exhibited a high level of phosphorylation of the exogenous protein, leading to a decreased growth and polyribosome abundance when compared to the control cell line. *Eh*IF2α-S59A had the highest growth rate and retained a high abundance of polyribosome. *Eh*IF2α-S59D exhibited the slowest growth rate and had a decrease in polyribosome when compared to control; however, *Eh*IF2α-S59D did exhibit the highest survival rate in over half the stress conditions tested. This may indicate the protective nature of phosphorylation of *Eh*IF2α during times of stress.

II. Author Summary

*Entamoeba histolytica* is a parasitic pathogen usually found in underdeveloped countries that lack proper water filtration and treatment plants. During the *E. histoltycia* life cycle, active trophozoites can reside in the large intestine, or invade the intestinal wall to cause extraintestinal infection. The parasite encounters demanding growth conditions in the host and must overcome these to survive. In other organisms, stress induces phosphorylation of the alpha subunit of the eukaryotic translation initiation factor 2 (eIF2α). This, in turn, inhibits protein translation allowing the cell to conserve energy. To determine the role of a putative eIF2α in *E. histolytica*, we have applied 6 different stress conditions to active trophozoites. One of these stress conditions, long term serum starvation, causes a significant increase in phosho-*Eh*IF2α levels. Consistent with this observation, long term serum starvation also reduces the abundance of polyribosomes, and important component
of the translational machinery. We have also created three transgenic cell lines to test mutant forms of \textit{E. helicotrichoides} \textit{IF2a}: \textit{E. helicotrichoides} IF2a-S59, \textit{E. helicotrichoides} IF2a-S59A, and \textit{E. helicotrichoides} IF2a-S59D. These cell lines overexpress the wildtype protein, a non-phosphorylatable protein, and a phosphomimetic protein, respectively. Due to the phosphorylation/dephosphorylation nature of the exogenous protein in \textit{E. helicotrichoides} IF2a-S59, both an increase in protein synthesis and a decrease in growth was observed. \textit{E. helicotrichoides} IF2a-S59A exhibited the highest growth, but additional studies are required to fully assess the translation machinery for this cell line. \textit{E. helicotrichoides} IF2a-S59D had the slowest growth and a decreased level of translation. However, \textit{E. helicotrichoides} IF2a-S59D had the highest survival in the majority of test stress conditions, indicating that phosphorylation of \textit{E. helicotrichoides} IF2a can be used to alleviate the cellular pressures attributed to stress.
III. Introduction

*Entamoeba histolytica* is an intestinal parasite that is the causative agent of amebic dysentery and amoebic liver abscesses. It is transmitted by the cyst form of the pathogen in fecally-contaminated food and water, making it prevalent in the developing world where sanitation practices are substandard. There are 173 million people living in regions with untreated water sources and one billion people carry out open defecation practices [1]. Thus, there is considerable risk for transmission of *E. histolytica*. *E. histolytica* is also considered a Class B bioterrorism agent as a water safety threat. These factors make the pursuit of knowledge regarding this parasite significant.

*E. histolytica* is passed from human to human without the utilization of an intermediate host during its life cycle. The parasite’s latent stage, a cyst, is able to withstand the extreme conditions in the external environment as well as the acidic pH of the host stomach. The cyst exits the stomach and enters the small intestine, where unknown triggers cause excystation. The emerging active trophozoites continue down the digestive system until they reach the large intestine, where the parasites divide by binary fission. Trophozoites are also responsible for extraintestinal complications of *E. histolytica* infections, including liver abscess. During infection, which includes invasion of the host intestinal tissue, the parasite may experience stress, in part due to immune pressure from the host. This stress can include heat shock, osmotic shock, nutrient deprivation, and/or exposure to reactive oxygen or nitrogen species, and high oxygen levels. The parasite must elicit a cellular response to counter these stresses and survive.
In many systems, stress is controlled, in part, by the phosphorylation of the alpha subunit of the eukaryotic initiation factor (eIF2α) [2]. Under normal conditions, eIF2α forms a protein complex that, when bound to GTP, delivers Met-tRNAi to the ribosome to initiate translation. Once Met-tRNAi is delivered, the bound GTP is hydrolyzed to GDP. To become reactivated, eIF2-GDP binds to the guanine exchange factor, eIF2B, and the GDP is released, allowing for the binding of GTP to start the cycle again. This is considered the rate-limiting step of translation initiation [reviewed in 2]. During stress, eIF2 kinases become activated and phosphorylate a key serine residue on the eIF2α subunit to generate a phosphorylated form of the protein (phospho-eIF2α). This phosphorylation induces a conformational change in eIF2, causing it to become the competitive inhibitor of eIF2B. This leads to a general decrease in protein translation; however, paradoxically, the expression of a subset of genes is up-regulated. This subset of genes assists the cell in surviving the affront.

