Suppressing Stress Granule Formation via Expression of AS3MT, TRX1, and FAIM

A Major Qualifying Project Report
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Abstract

Stress Granules (SGs) are cytoplasmic aggregates of mRNA, protein, and other cellular components that form as a result of different types of cellular stress. Many heavy metals, such as arsenic, exert toxicity on cells via oxidative stress, causing stress granules to form. Arsenic Methyltransferase (AS3MT) can methylate arsenic, reducing the oxidative stress it causes. Thioredoxin (TRX1) acts as a general sink for oxidative species, sequestering arsenite and reducing the oxidative stress it causes. Fas Apoptotic Inhibitory Molecule (FAIM) can sequester disordered proteins. It is hypothesized that the characteristics of AS3MT, TRX1 and FAIM can prevent SG formation through prevention of aggregation via G3BP1, one of the primary protein drivers for SG formation. To test this hypothesis, AS3MT, TRX1 or FAIM was expressed by transient transfection in cultured human cells (HeLas), and SG formation was assessed by fluorescence microscopy.. The results showed preliminary evidence that these proteins suppress SG formation, although more testing with refined experimentation is needed to fully assess the degree of suppression. Additionally, several proteins related to the alleviation of oxidative stress, including TRX1, TXNRD1, GPX4 and GSR, were found to co-localize with G3BP1 in SGs, indicating that these proteins may be important functional components of SGs. Overall, the results of this experiment and refined protocol will greatly help understanding of SG regulation in response to oxidative stress.

1. Background

1.1 Stress Granules and Oxidative Stress

1.1.1 What Are Stress Granules?

Stress granules (SGs) are reversible and dynamic membrane-less cytoplasmic protein/RNA complexes which form in response to environmental stress as a protective mechanism to temporarily arrest translation and favor only the synthesis of cytoprotective proteins (Kedersha et al., 1999). Some of these environmental stressors include heat shock, viral infection, oxidative stress, UV radiation, and hypoxia (Anderson & Kedersha, 2006).

The primary effect of SGs is the arrest of protein translation. Protein translation is an extremely important factor in cellular maintenance, but also is responsible for consuming a substantial amount of resources. Initiation of protein translation involves a number of factors, such as eIF2, which is responsible for delivering the GTP required for initiation of protein translation to the ribosome pre-initiation complex (Wek, 2018). This process consumes GTP and produces GDP in order to start protein translation. After translation is initiated, this GDP needs to be exchanged for a GTP, a process catalyzed by eIF2B (Wek, 2018). When phosphorylated, eIF2α is able to tightly bind to eIF2B in response to cellular stress, inhibiting it from exchanging GDP for GTP, and thus globally decreasing translation of proteins in order to conserve cellular resources (Wek, 2018).

A family of 4 kinases is responsible for phosphorylating eIF2α: PKR, HRI, GCN2 and PERK. Heme-regulated inhibitor Kinase (HRI) is activated by a lack of heme, the prosthetic group of hemoglobin. HRI is the kinase responsible for phosphorylating eIF2α upon exposure to arsenite, the primary causative factor of oxidative stress utilized in this experiment (McEwen et al., 2005). Once HRI is activated in response to oxidative stress, phosphorylation of eIF2α causes formation of stress granules through the mechanism discussed above (McEwen et al., 2005).

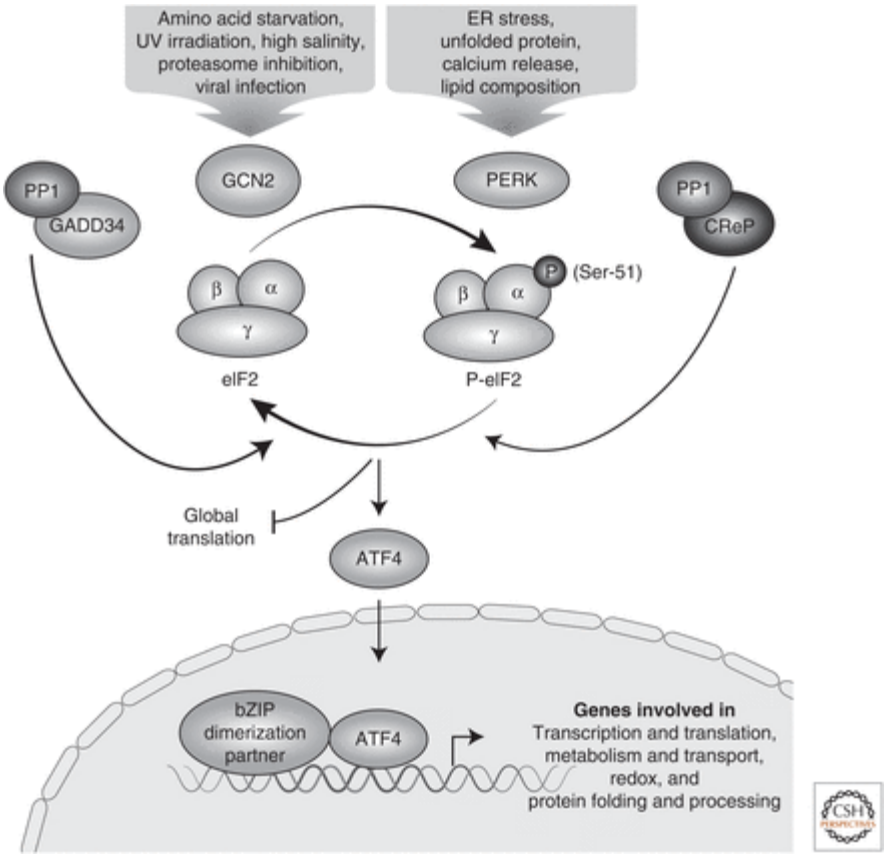

Figure 1

The pathway involving eIF2, as shown by activation through GCN2 and PERK, two of the other cell mediated stress response kinases orthogonal to HRI. Phosphorylation of the alpha subunit of eIF2 results in global translational arrest. (Figure from Wek, 2018)

1.1.2 Relationship of SGs to Oxidative Stress

The Heme-Regulated Inhibitor Kinase (HRI) pathway is one of the four main cell mediated stress response pathways. It primarily responds to oxidative stress (McEwen E, et al., 2005), such as high concentrations of heavy metals, like arsenic. When this pathway is activated, it phosphorylates the translation initiation factor eIF2 alpha subunit (eIF2α), impairing the GDP-GTP exchange and causing buildup of eIF2-GDP complexes that are unable to deliver $tRNA_i^{met}$ to the ribosome, causing translation to stop (Fig. 1) (Wek, 2018). Once translations stop, these complexes begin to accumulate and condense. With the help of RNA-binding proteins, they will become phase separated SGs, which fuse to form larger SGs (Jovanovic B et al., 2021).

