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Phosphorylation of Eukaryotic Initiation Factor 2-α in Response to Endoplasmic Reticulum and Nitrosative Stress in the human protozoan parasite, *Entamoeba histolytica*

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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 protein translation, which is regulated by phosphorylation of eukaryotic initiation factor (eIF-2α). Previous work has demonstrated that phosphorylation of the *E. histolytica* eIF-2α (*Eh*eIF-2α) increases significantly when exposed to long-term serum starvation, oxidative stress, and long-term heat shock. However, the effects of reagents that are known to induce nitrosative or endoplasmic reticulum (ER) stresses, on *Eh*eIF2α 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 known to induce nitrosative stress (DPTA-NONOate and SNP) or ER stress (BFA and DTT). We examined the morphology of the ER, tracked phosphorylation of *Eh*eIF-2α, and assessed protein translation in 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 eIF-2α were also highly sensitive to DTT-stress. Since protein translation decreased in the absence of phosphorylation of eIF-2α (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.*

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Keywords

Entamoeba histolytica; nitrosative stress; ER stress; eukaryotic initiation factor-2 alpha; translational control

1. 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 form. *E. histolytica* cysts are transmitted via fecally-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. Thus, there is considerable risk for the spread of this disease (Grebmer *et al.*, 2015). Globally, more than 50 million people become infected with the parasite, with over 100,000 deaths annually (Gunther *et al.*, 2011).

E. histolytica is ingested as a latent cyst and travels through the digestive system until unknown cues trigger the excystation of 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 (Hendrick *et al.*, 2016). In addition, increased protein demand caused by physiological and pathological stressors could induce endoplasmic reticulum (ER) stress (Oslowski and Urano, 2013). To be a successful parasite, *E. histolytica* must be able to counter these various stressful conditions. Understanding the stress response of *E. histolytica* may uncover new targets 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* (Hendrick, *et al.*, 2016) and other systems such as *Toxoplasma gondii* (Joyce *et al.*, 2013), *Plasmodium* (Zhang *et al.*, 2012), and *Leishmania* (Cloutier *et al.*, 2012). 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 a conserved serine residue 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, while simultaneously up-regulating a small subset of stress specific genes that aid in the stress response (Wek and Cavener, 2007; Hendrick *et al.*, 2016).

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E. histolytica experiences both oxidative stress and nitrosative stress in the human host. Reactive nitrogen species (RNS) and reactive oxygen species (ROS) are released by neutrophils and macrophages as an immune defense response (Nagaraja and Ankri, 2018). Both RNS and ROS attack cellular components, such as proteins, lipids, and nucleic acids, inducing nitrosative and oxidative stress in invading pathogens. *E. histolytica* has detoxification enzymes and repair systems that cope with both reactive species in a similar fashion (Vicente *et al.*, 2009). In the human host, *E. histolytica* may also encounter physiological stressors that are known to induce endoplasmic reticulum (ER) stress, such as glucose deprivation, high protein demand, and inflammatory cytokines (Oslowski and Urano, 2013). It is well-documented that the large intestine, which is the site of infection, has very low levels of glucose (Tovy *et al.*, 2011). Furthermore, during amebic invasion, host endothelial cells release inflammatory cytokines as part of the immune response (Ximenez *et al,* 2018). Stresses imparted by glucose deprivation and inflammatory cytokines, ultimately increases protein demand (Oslowski and Urano, 2013).Therefore, it is likely that *E. histolytica* may face ER stress while invading the human host. It has been shown that phosphorylation of eIF-2α occurs in *E. histolytica* after long-term serum starvation, long-term heat shock, and oxidative stress (Hendrick *et al.*, 2016). However, an eIF2-based response to ER and nitrosative stress has yet to be investigated.

In this study we investigated the phosphorylation status of *E. histolytica* eIF-2α (*Eh*eIF-2α) during nitrosative and ER stress. We used known rapid nitric oxide (NO) donors, dipropylenetriamine NONOate (DPTA-NONOate) and sodium nitroprusside (SNP) or known ER stress-inducing reagents, dithiothreitol (DTT) and Brefeldin A (BFA) to impart stress on the parasite. We examined the morphology of the ER, tracked phosphorylation of *Eh*eIF-2α, and assessed protein translation in control and stressed cells. We also examined the viability of cell lines that conditionally express mutant versions of *Eh*eIF2α. 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 induces ER stress in *E. histolytica* and that this stress is managed by the eIF2α-based system. This is supported by the observation that cells expressing a non-phosphorylatable version of eIF2α were also extremely sensitive to DTT-stress. Overall, our study further illuminates the nitrosative and ER stress responses in *E. histolytica.*

2. Material and Methods

2.1. 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 h and were grown in 15 mL glass screw cap culture tubes (Diamond, Harlow, and Cunnick, 1978). The generation of transgenic cell lines overexpressing exogenous wildtype or mutant forms of *Eh*eIF-2α was previously described by Hendrick, *et al.*, 2016. 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 hours prior to all

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experiments, expression of exogenous *Eh*eIF-2α was induced by adding 5 μg mL^{-1} tetracycline to the culture medium (Hendrick *et al.*, 2016).

2.2. 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) (Delgado-Corona *et al.*, 2002; Manning-Cela *et al.*, 2003; Oslowski and Urano, 2013) in 13 mL of TYI-S-33 culture medium for 1 h at 37°C. Control cells were incubated with appropriate diluents, sterile water or dimethyl sulfoxide (DMSO; MP Biomedicals, Solon, OH, USA) for DTT and BFA, respectively. To induce nitrosative stress, log-phase trophozoites were incubated with either sodium nitroprusside (SNP; Sigma-Aldrich) (Santi-Rocca *et al.*, 2012) or dipropylenetriamine NONOate (DPTA NONOate; Enzo Life Sciences, Farmingdale, NY, USA) (Vicente *et al.*, 2009) in 13 mL of TYI-S-33 culture medium for 1 h at 37°C. After each treatment, trophozoites were incubated on ice for 8 min after stress induction to detach the cells from the glass culture tube. Viability was immediately assessed using microscopy and Trypan Blue (VWR, Radnor, PA, USA) exclusion.