In other eukaryotic pathogens, one possible outcome of stress is stage conversion to a latent form. For example, under stress, Toxoplasma gondii will convert from an active tachyzoites form to a latent bradyzoite form. Phosphorylation of eIF2α is necessary for this stage transition [3]. Phospho-eIF2α also regulates the formation of latent sporozoites in Plasmodium spp. [4] and the transition of promastigotes to amastigotes in Leishmania [5]. In non-parasitic organisms, such as yeast [6] and Dictyostelium [7], phosphorylation of eIF2α stimulates the formation of latent spores. Genomic data suggest that E. histolytica and E. invadens, a related reptilian intestinal parasite that can undergo encystation in vitro,
possess the components of this stress-response system [8]. However, the role of eIF2α phosphorylation in the *E. histolytica* stress response has never been characterized.

In this study, we show that phosphorylation of *EheIF2α* occurs in *E. histolytica* in response to one stress condition, namely long term serum-starvation. This is accompanied by a reduction in global protein translation. We also demonstrate that expression of non-phosphorylatable or phosphomimetic forms of *EheIF2α* influences growth, protein translation, and the ability to counter stress. Together, these data support the hypothesis that *E. histolytica* possesses an eIF2α-based stress response system that controls protein translation.

IV. Results

eIF2α possesses conserved amino acid residues around the key phosphorylated serine residue

An alignment of the *E. histolytica* eIF2α (*EheIF2α*) amino acid sequence with that of six different organisms showed that it shared low sequence identity and moderate sequence similarity across the entire protein, even when compared to the factor from other eukaryotic pathogens (Fig 1A and B). The eIF2α with the highest homology to the *EheIF2α* was that from *E. invadens*. Though overall shared homology was low, there was strong sequence identity surrounding the key phosphorylated serine residue, which occurs at amino acid position 59 in *E. histolytica*, (Fig 1C). Thus, this serine residue is likely to be
Figure 2.1: Alignment of the eukaryotic translation initiation factor 2, alpha subunit

Protein identity (A) and similarity (B) matrices were generated using BLOSUM 62 algorithm and Protein Blast. (C) The amino acids around the key serine residue, occurring at position 59 in *E. histolytica* were aligned using a Standard Protein BLAST. The key serine residue that becomes phosphorylated is indicated by shading. Fully conserved residues are noted by an asterisk (*) below the residues. Residues showing strongly similar properties are indicated by a colon (:). Amino acid sequences were identified using UniProtKB; UniProtKB accession number identified. Eh, *Entamoeba histolytica* (accession no. C4M0A4); Ei, *E. invadens* (accession no. S0AZW3); Tg, *Toxoplasma gondii* (accession no. S8GC56); Pf, *Plasmodium falciparum* (accession no. Q8IBH7); Sc, *Saccharomyces cerevisiae* (accession no. P20459); Dm, *Drosophila melanogaster* (accession no. P41374); Hs, *Homo sapiens* (accession no. P05198)
the residue phosphorylated during stress. Conservation around this residue also suggests that the machinery for an eIF2α-based stress-response system is present in *E. histolytica*.

**Serum-starvation elicits an increase in the level of phospho-eIF2α**

To determine if the level of phospho-eIF2α changes during stress, cells were exposed to a variety of stress conditions: short and long term serum starvation [9], short and long term heat shock [10], glucose deprivation [11], and oxidative stress [12]. Viability was assessed and the level of total and phospho-eIF2α was tracked using Western blotting. Long term serum starvation and oxidative stress resulted in significant cell death (Fig 2). While the ratio of total eIF2α to actin showed some changes, none were significant (Fig 3A). There was a basal level of phosphorylated *Eh*IF2α in control unstressed trophozoites; however, only trophozoites that were serum-starved for 24 h exhibited enhanced phosphorylation of *Eh*IF2α compared to unstressed control cells (Fig 3B). This was not simply due to cell death, as oxidative stress, which was accompanied by a similar reduction in viability, did not result in a significant increase in phospho-*Eh*IF2α. Although cells that experienced long-term heat shock also exhibited an increase in phospho-*Eh*IF2α, the increase was not statistically significant. These data suggest that an eIF2α-based stress-specific response system exists in *E. histolytica* and is activated in a stress-specific manner.
Figure 2.2: Viability of trophozoites during stress

Log-phase trophozoites were incubated in a variety of stress conditions as described in the text. Cells were collected by centrifugation and live/dead cells were enumerated via microscopy and Trypan blue exclusion. Percent viability was determined using the ratio of live cells to the total cell population counted. The data represent the mean (± standard error) for at least three separate trials. *** indicates a highly significant result ($P<0.001$).
Figure 2.3: Western blot analysis of phospho-eIF2α and total eIF2α levels in control and stressed cells