As these SGs aggregate, they impact the overall function of the cell, controlling protein function and gene expression through sequestration of proteins and mRNA (Jovanovic B et al., 2021). It is hypothesized that by stopping many non-essential cellular processes, these SGs possess anti-apoptotic properties, allowing cells to survive through periods of elevated perturbation, though this idea has not been strongly proved through evidence (Amen T et al.,

2021). SGs have also been connected to the formation of pathogenic protein aggregates in neurodegenerative disorders, indicating that their presence may not be strictly beneficial (Wolozin & Ivanov, 2019).

As of now, many aspects of SG formation and function are not understood. Researching the ways in which changing oxidative stress impacts the formation of SGs would allow them to be better controlled, leading to a potential method to induce or suppress SGs in physiological settings. Not only would this allow for SGs to be studied with more convenience, but may also be used to harness the benefits of SGs while limiting the potential harms they may cause.

1.1.3 Arsenic Exposure and Oxidative Stress

Arsenic is a naturally occurring heavy metal. When in the inorganic, trivalent form, it is a strong oxidizer and can form reactive oxygen species (ROS) when it is exposed to biological systems. These ROS put significant strain on the cells by causing oxidative stress, which causes thiol linkages to form between cysteine residues in proteins, hindering their ability to function properly (Shao D et al., 2014). This in turn, prompts the HRI pathway to be activated, causing SGs to form and aggregate (Jovanovic B et al., 2021). For this reason, it is a good testing condition to apply to cells to stimulate oxidative stress. Through the HRI pathway, oxidative stress can lead to the formation of SGs. Because oxidative stress is directly causative of SG formation, it is possible that proteins that modulate oxidative stress may also modulate SG formation. For this reason, it is important to rely on a molecule such as arsenic which is able to consistently apply oxidative stress and lead to SG formation when studying these proteins.

1.2 Proteins that Modulate Oxidative Stress

1.2.1 Thioredoxin (TRX1)

In the human body, there are two main protein families responsible for maintaining redox homeostasis: thioredoxin and glutathione (Wang Z et al., 2007). Thioredoxin is important in its ability to perform reduction reactions, counteracting the effects of ROS and oxidative stress that is placed on the cell by compounds like arsenic (Shao D et al., 2014). The family is characterized by an extremely well conserved Cys-Gly-Pro-Cys sequence in the redox site of the proteins (Wang Z et al., 2007). This redox site allows TRX1 to cleave the thiol linkages that are formed in proteins as a result of oxidative stress, restoring function of these proteins (Fig. 2) (Shao D et al., 2014).

Thioredoxin 1 (TRX1) is one of the proteins in the thioredoxin family, which has been observed to localize at stress granules, specifically, those induced by arsenic exposure. In addition, TRX1 has been shown to play a part in SG formation but not in activation of HRI, indicating that the role it plays in this process likely occurs downstream of activation of the pathway (Jovanovic B et al., 2021).

TRX1 has also been shown to protect against the formation of cytoplasmic aggregates, namely by preventing thiol linkages from forming between proteins (Shao D et al., 2014). Although the relationship between TRX1 and the formation of these aggregates is not entirely understood, TRX1 has been shown to co-localize with SGs (Jovanovic B et al., 2021).

1.2.2 Arsenic Methyltransferase (AS3MT)

Arsenic Methyltransferase (AS3MT) is another protein which is able to assist cells in resisting oxidative stress. As indicated by the name of the protein, it has a much more specific purpose, reacting only with arsenic rather than the broad range of effect that TRX1 has (Fig. 2). Arsenic exists in many forms, often categorized into inorganic and organic arsenic. The primary difference between the two is the presence of methyl groups. The more methyl groups that are attached to an arsenic, the more "organic" that arsenic is. Inorganic arsenic is more toxic and reactive than organic arsenic, and as a result, poses greater threat to the cell. Additionally, organic arsenic can be excreted from the cell much more easily than inorganic arsenic, allowing for much faster clearance from the cell and in turn, prevention of oxidative stress. In line with this, AS3MT catalyzes a reaction in which methyl groups are added to inorganic arsenic, transforming it into an organic form and preventing oxidative stress on the cell upstream of where TRX1 acts in the stress pathway (Dheeman et al., 2014).

1.2.3 Fas Apoptotic Inhibitory Molecule 1 (FAIM1)

Fas Apoptotic Inhibitory Molecule (FAIM) is a family of proteins which is highly conserved (Kaku & Rothstein et al., 2020). It has been shown to confer resistance to apoptosis by blocking the Fas/Fas Ligand complex from forming, stopping the cascade to trigger apoptosis from occurring (Planells-Ferrer & Urresti et al., 2016). In addition to this function, FAIM has also been shown to protect against stress induced apoptosis by binding to protein aggregates (Kaku & Rothstein et al., 2020). FAIM is able to bind to protein aggregate complexes as a result of its unstructured N terminus region, which has been shown to play an important role in binding aggregate prone targets, preventing them from aggregating further (Fig. 2). (Hemond et al., 2009). Additionally, FAIM contains a proportionately low amount of cysteine residues (Kriehuber et al., 2010). Cross-linkage of cysteine residues occurs during exposure to oxidative stress and can cause proteins to lose function and aggregate. The lack of residues in FAIM allows it to function while the cell faces periods of increased oxidative stress (Kaku & Rothstein et al., 2020). Because of these factors, it is hypothesized that expression of FAIM1 in cells facing oxidative stress may suppress stress granule formation.