2.3. 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, 350 μM BFA, or vehicle control for 1 h at 37°C and then were fixed with 4% (vol/vol) paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room (RT). After permeabilization with 0.2% (vol/vol) Triton X-100 in PBS for 5 min at RT, nonspecific binding sites were blocked by incubation with 3% bovine serum albumin-10% goat serum-PBS for 30 min 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 min 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 in the Clemson Light Imaging Facility (CLIF).

2.4. 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 (3×10^5) were pelleted by centrifugation at 500 × *g* for 5 min. Cells were resuspended in NuPAGE LDS sample buffer (Life Technologies), heated for 5 min at 100°C, and loaded on a precast NuPAGE 12% Bis-Tris Gel (Life Technologies). The gels were electrophoresed at 200 V for 45 min and separated proteins were transferred using a blotter apparatus to polyvinylidene difluoride membranes (PVDF; Life technologies) at 12 V for 1.5 h 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 min at 37°C. Membranes were incubated overnight at 4°C in either anti-total eIF-2α (diluted 1:1000 in TBST) or anti-phosphorylated

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eIF-2α (diluted 1:1333 in TBST) antibodies, which were developed and tested as previously described (Hendrick *et al.*, 2016). The membranes were then washed in TBST for 45 min with 6 buffer changes, incubated for 1 h at RT with commercially available horseradish peroxidase-conjugated goat anti-rabbit (dilution factor 1:5000 in TBST) (Fisher Scientific), and washed again for 45 min 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). Matching gels were stained with Bio-Safe G250 Coomassie Stain (Bio-Rad Laboratories) to measure load.

2.5. SUrface SEnsing of Translation (SUnSET)

SUnSET was previously used to measure levels of general protein translation in *E. histolytica* (Hendrick *et al.*, 2016). Wildtype trophozoites were exposed to vehicle control, 10 mM DTT, 1 mM SNP, 300 μM DPTA-NONOate, or 350 μM BFA for 1 h at 37°C. Then, to assess levels of general protein levels in stressed and unstressed cells, we incubated cells (2×10^5) 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. Next, cells were pelleted, and proteins were precipitated using 20% (v/v) TCA and incubating on ice for 10 min. Proteins were separate by centrifugation at 2200 × *g* for 5 min (4°C) and washed with 5% (v/v) TCA. The protein pellet was resuspended in 2X SDS running buffer and incubated at 100°C for 10 min. The lysates were immediately analyzed using western blotting as described above. Primary mouse anti-puromycin monoclonal antibodies (Sigma-Aldrich) were used at a dilution of 1:100 and secondary horseradish peroxidase-conjugated goat anti-rabbit antibodies (Fisher Scientific) were used at a dilution of 1:2500. As a loading control, matching PVDF membranes were stained with Bio-Safe Coomassie G-250 Stain (Bio-Rad Laboratories) or Ponceau Stain (Sigma-Aldrich).

2.6. 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.

3. Results

3.1. DTT induces ER Stress in *E. histolytica*

DTT and BFA have been shown to induce ER stress in mammalian cell culture (Oslowski and Urano, 2013), but only BFA has been shown to induce the formation of tubular structures that colocalize with ER marker, protein disulfide isomerase (PDI) in *E. histolytica* (Welter and Temesvari, 2009). In addition, Manning-Cela *et al.* (2003) showed that treatment with 356 μM BFA resulted in the organization of two ER markers, ERD2 and PDI. However, 5 mM DTT has been shown to induce ER stress in the related parasite, *Toxoplasma gondii* (Nguyen *et al.*, 2017). Additionally, Santi-Rocca, *et al.* (2012) demonstrates that exposure to

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1 mM SNP (Santi-Rocca *et al.*, 2012) induces ER stress in *E. histolytica.* Furthermore, exposure to 200 μM DPTA-NONOate induced morphology changes associated with stress (Vicente *et al.*, 2009). Therefore, we chose a small range of concentrations around these published values for each reagent to determine if DTT, BFA, SNP, or DPTA-NONOate induces ER stress in *E. histolytica.* Trophozoites were exposed to various concentrations of DTT, BFA, SNP, or a second NO donor, DPTA-NONOate, for 1h at 37°C, and viability was assessed (Fig. 1). The concentrations of DTT, BFA, and SNP, used in this study, did not induce significant cell death. DPTA-NONOate produced significant cell death at 500 μM; therefore, we did not use that concentration in subsequent experiments, so as not to confound our conclusions.

For our immunofluorescence microscopy studies, we chose a single concentration of each reagent that did not significantly affect viability (Fig. 1). We treated the parasites with 1mM SNP, 300 μM DPTA-NONOate, 10mM DTT, or 350 μM for 1 hr at 37°C. After treatment, we carried out immunofluorescence microscopy with an anti-KDEL antibody on stressed and unstressed cells. KDEL is a tetrapeptide at the C-terminus of proteins that are destined to remain in the ER, and is commonly used as an ER marker in many organisms, including this parasite (Teixeira and Huston 2008; Santi-Rocca *et al.* 2012). For example, amebic BiP has this C-terminal signal peptide (Ghosh *et al.*, 1999). 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 (Santi-Rocca *et al.*, 2012). We captured confocal images of trophozoites exposed to DTT, BFA, SNP, DPTA-NONOate, or the appropriate vehicle control (Fig. 2A). To avoid bias, images were blindly scored (n=100 cells per each treatment and control group), and the cells were classified as “stressed” or “unstressed” based on the fragmentation of the ER within each condition. Fig. 2A shows representative images of cells exposed to each reagent. Treatment with DTT, DPTA-NONOate, or SNP resulted in a higher percentage of cells with fragmented ERs (Fig. 2B). 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 (albeit not statistically significant) with fragmented ERs when compared to its DMSO control (Fig. 2A,B). Furthermore, the DMSO control showed a higher incidence of ER fragmentation compared the dH_2O control. Although we were careful to choose a DMSO concentration that did decrease viability (Fig. 1B), we cannot rule out that DMSO alone is causing some cytotoxicity, which may explain the phenotypic change in the presence of DMSO alone. While BFA causes ER stress in mammalian cells (Oslowski and Urano, 2013), it does not seem to cause canonical ER stress in this parasite based on microscopic observations of ER morphology, when compared to its DMSO control.