Control or stressed cells were subjected to Western blot analysis using antibodies specific for actin, total eIF2α, phospho-eIF2α. The proportion of (A) total eIF2α to actin or (B) phospho-eIF2α to total eIF2α was determined by scanning densitometry and image analysis (Image J, NCBI). The data represent the mean (± standard error) for at least three separate trials. * indicates a significant result ($P < 0.05$). Only long term serum starvation induced a statistically significant (>2-fold) increase in the level of phospho-eIF2α.
Reduced protein translation accompanies phosphorylation of eIF2α

In other systems, eIF2α-based control of stress is accompanied by a reduction in global protein translation [3]. Therefore, we examined global protein translation by characterizing the abundance of polyribosomes using sucrose gradient ultracentrifugation after long term serum-starvation, or after glucose starvation. Serum-starvation, which induces phosphorylation of \(Eh\)eIF2α, resulted in a significant reduction in dense polyribosomes and an increase in free ribosomes and monosomes (Fig 4A and B). On the other hand, parasites subjected to glucose starvation, which does not induce phosphorylation of \(Eh\)eIF2α, did not exhibit a decrease in dense polyribosome-bound transcripts (Fig 4C). These results support the premise that \textit{E. histolytica} possesses an eIF2α-based stress response system that reduces protein translation.

Expression of mutant eIF2α alters global protein translation and growth

To further examine the function of phospho-\(Eh\)eIF2α, we generated cell lines that conditionally overexpress non-phosphorylatable or phosphomimetic forms of \(Eh\)eIF2α. The cDNA encoding \(Eh\)eIF2α was mutagenized in two ways using PCR. The codon for serine (S) at position 59 was changed to that of alanine (A) or aspartic acid (D) to produce non-phosphorylatable \((Eh\)eIF2α-S59A) or phosphomimetic \((Eh\)eIF2α-S59D) forms of \(Eh\)eIF2α, respectively [13]. To distinguish exogenous \(Eh\)eIF2α from the endogenous form, the PCR product was modified to include a FLAG epitope peptide sequence \((DYKDDDDK)\) [14] followed by a 5-glycine flexible region at the N-terminus.
Figure 2.4: Polyribosome abundance in control and stressed cells

Total RNA from control (A), serum-starved (B), or glucose-starved (C) cells were resolved by sucrose gradient (15-45%) ultracentrifugation, separating free ribosomes and monosomes into the lightest of fractions, from the polysomes in the densest fractions. Fractions were taken and analyzed by UV spectrometry (254 nm). Representative profiles of at least 3 separate trials are shown. Only long term serum starvation led to a decrease in large polysome abundance.
Modified PCR products were inserted into the *E. histolytica* expression vector pGIR209, which confers G418 (neomycin) resistance and allows for tetracycline-inducible expression of exogenous genes when introduced into trophozoites [15]. A standard electroporation protocol [16] was utilized to introduce the expression vector into trophozoites which had been previously transfected with an additional plasmid, pGIR308. This partner plasmid encodes the tetracycline repressor protein, which is necessary for tetracycline inducibility. Authentic transfection was confirmed by purification and sequencing of the episomal expression plasmids from stably transfected cell lines [17]. 

A previously established cell line that conditionally expresses an irrelevant protein, luciferase, was used as a control [15]. Expression of exogenous proteins was induced by the addition of 5 µg mL⁻¹ tetracycline to the culture medium for a minimum of 24 h. Western blot analysis using anti-FLAG and anti-*Eh* eIF2α showed successful induction of the protein with little to no expression of the exogenous proteins prior to the addition of tetracycline (Fig 5). Interestingly, the *Eh* eIF2α-S59 exhibited a high level of phosphorylation of the exogenous protein. This result suggests that the FLAG-tagged variant is functional.

To confirm that the exogenously expressed *Eh* eIF2α variants were functional, we monitored polyribosome abundance in the transgenic cell lines after 24 or 72 h of tetracycline induction. After 24 h of induction, there was no change in polyribosome abundance (Fig S1). However, after 72 h of induction, polyribosome abundance remained high in the control 209-Luc cell line (Fig 6A). While high density polysomes were present
Figure 2.5: Western blot analysis confirming exogenous protein expression in mutant cell lines