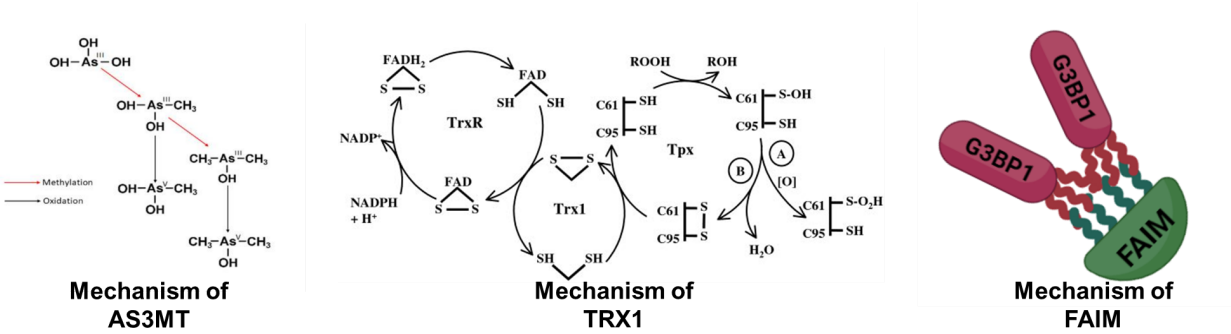

Figure 2

Visualization for the mechanism and chemistry for the 3 main proteins studied in this project.

1.3 Research Hypothesis and Objectives

This project is based around exploring stress granule formation. The core idea behind studying AS3MT and TRX1 relies on their relation to oxidative stress. If oxidative stress causes SG formation, then it follows that proteins which modulate oxidative stress, such as AS3MT and TRX1 should also modulate the formation of SGs. The rationale behind studying FAIM is similar, but slightly different, since FAIM acts downstream of formation of ROS. Instead, FAIM will bind to protein aggregates, sequestering them and preventing them from binding to other aggregate prone bodies. This, in turn, prevents further protein aggregation which leads to the formation of SGs.

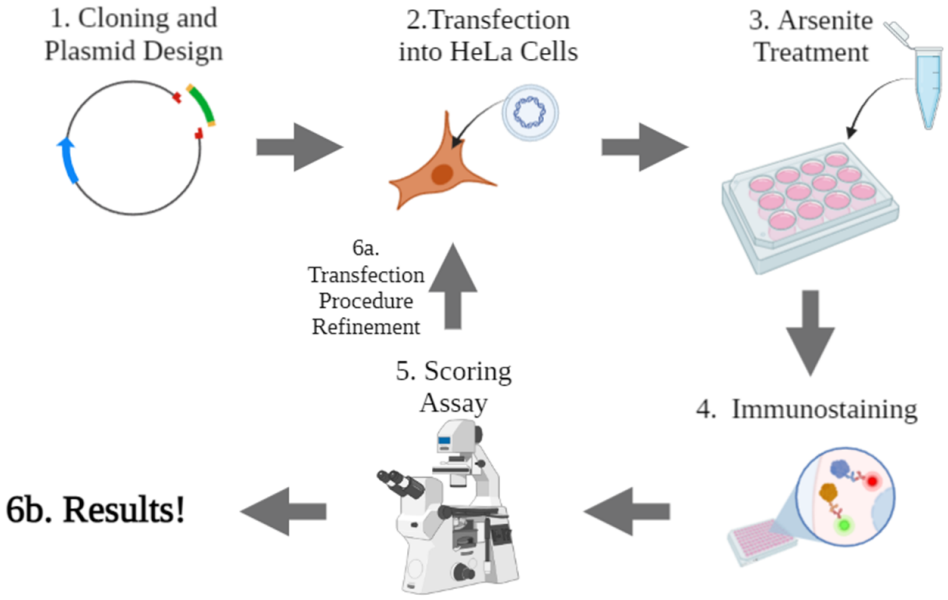

Figure 3

An image of the experimental workflow designed to assess the ability of these proteins to suppress SG formation.

In order to study these ideas, the aforementioned proteins will be transiently expressed in mammalian epithelial cells. The cells will then be exposed to arsenite in order to induce oxidative stress on the cells. Immediately following this, the cells will be fixed in order to observe the state of each of the cells directly following the stress event. The cells will then be imaged in order to analyze the presence or absence and morphology of the SG formed (Fig. 3). This process should elucidate the relationship between expression of the three proteins being analyzed and SG formation.

2. Methods

2.1 Cell Plating

All cell lines were cultured in 1X DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin solution, and 2mM glutamine. Cells were maintained at 37℃ and 5% CO_2. Cells of chosen strain (COS7, U2OS, HeLa), were plated in a 6 well plate at a density of $1x10^5$ cells/well, and given 24 hours to settle.

2.2 Plasmid Cloning and Use

Genetic sequences for proteins used in this experiment were adapted from the Addgene database and cloned into pcDNA3 plasmid backbones. The GFP plasmid used was pEGFP-N3. The AS3MT coding region was synthesized by IDT DNA. The TRX1 coding region was from pcDNA3-flag-Trx1 (WT), Addgene 21283. The FAIM coding region was adapted from pDONR223-FAIM-WT-V5, Addgene 82930.

2.3 Transient Transfection, Initial Protocol

Approximately 24 hours after cells were plated, transient transfection was performed. For each sample, a tube was created with 100 µL of serum-free media. Following this, 1.5 µg of the desired plasmid was added to each tube, and 10 µL of Qiagen Polyfect reagent was added to each tube. Immediately after, tubes were vortexed for 10 seconds, then incubated at room temperature in a sterile environment for 10 minutes. While these tubes were incubating, the growth media was extracted from the cells, the cells were rinsed with 1x PBS and 1.5 µL of new growth media was applied. Once the 10 minute incubation period had elapsed, 600 µL of complete media was added to each of the tubes containing transfection mixture. The mixture was pipetted gently up and down to mix, and the total volume of transfection. Approximately 24 hours were allowed to pass, and the cells were moved to a 12 well plate with coverslips, and fresh growth media was added. The format used for plating to 12 well plates is shown below in Fig. 4.