3.2. 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 (Fig. 3) (Hendrick *et al.*, 2016). We observed a basal level of phosphorylated *Eh*eIF-2α in control unstressed trophozoites, which was consistent with

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previous findings (Hendrick *et al.*, 2016). Treatment with DTT resulted in a significant increase in the phosphorylation of *Eh*eIF-2α (Fig. 3A). 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, although the increase was not statistically significant (Fig. 3B). Overall, this is consistent with our conclusion that BFA may not cause ER stress in this parasite. Surprisingly, nitrosative stress, imparted by NO donors, 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 (Fig. 3C,D). In the case of the NO donor, DPTA-NONOate, the decrease in the ratio of phosphorylated *Eh*eIF-2α and total *Eh*eIF-2α was statistically lower (Fig. 3C). One explanation for this interesting result is that the machinery necessary for phosphorylation of *Eh*eIF-2α may be damaged. Reactive nitrogen species are known to damage proteins, lipids, and other cellular components, by S-Nitrosylation (Nagaraja and Ankri, 2018).

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

A common cellular stress response is to reduce global protein translation, which allows 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 this decrease in protein translation during stress and this system seems to be operational in *E. histolytica* (Hendrick *et al.*, 2016).

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 (Hendrick *et al.*, 2016; Rivi Hertz *et al.*, 2014). Briefly, after incubation with 10mM DTT, 350 μM BFA, 300 μM DPTA-NONOate, 1 mMSNP, 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 first 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). Cycloheximide inhibits translation by blocking translocation of the tRNA molecules during protein translation (Schmidt *et al.*, 2009) . The absence of protein bands in the sample treated with cycloheximide and puromycin (Fig. 4A, ddH_2O+Puro+Cyclo) indicates that our antibody is specific for puromycin incorporation and that this method of measuring translation is accurate. The bands seen in ddH_2O lanes for Fig. 4C and Fig. 4D are non-specific bands that we occasionally see in this control depending on the exposure time to the film. For all four stress treatments, diminished protein band intensities on the western blots indicates that protein translation was 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

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under the same conditions. However, we were surprised to see a decrease in protein translation after 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 unknown alternate stress response pathways in *E. histolytica* that act independently of the *Eh*eIF-2α pathway, yet still affect the rate of protein translation.

3.4. Phosphorylation of *Eh*eIF-2α is required to counter ER stress

Since treatment with DTT induced phosphorylation of *Eh*eIF-2α, we tested if phospho-*Eh*eIF-2α was necessary to counter DTT-induced ER stress. We exposed three transgenic cell lines that overexpress wildtype or modified versions of *Eh*eIF-2α in a tetracycline-inducible fashion (Hendrick *et al.*, 2016) to DTT and measured viability. The cell lines included one that expresses a phosphomimetic version eIF-2α (D59), in which the serine at position 59 was mutated to aspartic acid, one that expresses a non-phosphorylatable version of eIF-2α (A59), in which the serine at position 59 was mutated to alanine, and one that overexpresses wildtype *Eh*eIF-2α (S59). In each of these cell lines, the exogenous versions of *Eh*eIF-2α have a FLAG epitope tag on the N-terminus to distinguish endogenous *Eh*eIF-2α from exogenous *Eh*eIF-2α. A cell line that expresses luciferase (Luc), an irrelevant protein, in a tetracycline-inducible fashion served as a control.

First, to confirm appropriate inducible expression of the exogenous proteins, the transgenic cell lines were incubated with (+) or without (−) 5 μg mL^{-1} tetracycline for 24 h 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 (Fig. 5A).

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 h at 37°C and viability was measured using trypan blue exclusion (Fig. 5B). If phosphorylation of *Eh*eIF-2α is necessary to counter DTT-induced ER stress that cells expression A59 should have decreased viability while cells expressing D59 and S59 transgenic may have increased viability. As expected the cell line expressing the nonphosphorylatable version of *Eh*eIF-2α, A59, exhibited a significant lower viability (p-value < 0.05) in the presence of DTT than control cells (Fig. 5B). However, cells expressing the phosphomimetic version of *Eh*eIF-2α, D59, or cells overexpressing wildtype *Eh*eIF-2α, S59, exhibited no difference in viability compared to control cells.

4. Discussion

This study is the first to investigate the phosphorylation status of *Eh*eIF-2α in the presence of reagents known to induce ER stress and nitrosative stress. Treatment with DTT induced distinct fragmentation of the ER, reminiscent of ER stress, and was accompanied by an increase in the phosphorylation of *Eh*eIF-2α. Furthermore, overexpression of a non-phosphorylatable version of *Eh*eIF-2α reduced the viability of *E. histolytica* trophozoites in the presence of DTT. Thus, DTT seems to induce a classical ER stress-response in *E. histolytica* and phosphorylation of *Eh*eIF-2α is necessary for coping with ER stress. In support of this, glucose deprivation induces ER stress in other systems (Garcia de la Cadena,

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et. al., 2014; Iurlaro, Raffaella *et al.*, 2017; Mulhern *et al.*, 2006) and glucose deprivation induces phosphorylation of eIF-2α in *E. histolytica* (Hendrick *et al.*, 2016). Interestingly, a similar outcome was not observed after treatment with another canonical ER-disrupting agent, BFA. Treatment with NO donors, SNP and DPTA-NONOate, did not induce phosphorylation of *Eh*eIF-2α suggesting that the eIF2α-based system may not play a role in nitrosative stress in *E. histolytica.*

While DTT is a reducing agent that prevents disulfide bond formation, 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 (Donaldson, 1992). In mammalian cells, this ER stress induces the phosphorylation of eIF-2α (Oslowski and Urano, 2013). Based on the current study, it cannot be discerned if BFA induces a canonical ER stress response in *E. histolytica.* Although we observed augmented levels of phosphorylated *Eh*eIF-2α after treatment with a high concentration BFA, the increase was not statistically significant. Furthermore, our microscopic observations showed only slight fragmentation of the ER in BFA treated parasites. The slight increase in phosphorylation of *Eh*eIF-2α and the slight fragmentation of the ER are not sufficient to surmise that BFA causes ER stress in this parasite.