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Trophozoites were transfected with plasmids encoding luciferase (209-Luc), wildtype eIF2\(\alpha\) \((E_{h}eIF2\alpha\)-S59\), the non-phosphorylatable variant \((E_{h}eIF2\alpha\)-S59A\), and the phosphomimetic variant \((E_{h}eIF2\alpha\)-S59D\). Protein expression was induced using 5 \(\mu g\) mL\(^{-1}\) tetracycline for 24 hours. Western blots of cell lysates were performed using antibodies specific for total, phspho-\(E_{h}eIF2\alpha\), the FLAG tag, or luciferase.
RNA was isolated from the four transgenetic cell line after incubation in 5µg/mL tetracycline for 72 h: the control cell line 209-Luc (A), the cell line overexpressing EhelIF2α (B), the cell expressing the non-phosphorylable form of EhelIF2α (C), and the cell line expressing the phosphomimetic form of EhelIF2α (D). The RNA was resolved by sucrose gradient (15-45%) ultracentrifugation, separating free ribosomes and monosomes into the lightest of fractions, with the polysomes in the densest fractions. Fractions were taken and analyzed by UV spectrometry (254 nm). Representative profiles that show the general data trend of at least 3 separate trials are shown for 209-Luc, EhelIF2α-S59, and EhelIF2α-S59D. n=1 for EhelIF2α-S59A.
in the cells expression *Eh*IF2α-S59, the peaks were not as well defined as those in the control cell line (Fig 6B). This may indicate a reduction in the abundance of polysomes, or a greater variety of different messages in the process of being translated. The former would be consistent with the higher level of phosphorylation of this cell line. This would be consistent with the high level of phosphorylation of the exogenous protein (Fig 5). *Eh*IF2α-S59A retained a high level of polysomes after 72 h of induction. However, this result requires verification, due to the low sample number (n=1; Fig 6C). *Eh*IF2α-S59D did not have any defined polypeptide peaks after 72 h induction (Fig 6D). Given that the phosphorylation of eIF2α down-regulates translation, it was not surprising to see a decrease in polyribosome abundance in the *Eh*IF2α-S59D cell line. Together, these data suggest that the exogenous proteins are functional. Interestingly, the data also show that although expression of exogenous protein is detectible at 24 h, it takes longer to detect any protein translation phenotype.

Since polyribosome profiling provides a snapshot of protein translation at the mRNA level, we wanted to confirm the alterations in translation in the mutants at the protein level. Therefore, we used a second method known as **SUrface SEnsing of Translation** (SUnSET). This non-isotopic technique uses anti-puromycin antibody for the immunological detection of puromycin-labelled proteins [18, 19, 20]. When added to live cell cultures, puromycin, a tyrosyl-tRNA analog, becomes incorporated into actively translating proteins. Subsequent Western blot analysis of whole cell lysates with anti-puromycin antibody reveals the extent of active protein translation in the cell.
To determine if SUnSET could be used to assess protein translation in *E. histolytica* in our hands, wildtype cells were incubated with puromycin before or after incubation with cycloheximide, a protein translation inhibitor. Western blotting using anti-puromycin antibody revealed that puromycin was readily incorporated into proteins in *E. histolytica* (Fig 7A). The incorporation was specific since there was minimal background staining of lysates from cells that were not treated with puromycin or from cells that were first treated with cycloheximide (Fig 7A).

Protein expression was induced in the transgenic cell lines and then they were subjected to SUnSET analysis. The overexpressing *Eh*elF2α-S59 cell line exhibited the highest incorporation of puromycin among the tested cell lines (Fig 7B). Since the *Eh*elF2α variant expressed in this cell line is wildtype in nature and can be phosphorylated or dephosphorylated, the increased translation may indicate a decreased pressure on the rate limiting step of protein translation. An increase in this initiation factor could, in theory, enhance protein translation. Consistent with the polyribosome profile (Fig 6), the incorporation of puromycin into the cell line expressing *Eh*elF2α-S59A was similar to that of the control cell line. Transgenic cells expressing *Eh*elF2α-S59D exhibited the lowest incorporation of puromycin. This supports the predicted function of the *Eh*elF2α-S59D as a phosphomimetic form of *Eh*elF2α, as both the polysome profile and the SUnSET analysis show a low level of protein translation.

To assess growth and viability in the transgenic cell lines, we generated a growth curve after inducing protein synthesis. Cells expressing luciferase (control) and *Eh*elF2α-S59A exhibited a similar rate of growth (Fig 8). This was not surprising since protein
Figure 2.7: SUnSET analysis of active protein translation in control and mutant cell lines