2.4 Transient Transfection, Refined Protocol

Approximately 24 hours after cells were plated, transient transfection was performed. For each sample, a "Lipofectamine" tube was created, and 125 µL of opti-mem media was added to each tube. After this, 3.75 µL of Invitrogen Lipofectamine 3000 was added and the tubes were vortexed for 3 seconds. After this a "DNA" tube was made for each sample, containing 125 µL of opti-mem media. 5 µg of total DNA was added to each tube, 10 µL of Invitrogen P3000 reagent was added, and the tubes were mixed gently by pipetting up and down. At this point, the contents of each "Lipofectamine" tube was added to the contents of each "DNA" tube, and the tube was incubated for 10-15 minutes. While these tubes were incubating, the growth media was extracted from the cells, the cells were rinsed with 1x PBS and 1.5 µL of new growth media was applied. Once the 10-15 minute incubation had elapsed, 250 µL of the transfection mixture was also added to the cells. Approximately 24 hours were allowed to pass, and the cells were moved to a 12 well plate with coverslips, and fresh growth media was added. The format used for plating to 12 well plates is shown below in Fig. 4.

2.5 Arsenite Treatment

A predetermined amount of time was allowed to pass following cells being transfected (48-72 hours after transfection, 24-48 hours after plating to cover slips), and cells were treated with arsenite in varying concentrations (100-500 µM). Cells were treated with arsenite for 30 minutes to 1 hour, depending on the interaction of the procedure used. Immediately following this, arsenite media was aspirated, and cells were fixed using 4% paraformaldehyde PBS, for 10 minutes while shaking. Following this, the paraformaldehyde was discarded, and 100% methanol was applied to the cells for 10 minutes while shaking at room temperature to finish the cell fixation process. The organization of arsenite treatment for each of the experiments performed is shown below in Fig. 4.

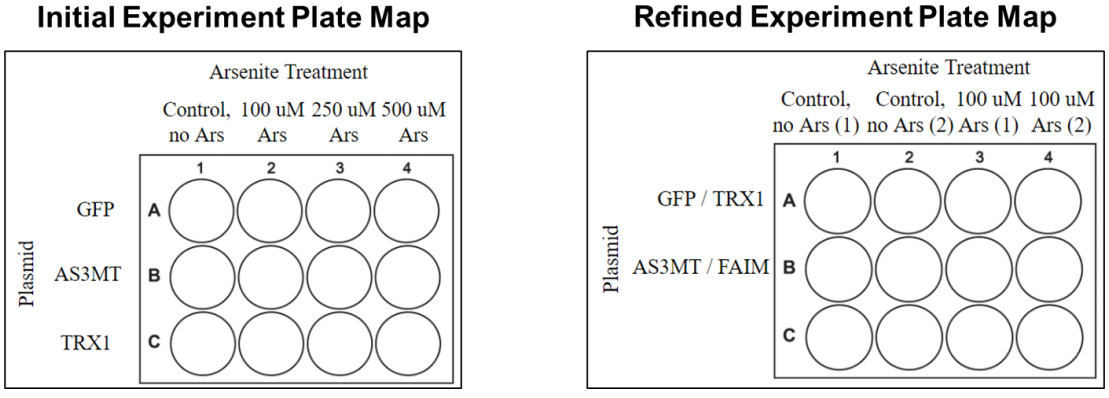

Figure 4

A plate map of the transfection conditions and arsenite treatments applied to each 12-well plate.

2.6 Cell Staining

Remaining solution in the wells was aspirated, and BSA-PBS was added to the wells and allowed to sit from 1 hour to overnight. Following this, a primary antibody was applied with BSA-PBS, and allowed to sit anywhere from 1 hour to overnight. Primary antibodies and concentrations used in this experiment are listed below in table 1. After this time had elapsed, the primary antibody solution was aspirated, and the cells were washed 3 times for 5 minutes each, using PBS. Following the wash, a secondary fluorescent antibody was applied to the cells, and allowed to sit for 1 hour. Secondary antibodies and concentrations used in this experiment are listed below in table 2. After the time had elapsed, the secondary antibody solution was aspirated, and the cells were again washed with PBS, 3 times for 5 minutes each. Then, the coverslips containing cells were mounted onto slides using vinol as a mounting media, blinded to minimize bias, and imaged.

Table 1- Primary Antibodies.

Antibody Name	Manufacturer	Product ID	Dilution
Rb-G3BP1	Proteintech	13057-2-AP	1:2000
Ms-FLAG	Proteintech	66008-3-Ig	1:2000
Ms-G3BP1	Proteintech	66486-1-Ig	1:2000
Rb-TXN	Proteintech	14999-1-AP	1:1000
Rb-TXNRD1	Proteintech	11117-1-AP	1:500
Rb-GPX4	Proteintech	14432-1-AP	1:200
Rb-GSR	Proteintech	18257-1-AP	1:200

Table 2- Secondary Antibodies

Antibody Name	Manufacturer	Product ID	Dilution
Alexa Fluor® 594-conjugated AffiniPure Donkey Anti-Rabbit IgG	Jackson ImmunoResearch	711-585-152	1:500-1:1000

2.7 Cell Scoring

Beginning on the fluorescent channel associated with the FLAG tag showing transfected cells, a transfected cell would be searched for. Once a cell had been found, the fluorescent channel was switched to the channel associated with G3BP1. The cell was then assessed for the presence or absence of stress granules. 200 cells for each trial were counted, and the ratio of cells with and without stress granules was recorded as the “Percent of cells with SGs”.

3. Results

3.1 Initial Results

To assess the impact of AS3MT and TRX1 on SG formation, transient transfection experiments were performed. At the beginning of the project, COS-7 cells were used because of their high transfection efficiency and their ability to replicate plasmids due to the presence of large T antigen. Four separate arsenite concentrations were used, from 0 μm to 500 μm, and the primary proteins observed in the study were AS3MT and TRX1. Green fluorescent protein (GFP) was also used as a convenient protein which could mark transfected cells while having no direct impact on SG formation. Half of the cells were exposed to arsenite media 24 hours following transfection, and the other half was exposed to arsenite media 48 hours following transfection.