A search of AmoebaDB (amoebadb.org) demonstrates that *E. histolytica* possesses multiple genes encoding conserved ARFs. Furthermore, X-ray crystallographic analysis of one of these ARFs, ARF1 (UniProt accession code C4LXL1), demonstrates that the *E. histolytica* ortholog has a conserved structure (Serbzhinskiy *et al.*, 2015). Thus, *E. histolytica* seems to have the targets for BFA. In a previous study, Welter *et al.* (2009) 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. Manning-Cela *et al.* (2003) showed that treatment with 356 μM, resulted in the organization of two ER markers, ERD2 and PDI. However, neither the Welter *et al.* (2009) study nor the Manning-Cela *et al.* (2003) study assessed the morphology of the ER using immunostaining and anti-KDEL antibodies. Therefore, in *E. histolytica* it is possible that BFA causes re-organization of the ER in a way that excludes the co-localization of KDEL-containing proteins and other ER markers. Future studies that attempt to co-localize KDEL-containing proteins and other ER markers before and after BFA treatment will provide insight into this question. Inducing ER stress with other reagents such as tunicamycin or calcium ionophores will also provide insight into this question.

Our data also showed that nitrosative stress may induce ER stress in *E. histolytica.* We observed some fragmentation of the ER upon treatment with DPTA-NONOate or SNP; however, the increase was not statistically significant. Santi-Rocca *et al.*, (2013) also showed that nitrosative stress leads to ER stress. A previous study showed that *E. histolytica* responds similarly to oxidative and nitrosative stress (Vicente *et al.*, 2009). It has also been shown that oxidative stress results in significantly increased levels of phosphorylated

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*Eh*eIF-2α (Hendrick *et al.* ,2016). Thus, we predicted that nitrosative stress would also result in the significant phosphorylation of *Eh*eIF-2α. Surprisingly, our data revealed decreased levels of phosphorylated *Eh*eIF-2α when trophozoites were exposed to DPTA-NONOate or SNP, both strong NO donors.

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 important cysteine residues. This modification leads to aberrant protein activity (Nagaraja and Ankri, 2018; Nakamura, T. and Lipton, 2007). Nitrosative stress also directly inhibits protein synthesis by inducing cleavage of ribosomal proteins (Rivi Hertz *et al.*, 2014). Therefore, one possible explanation for a decrease in phosphorylation of *Eh*eIF-2α and a simultaneous decrease in general protein translation could be a damaged phosphorylation machinery and cleavage of ribosomal proteins through aberrant protein function and/or levels. To confirm this, it will be necessary to expose cells sequentially to nitrosative stress followed by a second stress (e.g., long-term heat shock or oxidative stress), known to induce phosphorylation of *Eh*eIF-2α (Hendrick *et al.*, 2016), and measure protein translation and phosphorylation of *Eh*eIF-2α to determine if the NO donors damage the *Eh*eIF-2α phosphorylation machinery, itself. Furthermore, examining the translatome of parasites exposed to nitrosative stress via RNA-sequencing could illuminate other possible mechanisms of nitrosative stress.

The eIF-2 mechanism is known to counter stress by reducing global protein translation, while simultaneously upregulating a subset of stress-specific genes (Wek and Cavenar, 2007). This reduction in protein synthesis allows the cell time to repair any damage caused by stress and avoid significant cell death (Kim *et al.*, 1998; Wek and Cavener, 2007). To further illuminate the eIF-2 mechanism in this parasite, we measured levels of protein translation in control and stressed trophozoites using SUnSET. 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. However, we observed reduced translation in trophozoites for all four stress conditions. Interestingly, despite reduced protein translation, parasite viability was not significant decreased in any treatment. Importantly, we only stressed our parasites for 1 hr. We cannot rule out that longer incubations may result in lower viability. Reduction of protein translation in the absence of phosphorylated *Eh*eIF-2α suggests that there may be an unknown stress-specific response pathway, independent of the eIF-2 mechanism. This is an important finding, as identifying another stress response pathway may present new targets for drug therapy. Furthermore, high parasite viability in the face of reduced protein translation demonstrates that these stress response mechanisms are working efficiently. However, we only stressed our cells for 1 hr. If parasites were incubated for longer periods of time, the system may become overwhelmed and significant cell death may be observed. This would be an interesting experiment for future studies.

In the case of nitrosative stress, that alternative mechanism may be cleavage of ribosomal proteins, which leads to a decrease in general protein translation (Hertz *et al.*, 2014). Since treatment with DPTA-NONOate or SNP does not lead to the phosphorylation of *Eh*eIF-2α,

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we expect that the decreased translation is due to damaged ribosomal proteins. However, Kim *et al.* found that nitrosative stress induces the phosphorylation of eIF-2α in murine macrophages and pancreatic islet cells (Kim *et al., 1998).* 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 DTT-induced ER stress in this parasite, we used transgenic *E. histolytica* cell lines that overexpress wildtype or express modified forms of eIF-2α in a tetracycline-inducible fashion (Hendrick *et al.*, 2016). In agreement with our prediction, the cell line expressing non-phosphorylatable (A59) *Eh*eIF-2α suffered significantly more cell death than control cells when exposed to DTT. However, cells overexpressing wildtype *Eh*eIF-2α or the phosphomimetic (D59) version of *Eh*eIF-2α did not exhibit reduce viability in the presence of DTT. This could not be explained by a ceiling effect because overexpression of wildtype of phosphomimetic (D59) *Eh*eIF-2α did not enhance viability even when higher concentrations of DTT up to 70 mM were used (data not shown).

While phosphorylated eIF-2α is known for reducing global protein synthesis, this mechanism paradoxically promotes the translation of stress-specific mRNAs (Castilho *et al.*, 2014). For example, Vicente *et al.*, (2009) demonstrated by transcriptional analysis that 443 genes were up-regulated and 593 genes down-regulated by DPTA-NONOate. However, the translatome of stressed *E. histolytica* cells has yet to be defined. A current goal of our laboratory is to isolate and identify those mRNAs that are part of the translatome by escaping this translational control. We predict that such gene products are critical to the stress response of this parasite. These data may reveal novel stress response pathways that function independently of the eIF-2α mechanism or may identify novel targets for drug and vaccine development.