(A) Wildtype cells were incubated in normal growth medium with 100 µg mL\(^{-1}\) cycloheximide (Cyclo), 10 µg mL\(^{-1}\) puromycin (Puro), or both (Cyclo + Puro). Cell lysates were subjected to SDS-PAGE and Western blotting using antibody specific for puromycin. Trophozoites readily incorporated puromycin into proteins (Puro). The incorporation was authentic given that it was blocked by cycloheximide treatment (Cyclo + Puro). This suggests that SUnSET can be used to assess protein translation in E. histolytica. (B) Protein expression was induced in the four transgenic cell lines by tetracycline for 72 h and SUnSET was performed. Cell overexpressing the wildtype \(Eh\text{eIF2}\alpha\) showed the highest level of protein translation over the incubation period. As expected, cells expressing the phosphomimetic protein (\(Eh\text{eIF2}\alpha\)-S59D) had the lowest amount of proteins synthesized, corresponding to the proposed shutdown in translation machinery. Data represent at least 3 separate trials. Protein loads were verified by total protein staining samples with Coomassie Blue (lower panels).
Exogenous proteins expression was induced by 24 hour incubation with 5 µg ml\(^{-1}\) prior. An original inoculum of 5x10\(^4\) cells was seeded into T-75 flasks in the presence of 5 µg ml\(^{-1}\) tetracycline. Cells were counted every 24 hours until the experiment was terminated after 72 hours. (A) Average cell count for each cell line, showing general trends. (B) Average cell counts and standard error bars for each individual cell line. Data represents the mean (± standard error) for at least three separate trials.
translation was seemingly unchanged in the *EheIF2α*-S59A-expressing cell line. Cells expressing the phosphomimetic form of eIF2α, *EheIF2α*-S59D exhibited a dramatic decrease in doubling time compared to the other cell lines (Fig 8). Cells overexpressing wildtype eIF2α, *EheIF2α*-S59, also grew more slowly compared to control (Fig 8). Increased protein translation could account for this slower growth due to the depletion of ATP and oxidative stress. Indeed, this is the case in mammalian cells that have increased protein translation [21].

**Expression of mutant eIF2α alters viability during stress**

The mutant cell lines were then assessed for their ability to survive the various stress conditions applied previously. After 24 h of induction, there was no statistical difference in survivability among the cell lines (Fig 9A). This is not surprising given that there was no protein translation phenotype at 24 hours (Fig S1). However, when serum starvation was carried out to 48 h, a distinct difference in viability was observed (Fig 9B). Cells expressing *EheIF2α*-S59D were better than the other cell lines at surviving this stress condition. Given that this variant is the phosphomimetic, it is possible that this cell line was pre-conditioned to handle stress.
Cell lines were exposed to various stress conditions for the determined time (see methods). (A) Cell viability after an initial 24 hours of induction with 5 µg ml\(^{-1}\) tetracycline, followed by the incubation in the control or stress condition (see Materials and Methods). The cell line expressing *Eh*IF2αS59D exhibited slightly higher viability after serum starvation, short term heat shock, and long term heat shock but these increases in viability were not statistically significant.  

(B) Protein expression was induced in the transgenic cells for 24 hours and then the cells were exposed to 48 hour serum starvation. The cell line expressing *Eh*IF2αS59D exhibited the highest viability.  

Data represents the mean (± standard error) for at least three separate trials. While no condition resulted in a significant difference, in four of the seven total tested conditions, the cell line expressing the phosphomimetic form of *Eh*IF2α had the highest survival rate (*Eh*IF2α-S59D).
V. Discussion

This study is the first to demonstrate that stress can induce phosphorylation of eIF2α in *E. histolytica*. Specifically, eIF2α was phosphorylated and protein translation was down-regulated as a result of long-term serum starvation. Transgenic cell lines expressing a phosphomimetic form of eIF2α exhibited higher viability during long-term serum-starvation than cell lines expressing wildtype eIF2α, a non-phosphorylatable mutant of eIF2α, or an irrelevant control protein, luciferase. This suggests that phosphorylation of eIF2α is beneficial to *E. histolytica* survival during stress.

The eIF2α response to stress appears to be specific to the condition applied since phospho-eIF2α did not increase in other presumptive states of stress including short-term serum starvation, heat shock, glucose deprivation or oxidative stress. We also cannot rule out the possibility that combinations of stresses could fully activate the eIF2α kinases as in other systems; for example, *Leishmania* requires the application of both heat shock and alkaline stress to significantly phosphorylate eIF2α [5].

Analysis of *E. histolytica* genome data suggests that this pathogen possess other components of this stress response system. There are putative homologs for eIF2β (EHI_153480) and eIF2γ (EHI_132880). Furthermore, *E. histolytica* possesses two presumptive eIF2α kinases (eIF2K) (EHI_109700, EHI_035950) [8]. Currently, these *E. histolytica* eIF2α kinases have not been authenticated, nor have the conditions that lead to their activation been discerned. Nonetheless, the occurrence of each of the three subunits of eIF2 as well as kinases in genome sequences indicates that this translation factor has a
conserved role in this pathogen in delivering Met-tRNA\textsubscript{i}s to the translation machinery. In support of this, cells expressing the phosphomimetic variant of EheIF2\textalpha\ exhibited reduced polyribosome abundance. Furthermore, long-term serum starvation, a condition that induces phosphorylation of eIF2\textalpha, also reduced polyribosome abundance. To date, there have been no proteomic or microarray analyses in serum-starved \textit{E. histolytica} cells. Therefore, the types of proteins that are downregulated during the stress remain to be seen.