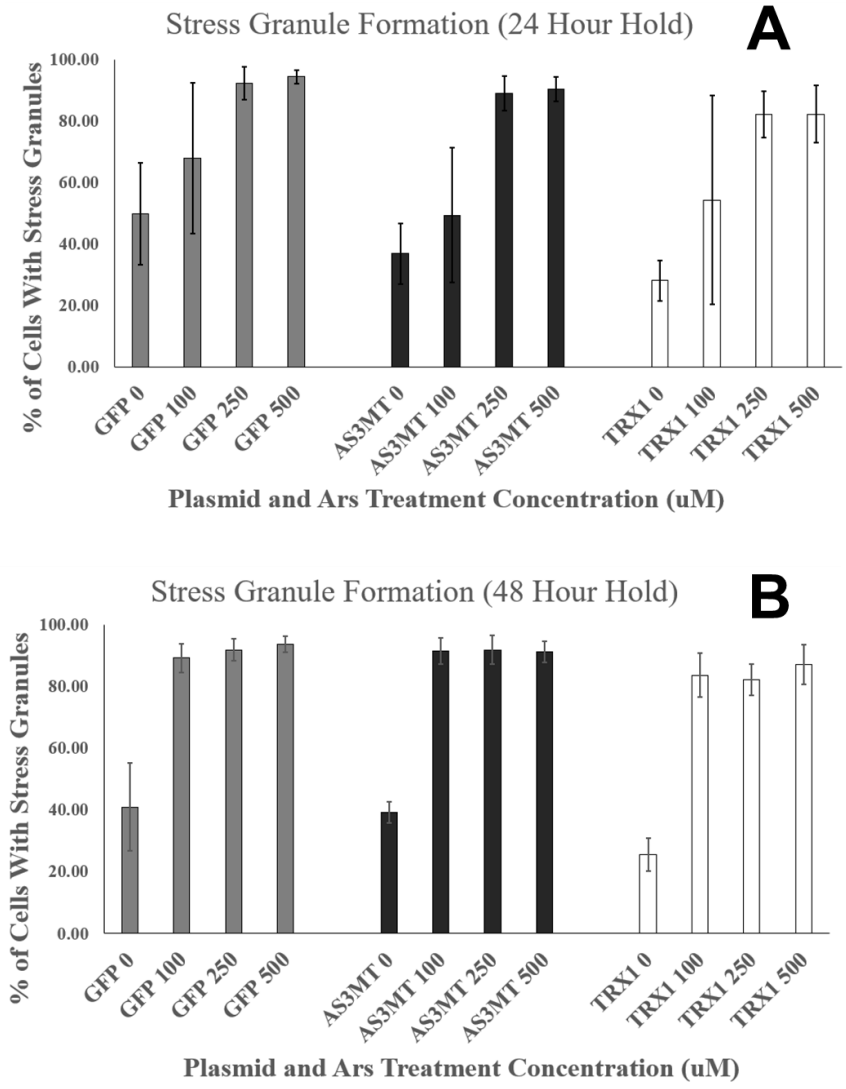

Figure 5

The initial trial examined SG formation in cells expressing AS3MT and TRX1 when exposed to arsenic. a.) shows the trials in which cells were exposed to arsenite 24 hours after transfection, and b.) shows trials where cells were exposed to arsenite 48 hours after transfection. In both cases, bars are grouped based on the protein being expressed (GFP, AS3MT or TRX1). Numbers represent the concentrations of arsenic exposed to the cells in μm. Each testing condition was scored in triplicate, and error bars represent the standard deviation for each of the conditions.

This experiment showed very high SG formation in cells that were not exposed to arsenite (Fig. 5). Additionally, all of the trials treated with 250 μm and 500 μm resulted in close to 100% SG formation across the population. Furthermore, all of the 100 μm trials in the 48 hour hold experiment also resulted in close to 100% arsenite formation. Large variance was also seen in the 100 μm trials from the 24 hour hold experiment. When analyzed using a two way ANOVA (shown in appendix 1 and 2, for the 24 and 48 hour hold respectively), the p value

showed statistical significance for the independent arsenite treatments. It also showed statistical significance between at least one of GFP, AS3MT and TRX1 shown in Fig. 5B , while showing there was no statistical significance between any of GFP, AS3MT and TRX1 in Fig. 5A.

3.2 Procedural Refinement

In order to refine the experiment, the issue of SG formation in the absence of arsenite was the first issue addressed. The cell line was switched from COS-7 to HeLa, in the hopes that lower protein expression would result in less spontaneous SG formation. Additionally, a new transfection reagent (Lipofectamine 3000) was employed. Using these new approaches, the experiment was repeated in the absence of arsenite exposure to assess spontaneous SG formation and transfection efficiency (Figure 6). The lowest SG formation was achieved using the “1x” lipofectamine treatment outlined in the reagent protocol and waiting 48 hours following transfection to fix the cells. This treatment also resulted in the lowest transfection efficiency, but since the transfection efficiency was still above the acceptable threshold, these conditions were used going forward to minimize spontaneous SG formation.