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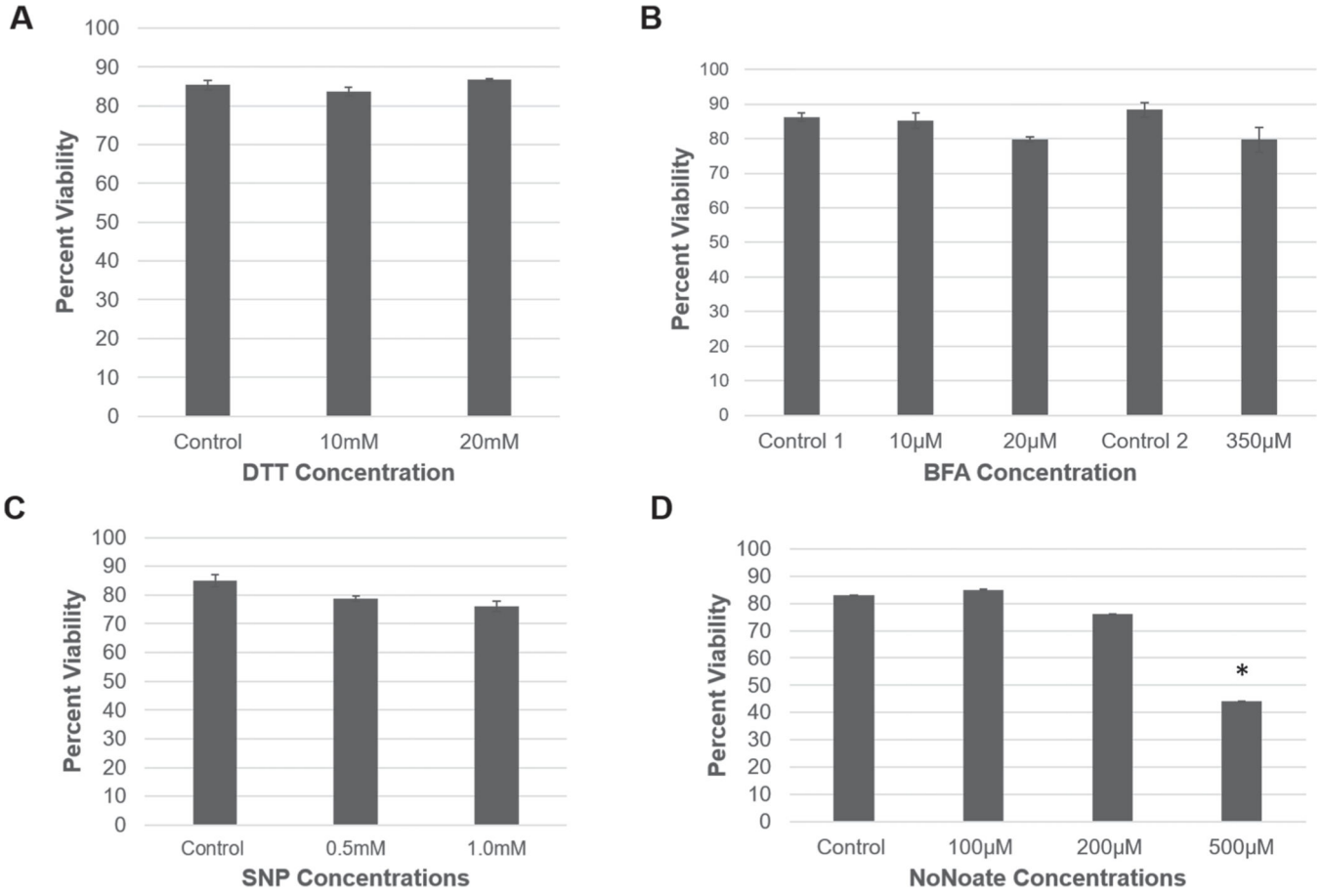

Fig. 1. Viability of stressed *E. histolytica* trophozoites.

E. histolytica trophozoites were exposed to DTT (A), BFA (B), SNP (C), or NONOate (D) and their viability was quantified using Trypan Blue exclusion. Sterile deionized water was used as the vehicle control for experiments using DTT (A), SNP (D), and DPTA-NONOate (E), while DMSO was used as the vehicle control for BFA (B). For Panel B, Control 1 corresponds to BFA concentrations 10 μM and 20 μM, while Control 2 corresponds to BFA concentration of 350 μM. The data represent the mean (± standard error) of at least 3 separate trials. Only 500 μM NONOate induced significant cell death (*, p value <0.05).