We did not observe an increase in the level of phospho-eIF2\textalpha as a result of oxidative stress. However, others have shown that protein translation is inhibited in this condition in \textit{E. histolytica}. Using SUnSET, Shahi \textit{et al.} (2016) demonstrated that exposure to 2.5 mM H\textsubscript{2}O\textsubscript{2} for 15 minutes was sufficient to strongly inhibit incorporation of puromycin. This observations suggests that down-regulation of protein synthesis during oxidative stress must occur in an eIF2\textalpha-independent fashion. Interestingly, Shahi \textit{et al.} also observed that the components of the parasite’s translation machinery, such as ribosomal proteins and elongation factors, were oxidized as a result of H\textsubscript{2}O\textsubscript{2} exposure [19]. Although, this could have contributed to the stress-induced inhibition of protein synthesis, a recent report suggests that a more likely mechanism is global, enzymatic down-regulation of almost all tRNA species [22]. Importantly, the concentration of H\textsubscript{2}O\textsubscript{2} use by Shahi \textit{et al.} was 5-fold higher than the concentration used in the current study. Thus, we cannot rule out the possibility that eIF2\textalpha could become phosphorylated in higher concentrations of H\textsubscript{2}O\textsubscript{2}.

In the current study, the expression of wild-type and mutant forms of eIF2\textalpha was exogenous. Due to polyploidy, methods for homologous recombination and gene replacement are not yet available for \textit{E. histolytica}. As such, endogenous eIF2\textalpha was
present. Despite this limitation, we observed phenotypes in the transgenic cell lines. For example, in cells expressing the phosphomimetic version of eIF2α, we observed a reduction in polyribosome abundance. This phenotype is expected given the known function of phospho-eIF2α in reduction of translation initiation. This suggests that the exogenous proteins are functional and studies of these transgenic cell lines are valuable.

Currently, it is unknown if the level of phospho-eIF2α increases during encystation of *E. histolytica*. It is also unknown if phosphorylation of eIF2α is sufficient to induce stage conversion to the cyst form. However, eIF2-based systems are widely used in eukaryotes for the conversion to latent or dormant forms. Phosphorylation of eIF2α is responsible, in part, for stage conversion in *Toxoplasma gondii* [3], *Plasmodium* spp. [4] *Leishmania* spp. [23], yeast [6], and *Dictyostelium discoideum* [7].

Several lines of evidence suggest that an eIF2-based system may play a role in stage conversion in *E. histolytica*. First, encystation is believed to be a stress-response. Second, encystation is accompanied by the aggregation of ribosomes into structures known as a chromatoid bodies and by a decrease in the incorporation of exogenous amino acids [24, 25]. Chromotoid bodies are RNA- and ribosomal-containing cytoplasmic granules that arise during stress. They are reminiscent of stress granules which accumulate in an eIF2-dependent manner in other systems [26]. Third, *E. invadens*, the encystation model, possesses a single eIF2α (EIN_242170) and two kinases (EIN_059080, EIN_0333330). One of the kinases (EIN_0333330) is developmentally regulated [27]. Specifically, there is a statistically significant increase in transcript at 24 h of encystation making it a candidate for regulating the levels of phospho-eIF2α during stage conversion. Further studies with
the model organism may shed light on the role of eIF2-based control of protein translation during encystation in *E. histolytica*.

In summary, this study demonstrates that an eIF2-based stress response pathway of phospho-eIF2α is functional in *E. histolytica*. *E. histolytica* continues to infect thousands of individuals worldwide every year and there is a desperate need for new drugs. Given the importance of the stress response system in *E. histolytica* survival, components of this pathway may one day serve as targets for novel therapies.

**VI. Materials and Methods**

**Alignment of eIF2a protein sequences**

Amino acid sequences of eIF2α for 7 model species were aligned individually to the *Entamoeba histolytica* eIF2α to examine sequences surrounding the key serine residue. The sequences were also analyzed using a Standard Protein BLAST v 2.3.1 (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) and the BIOSUM 62 algorithm for a “positive” or similarity score, as well as an identity score to form a similarity and an identity matrix.

**Cell culture and stress**

*Entamoeba histolytica* (strain HM-1:IMSS) was cultured axenically in TYI-S-33 medium in 15 mL glass screw cap tubes at 37°C [28]. Cells were passaged into fresh media every 72 to 96 h.
Log-growth trophozoites were incubated on ice for 10 min to release the cells from the glass surface. Centrifugation was performed at 500 x g for 5 min to pellet cells. The cell pellet was resuspended in the appropriate stress medium as follows and incubated at 37°C unless otherwise noted. To induce short term serum starvation, cells were cultured in TYI-S-33 medium without the addition of adult bovine serum, penicillin-streptomycin, and Diamond’s Vitamins for 1 h prior to analysis. To induce long term serum starvation, cells were incubated in the same medium for 24 h prior to analysis [9]. Short term heat shock was induced by incubating trophozoites in complete growth medium in a 42°C water bath for 4 h [10]. To assess long term heat shock, cells were incubated at 39°C for 24 h. To induce glucose deprivation, trophozoites were incubated for 12 h in TYI-S-33 medium without glucose [11]. To induce oxidative stress, 500 µM hydrogen peroxide (Fisher Scientific) was added to the normal TYI-S-33 medium and incubated for 45 min [12]. Viability was determined with microscopy using Trypan Blue exclusion (VWR).