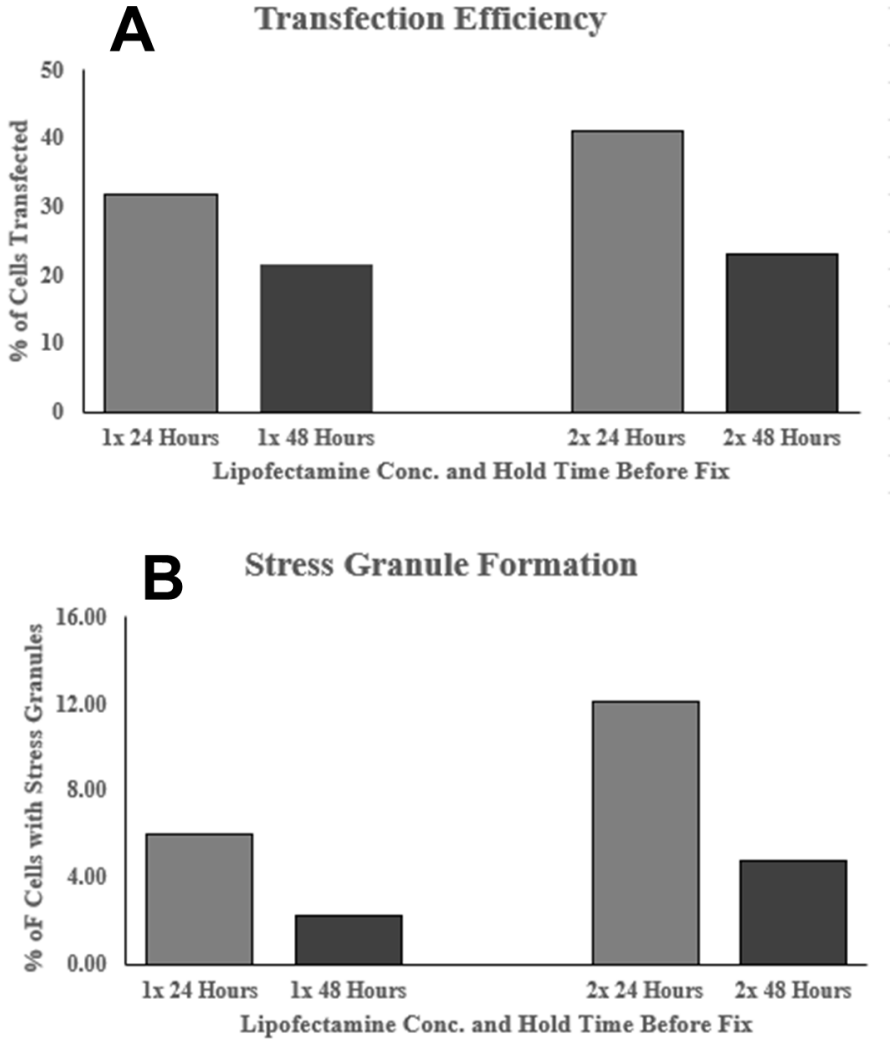

Figure 6

Refinements examined spontaneous SG formation in HeLa cells, testing varying reagent concentrations and hold times between transfection and arsenite treatment. The graphs are arranged to show a.) the % of transfected cells and b.) the % of cells in a 200 cell population with SGs.

3.3 Procedural Re-Implementation

To assess the impact of AS3MT, TRX1 and GFP on SG formation, the initial protocol was revisited with the new implementations to lower spontaneous SG formation. Across all trials, spontaneous SG formation was reduced, as shown by Fig. 7a. The percentage of cells with SGs in the absence of arsenite was below 10% in all trials, contrasted with the initial protocol where at lowest SG formation in the absence of arsenite was 20%.

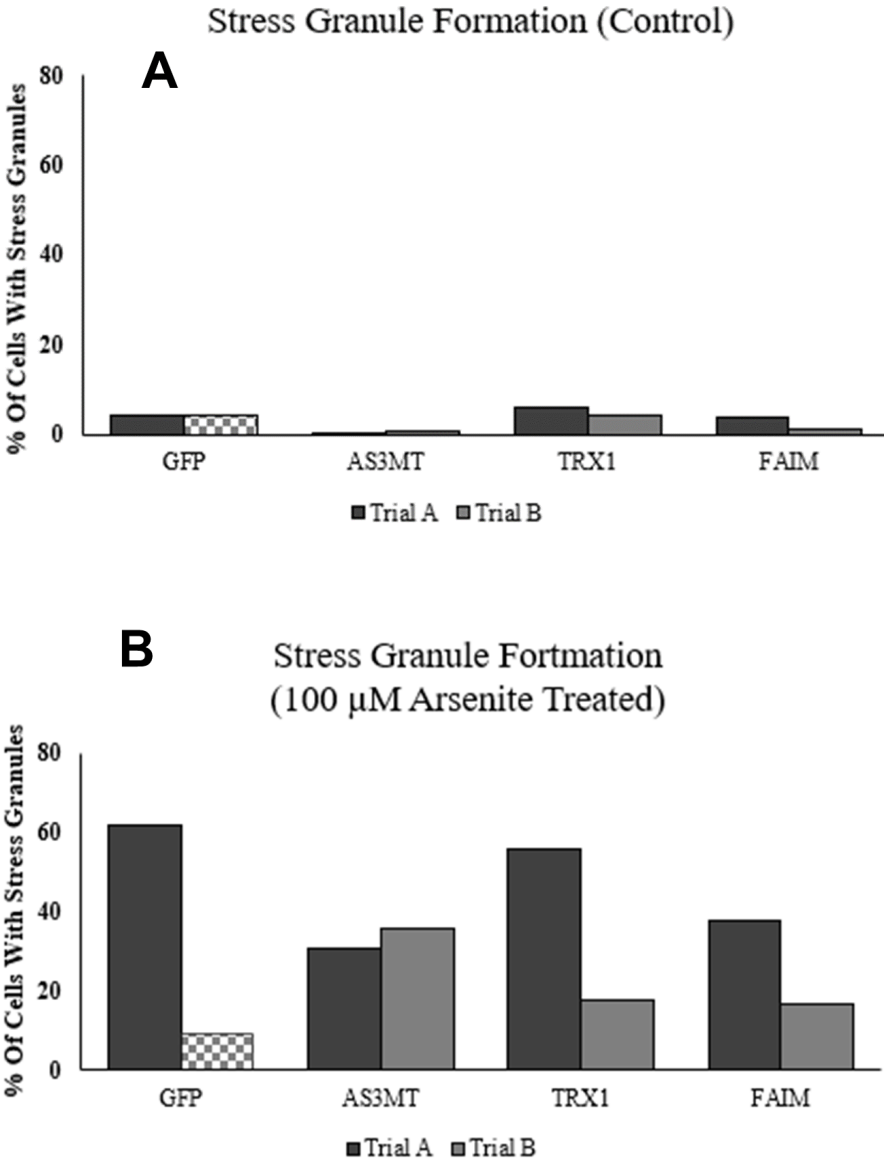

Figure 7

The results of the final trials performed within the span of the experiment. These trials show the percentage of cells exhibiting SGs within a population in a.) untreated cells and b.) cells treated with 100 µm arsenite for 1 hour. Trial A represents only 1 sample due to cell death, while trial B represents 2 samples transfected in parallel for each testing condition, averaged. For trial B, GFP scoring thresholds were only reached for 1 of the control trials, while the other control trial and both of the 100 µm trials did not have enough cells. To illustrate this, the GFP bar for trial B has been shaded to reflect the shortcomings of this data.