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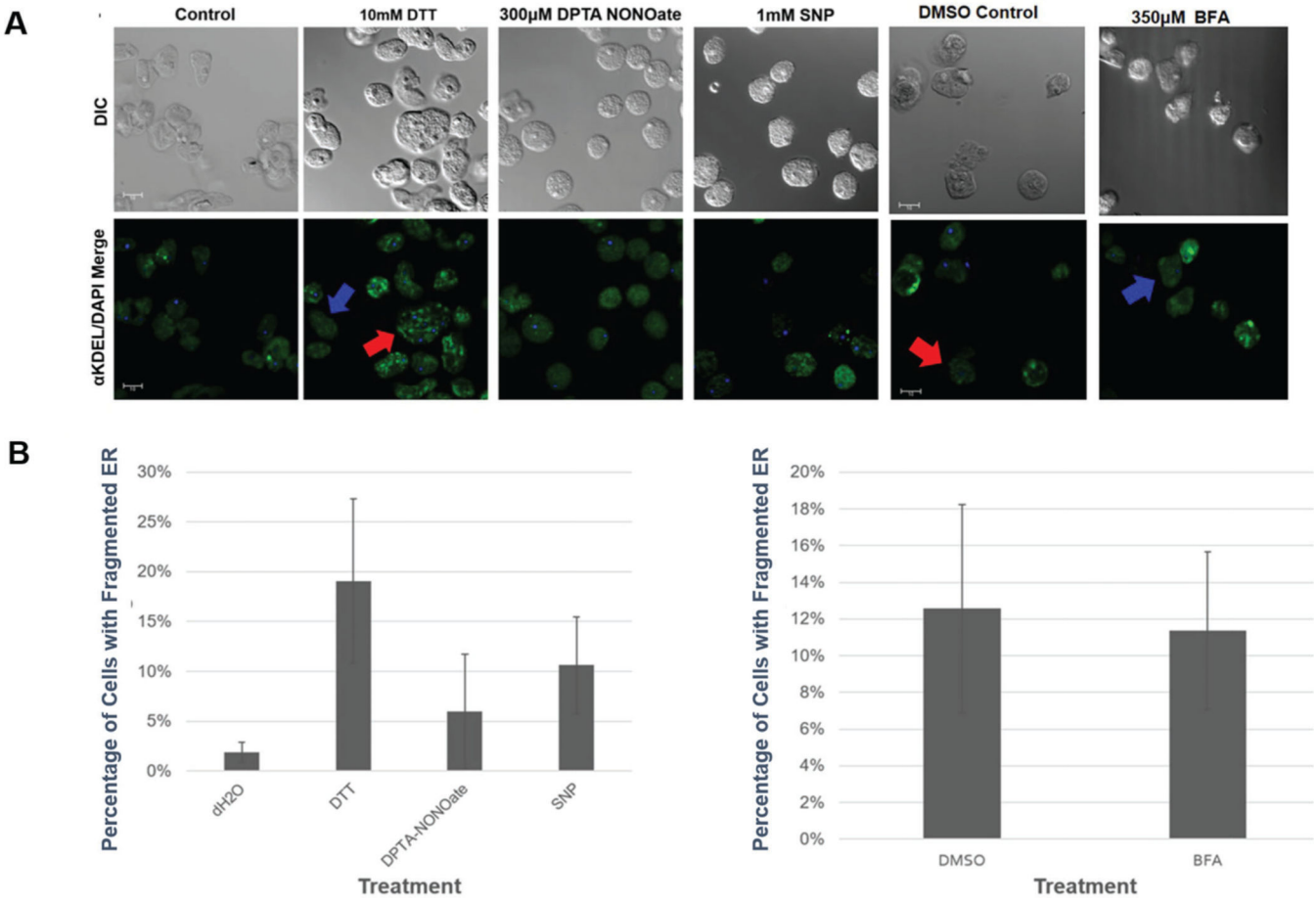

Fig. 2. Fragmentation of the ER during stress.

(A) Trophozoites were incubated with 10 mM DTT, 300 μM DPTA-NONOate, 1 mM SNP, 350 μM BFA, or the appropriate vehicle controls. Cells were stained with anti-KDEL antibody (green) to visualize the ER and DAPI (blue) to visualize the nucleus. Cells where then mounted on glass slides for fluorescence microscopy. Top Row: DIC images. Bottom Row: Corresponding anti-KDEL/DAPI merged images. The red arrows indicate representative cells with a stressed ER, while the blue arrows indicate representative cells with an unstressed ER. DTT-treated cells showed a higher incidence of fragmented ERs than other conditions. Scale bars represent 10 μm. (B) At least 100 cells from each trial with fragmented ERs (“stressed”) or continuous ERs (“unstressed”) were blindly scored. 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); however, the increase in fragmented ERs seen after incubation with DTT approached significance (p-value=0.0660). The data represent the mean (± standard error) of at least 3 separate trials.

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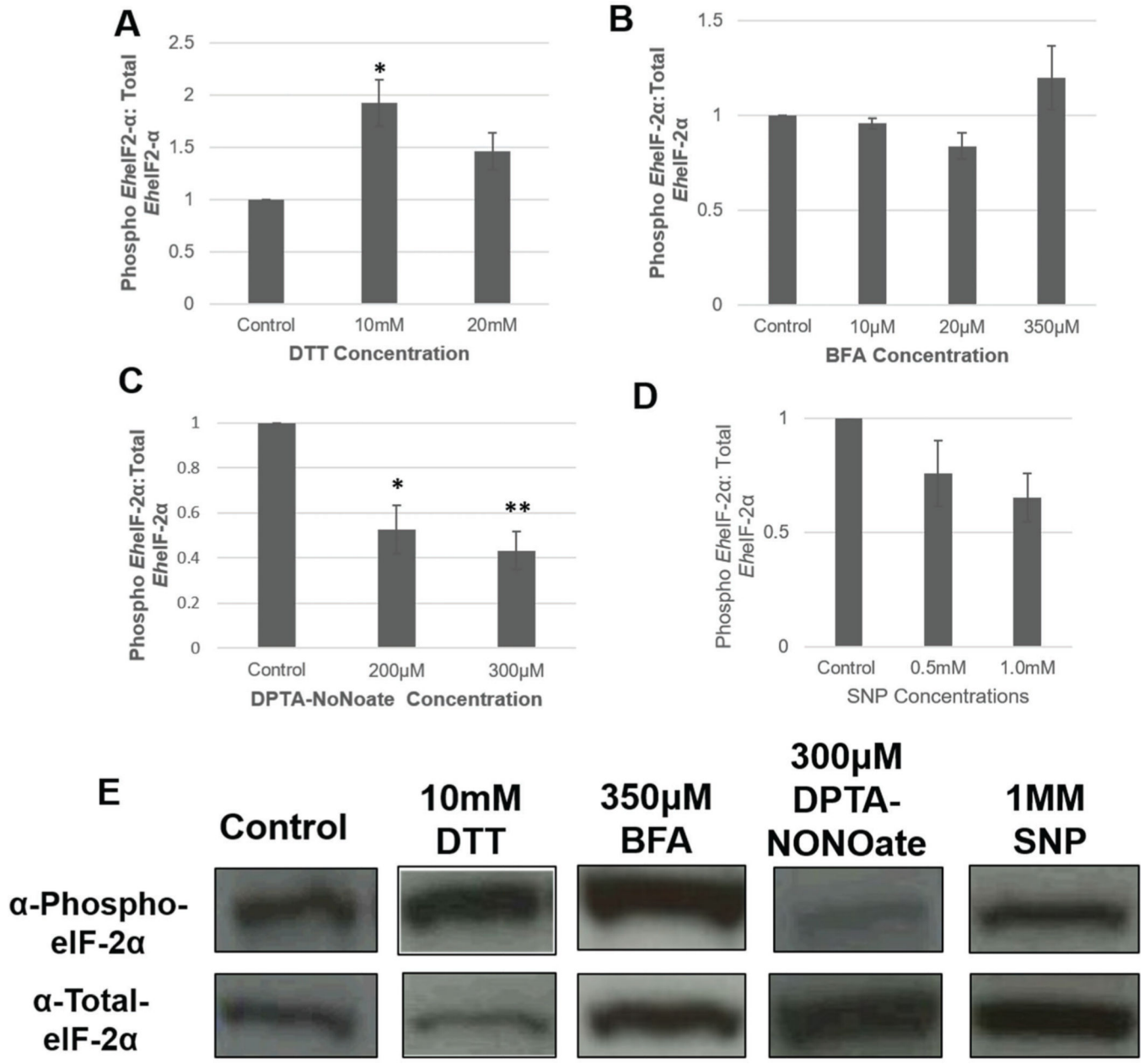