**Antibody development**

Antibodies were developed in rabbits against the synthetic phosphorylated polypeptide- ILMSEL(pS)KRRFRS and against the unphosphorylated polypeptide EMGTYVALKEYDDIQGMIP targeting the phosphorylated and total eIF2α levels, respectively (Pierce Biotechnology, Inc., Rockford, IL, USA). These antibodies purified by ELISA and confirmed against the synthetic polypeptide used in the initial immunization.
Western Blot of total and phospho-eIF2α

SDS-PAGE and Western blot analysis were performed as described previously [29]. Stressed and unstressed trophozoites (5×10⁴) were collected by centrifugation and resuspended in NuPage LDS buffer (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Samples were then heated for 10 minutes at 70°C and loaded onto a precast 12% Bis-Tris polyacrylamide gel (Life Technologies, Carlsbad, CA, USA). The gel was electrophoresed at 200V and the separated proteins were transferred to a polyvinylidene difluoride membrane (PVDF; Invitrogen, CA, USA) for 1.5 hours at 12V in Towbin buffer. The membrane was then blotted with 5% w/v powdered milk in TBS/0.5% Tween 20 solution (50mM Tris, 150mM NaCl) for 30 minutes at 37°C. Total and phospho-eIF2α antibodies were used at a dilution of 1:1000. To control for loading, mouse anti-actin commercial antibodies were used at a dilution of 1:5000 (Abcam, Cambridge, MA, USA). The membrane was incubated in primary antibody overnight at 4°C, followed by extensive washes in TBS/0.05% Tween 20. The blots were then incubated for 1 hour at 20-22°C with commercially available secondary antibody: peroxidase-conjugated goat anti-rabbit (dilution factor 1:10,000) or anti-mouse (1:5000) (Fisher Scientific, Fair Lawn, NJ, USA). After washing with a TS/0.05% Tween 20 the membrane was developed using the Enhanced ChemiLuminescence Western blotting detection system (Thermo Scientific, Hercules, CA, USA) according to the manufacturer's instructions. Protein was quantified by scanning densitometry (ImageJ, version 1.47, National Institute of Health, USA) and normalized to the actin load control.
Polyribosome analysis of stress conditions

To halt protein translation, stressed and unstressed E. histolytica trophozoites were treated with cycloheximide (100 µg mL⁻¹) for 10 min at 37°C. Cells were collected by centrifugation (500 x g for 5 min at 4°C). The cell pellet was suspended with cold 1X PBS buffer, washed and resuspended in Breaking/Polysome Buffer (BPB) (10mM Tris-HCl (pH 7.4), 300 mM KCl, 10 mM MgCl₂, 1% (v/v) Triton-X-100, 2 mM DTT, 1 mg/ml heparin, 50 µg/ml cycloheximide, and 0.04 units/μl RNase Out) in the presence of protease inhibitors. Lysis was achieved by passing cells twice through a 27 gauge syringe needle. Lysates were clarified by centrifugation at 14,000 × g for 5 min. Samples were loaded onto a 15–45% sucrose gradient in BPB without RNase Out, heparin, DTT or Triton as described previously [30, 31]. Ultracentrifugation was performed at 230,000 x g for 2 h. Gradients were fractionated and the fractions were analyzed for polyribosome abundance by spectrophotometry (254 nm) and the absorbance was corrected for cell count.

Mutagenesis of eIF2α and transfection of E. histolytica trophozoites

The E. histolytica eIF2α gene is predicted to be intron-less. Therefore, genomic DNA was purified (Wizard Genomic DNA Purification Kit, Promega) and the Eh eIF2α gene was isolated by PCR. During PCR, nucleotides encoding a BglII restriction site, a FLAG tag [14], and a 5-glycine flexible region were added to the 5’ end of the gene and nucleotides encoding SalI were added to the 3’ end of the gene.

Site-directed mutagenesis of the codon for serine (S) (TCA; amino acid position 59) to the codon for alanine (A) (GCA) or aspartic acid (D) (GAT) was carried out using a
PCR-based protocol using the QuikChange Kit (Stratagene) according to manufacturer’s instructions. Successful mutagenesis was confirmed by sequencing. Wildtype and mutated \textit{Eh}eIF2\textalpha{} coding sequences were digested with BglIII and SalI and ligated into the \textit{Entamoeba histolytica} expression vector, pGIR209 [15] (gift of Dr. W. A. Petri, University of Virginia, Charlottesville, VA), which had been digested with BglIII and SalI. This vector allows for the inducible expression of exogenous proteins via the addition of tetracycline to the medium and is co-transfected with a second vector, pGIR308, which encodes the tetracycline repressor.