Despite the improvement in lowering spontaneous SG formation, both trial A and B both faced significant issues. Following transfection, the population used in trial A experienced a large amount of cell death. This cell death allowed only half of the cells to be scored. In trial B,

transfection of the plasmid carrying GFP was extremely inefficient. The 200 cell threshold outlined in the scoring procedure was only met in 1 of the 4 transfection trials. The data has been displayed, but shows drastic differences from the data collected in trial A (Fig. 7). Additionally, the TRX1 and FAIM trials show a relatively high difference in SG formation between trial A and trial B. Despite this, all 6 of the trials in the proteins of interest, AS3MT, TRX1, and FAIM show reductions in SG formation compared with trial A GFP.

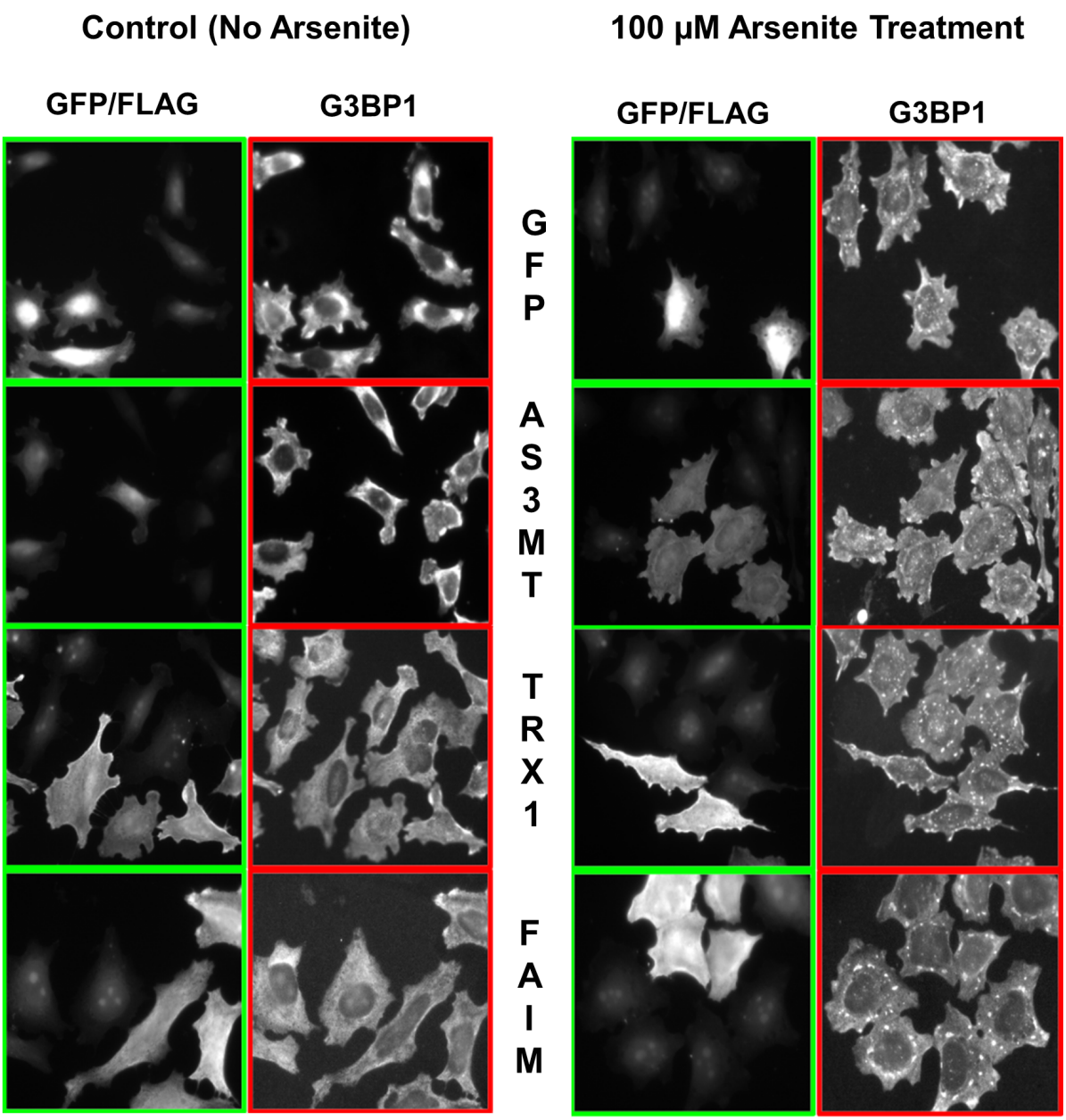

Figure 8

Both columns of the image contain HeLa cells, showing the GFP/FLAG flag channel bordered in green, where cells expressing the proteins are the brightest. The G3BP1 channel is bordered in red, and shows G3BP1, a protein which drives SG formation, as bright points within a cell. The left column shows untreated cells, while the right column shows cells treated with 100 μm arsenite. Fields of vision are the same in all pairs of images to allow for qualitative comparison of SG formation in transfected and untransfected cells.

In order to assess the qualitative morphology of the SGs forming in transfected cells and as a complement to the aforementioned refined results, images of each of the test conditions were taken and displayed in Fig. 8. These figures show the morphology changes that occur in each of the cells in response to expression of these proteins. Overall, morphology differences in cells expressing stress granules are slight, but in some cases SGs are smaller or less frequently occurring in cells which express these proteins.