Fig. 3. Levels of phosphorylated and total *Eh*eIF-2α during stress.

Cells were treated with DTT (A), BFA (B), DPTA-NONOate (C) or SNP (D) and the levels of total and phosphorylated *Eh*eIF-2α were measured using western blotting. The ratios of phospho-*Eh*eIF-2α: total *Eh*eIF-2α were calculated using scanning densitometry after the protein 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. Trophozoites exposed to 10 mM DTT exhibited a significant increase in phosphorylation of *Eh*eIF-2α compared to control cells (*, p-value<0.05) (A). Interestingly, a higher concentration of DTT did not induce significant phosphorylation of *Eh*eIF-2α (A), perhaps due to off-target effects. Trophozoites exposed to 200 μM and 300 μM DPTA-

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NONOate exhibited a significant decrease in phospho-*Eh*eIF-2α (*, p-value <0.05; **, p-value<0.01) (C). There was no significant change in the level of phospho-*Eh*eIF-2α in cells exposed to BFA (B) or SNP (D). These data represent the mean (± standard error) of at least 3 separate trials. (E) Representative western blots, showing total and phosphorylated *Eh*eIF-2α are shown for each stress condition. Due to the lability of phosphorylated *Eh*eIF-2α, it was necessary to perform western blots for the various conditions at different times. As such, differences in the intensities of bands representing total *Eh*eIF-2α are due to variations in exposure to film from trial to trial.

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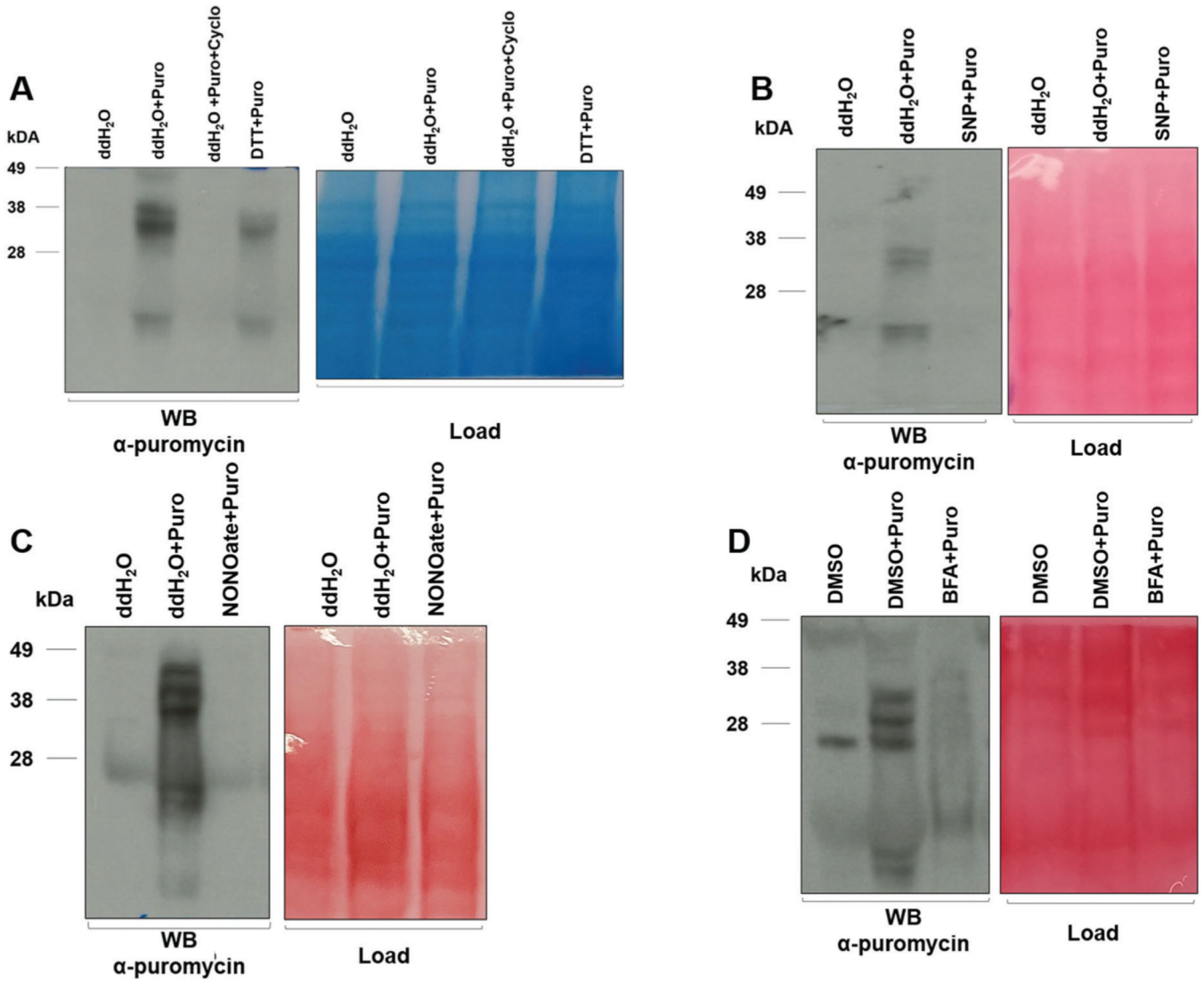

Fig. 4. SUnSET demonstrates reduced protein translation during stress.