Exponentially growing trophozoites of \textit{E. histolytica}, harboring pGIR308, were transfected with the engineered pGIR209 vector as described [16]. As a control, amoebae were also transfected with pGIR209 containing the gene encoding luciferase [15]. Transfected amoebae were maintained by adding 6 \(\mu\)g mL\(^{-1}\) G418 (pGIR209) and 15 \(\mu\)g mL\(^{-1}\) hygromycin (pGIR308) selection agents to the medium.

Mutant eIF2\textalpha{} expression was induced by the addition of 5 \(\mu\)g mL\(^{-1}\) tetracycline to the culture medium for 24 to 72 h prior to all studies and confirmed by Western blotting was performed as described [29] with rabbit anti-luciferase (Invitrogen), anti-FLAG (Sigma), anti-eIF2\textalpha{} and anti-phospho-eIF2\textalpha{} at dilutions of 1:2500, 1:5000, 1:1000, or 1:1000, respectively.

Polyribosome analysis, as described above was used to assess the polysome profile of the mutant cell lines after 24 h or 72 h post induction of exogenous protein translation.
SUnSET

SUnSET analysis has been previously used to assess translational machinery in *E. histolytica* [19, 20]. To determine if SUnSET could be used to assess protein translation in *E. histolytica* in our hands, we assessed the incorporation of puromycin into wildtype trophozoites. Cells (2x10⁶) were incubated with 10 µg mL⁻¹ puromycin (Sigma-Aldrich) for 15 min before or after incubation with 100 µg mL⁻¹ cycloheximide for 10 min. All incubations were held at 37°C. Cells were then pelleted and proteins were precipitated using 20% (v/v) TCA and incubating on ice for 10 min. Proteins were isolated via centrifugation at 2200 x g for 5 min and washed with 5% (v/v) TCA. The protein pellet was resuspended in 2X SDS running buffer and incubated in boiling water for 10 min (Ref Works 118). The lysate was frozen at -80°C until analyzed via Western blot as described above. Mouse anti-puromycin monoclonal antibodies (Sigma-Aldrich) were used at a 1:2500 dilution. As a loading control, samples were stained with Bio-safe Coomassie (Bio-Rad Laboratories). The protocol was repeated in the transgenic cell lines after a 72 h induction period.

Growth assays and viability studies of mutant cell lines

Cells (5x10⁴) expressing luciferase, *EheIF2α*-S59, *EheIF2α*-S59A, or *EheIF2α*-S59D were seeded into 15 mL glass screw cap tubes with TYI-S-33 media with selection agents and tetracycline. At 24 hour intervals, cells were enumerated by microscopy with Trypan exclusion.
The viability of the four mutant cell lines was also assessed as described above. An additional stress of 48 h incubation without Diamond’s Vitamins, PenStrep, or adult bovine serum, was also used to assess any viability differences.

**Statistical analysis**

All values are given as means ± standard error of at least 3 trials. To compare means, 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 0.001 were considered highly statistically significant, while $P$ values of less than 0.01 or 0.05 were considered statistically significant.
Supplemental Fig 2.S1: Polyribosome profiles for mutant eIF2α cell lines after 24 h induction

Ribosomes were isolated from four different cell lines after incubating with 5µg/mL tetracycline for 24 h: the control cell line 209-Luc (A), the cell line overexpressing *Eh* IF2α (B), the cell expressing the non-phosphorylatable form of *Eh* IF2α (C), and the cell line expressing the phosphomimetic form of *Eh* IF2α (D). These isolates were purified using sucrose gradient (15-45%) ultracentrifugation, separating free ribosomes and monosomes into the lightest of fractions, with the polysomes in the densest fractions. Fractions were taken and analyzed by spectrometry (254 nm). Representative profiles of at least 3 separate trials are shown. All cell lines exhibited a high level of free ribosomes and monosomes. However, all cells still had multiple ribosomes attached to mRNAs, as indicated by the increased absorbance in higher sucrose fractions. As no growth phenotype was observed after a 24 h induction, the induction time was increased to 72 h (see Fig 6).
VIII. Acknowledgements

The authors would like to thank the Temesvari lab for support and assistance. We also acknowledge the Eukaryotic Pathogens Innovations Center and its members for helpful assistance and discussion. A special thanks to Dr. W. A. Petri at the University of Virginia, Charlottesville, VA for the tetracycline inducible vectors pGIR308/pGIR209.

IX. Literature Cited