(A) Wildtype trophozoites were incubated with vehicle control (1 h) [Control], vehicle control (1 h) + 10 μg mL-1 puromycin (15 min) [ddH_2O + Puro], vehicle control (1 h) + cycloheximide (10 min), + 10 μg mL-1 puromycin (15 min) [ddH_2O + Puro + Cyclo], and 100μg mL-1, or 10 mM DTT (1 h) +10 μg mL-1 puromycin (15 min). Subsequent western blotting was performed using an anti-puromycin antibody. The lack of bands seen in the cycloheximide treated sample shows that we can visualize reduced translation and that the anti-puromycin antibody is specific. Also, the decrease in band intensity between the ddH_2O l+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. The bands seen in ddH_2O lanes for panel C and D are non-

specific bands that we occasionally see in this control depending on the exposure time to the film. 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.

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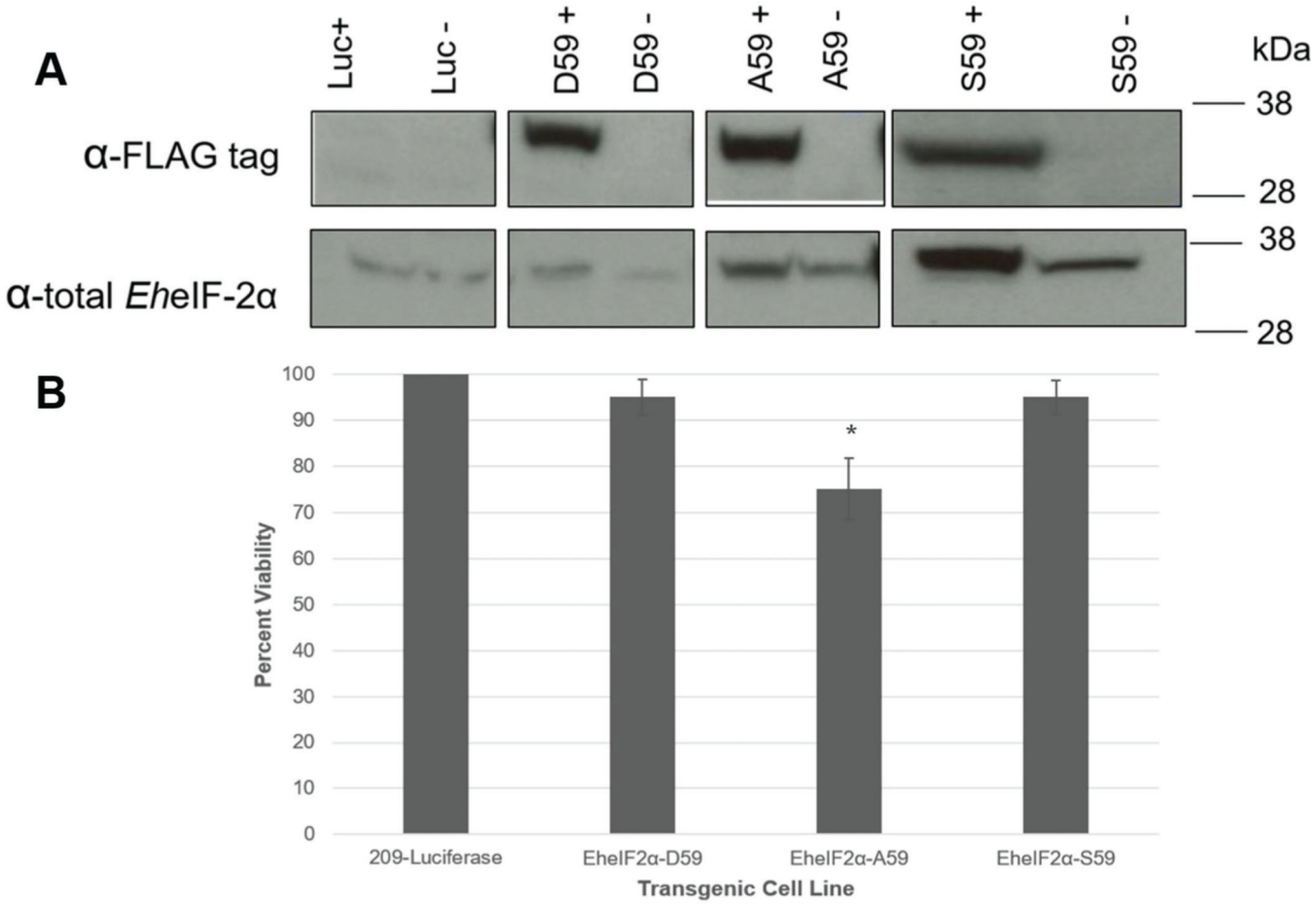

Fig. 5. Viability of *Eh*eIF-2α mutants during stress.

(A) *E. histolytica* transgenic cells lines overexpressing a FLAG-tagged phosphomimetic version of *Eh*eIF-2α (D59), a FLAG-tagged non- phosphorylatable version of *Eh*eIF-2α (A59), or a FLAG-tagged unmutated version of *Eh*eIF-2α (S59) were exposed to 5 μg mL-1 tetracycline for 24 h and the cell lysates were assessed by western blotting with an anti-FLAG tag antibody or an anti-total *Eh*eIF-2α antibody. As a control, a cell line that expresses luciferase (Luc) in a tetracycline inducible fashion was included. Exposure to tetracycline (+) induces clear expression of a FLAG-tagged protein of correct size that also cross-reacts with the *Eh*eIF-2α antibody. This exogenous band is not seen in the absence (−) of tetracycline nor in the control cell line (Luc). (B) Trophozoites were incubated with 10 mM DTT for 1 h at 37°C. Viability was quantified using Trypan Blue exclusion. The viability of cells overexpressing the phosphomimetic (D59) or wildtype (S59) versions of *Eh*eIF-2α was not significantly different from the luciferase expressing control. However, viability of the cells expressing the non-phosphorylatable (A59) version of *Eh*eIF-2α was significantly lower than that of control cells (*, p-value <0.05). These data represent the mean (± standard error) of at least 3 separate trials.