3.4 TRX1, TXNRD1, GPX4 and GSR Co-Localize with G3BP1

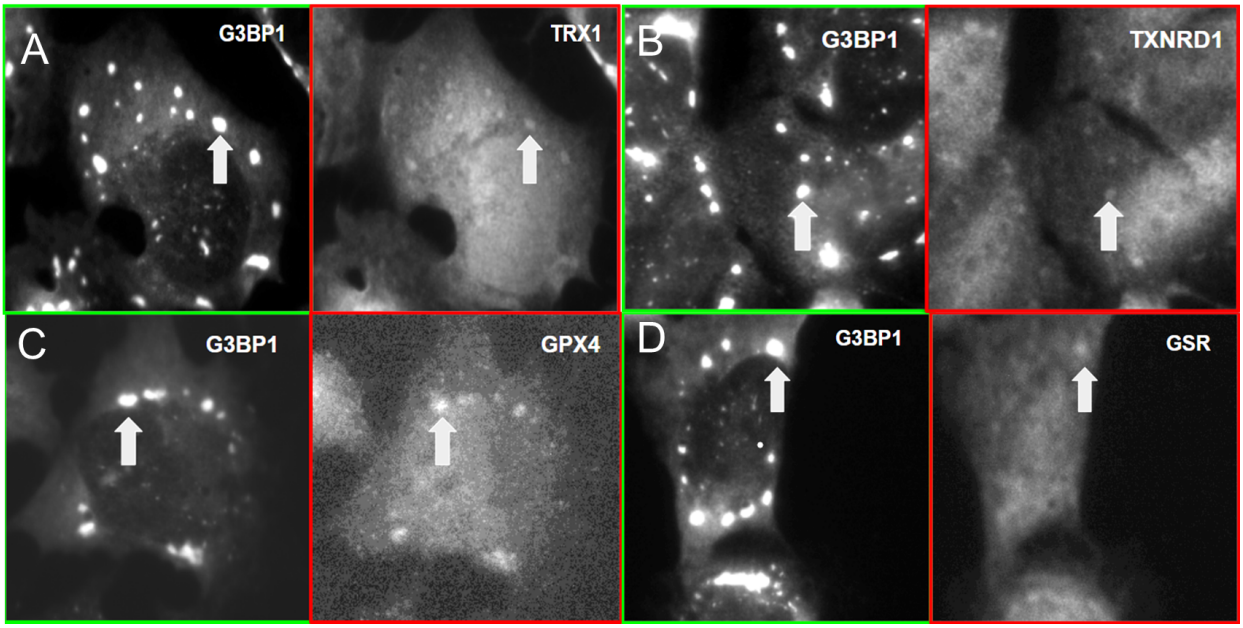

Figure 9

Pairs of images taken with the same frame of reference showing G3BP1 co-localization with a.) TRX1 b.) TXNRD1 c.) GPX4 and d.) GSR. White arrows are included to mark points of strong co-localization determined through qualitative microscopy.

To assess the localization of key oxidative stress proteins during arsenite stress, co-stains were performed between G3BP1 and TRX1, TXNRD1, GPX4 and GSR. The results of these stains are shown above in Fig 9. The results are strictly qualitative, but show that all 4 proteins in varying degrees co-localize with G3BP1 at SGs. The strongest localization is in GPX4, while TRX1 and TXNRD1 localize to a slightly less degree. Localization with GSR appears to be present to a far lesser degree than the other 3 proteins, and only occurs in some SGs viewed in the G3BP1 channel. The results confirm that proteins that deal most directly with oxidative protein damage, TRX1 and the associated TRX1 reductase TRXRD1, are localized to SGs, whereas the proteins of the glutathione pathway may be less involved with SG formation.

4. Discussion

This study was primarily focused on procedural refinement, where it was able to accomplish substantial improvements relating to the study of AS3MT, TRX1 and FAIM expression in relation to SG suppression. Unfortunately, due to major time constraints, results from refined experimentation were not collected, partially as a result of necessity for further refinement. In trial A of the refined experiment, many of the cells experienced death following transfection. Although this did not appear to significantly impact the results of the trial, it resulted in only half of the samples being able to be scored. In trial B the transfection of GFP proved to be extremely inefficient, resulting in far fewer countable cells than the desired experimental threshold. As a result of this, the data obtained for the control in trial B varied drastically from that shown in trial A, making it difficult to draw distinct conclusions from either of the experiments. Despite this, comparing the AS3MT, TRX1 and FAIM to the control GFP from trial A shows that all 3 were able to decrease SG formation in varying degrees. This is a promising result, and should be able to be supported or refuted with relative ease now that the experiment has been further refined.

Another important result that came from this project was the confirmation that TRX1 co-localized with G3BP1, and showed that G3BP1 also co-localized with TXNRD1, GPX4 and (to a lesser extent) GSR. All of these proteins deal with relieving oxidative stress, and as a result may be related to SG neutralization. Now that their localization has been confirmed, the next step in this process will be to determine if these proteins are catalytically active at the SGs, or if they are simply being sequestered along with the rest of the proteasome.

This project showed that transfection could be achieved with very low spontaneous SG formation, which will further enable transient transfection to be used to assess the function and morphology of SGs in the future. SGs will be able to be better characterized and understood, and their overall biological effects can be better elucidated.

Additionally, because of arsenic's global prevalence and the negative impacts of chronic arsenic exposure, examining the abilities of these proteins to lower SG formation in cells may also reveal their ability to counteract the negative biological effects of arsenic. At this point, nothing can be said for certain about any of the protein's abilities to minimize SG production caused by arsenite, or if they can counteract the effects of the molecule. However, if this hypothesis is confirmed, and the proteins are able to counteract SG formation through alleviation of oxidative stress, perhaps they may find applications in treatments for chronic arsenic exposure, experienced in many parts of the world where arsenic is prevalent in groundwater.

References

Amen, T.; Kaganovich, D. Stress Granules Inhibit Fatty Acid Oxidation by Modulating Mitochondrial Permeability. *Cell Reports* **2021**, *35* (11), 109237.

Anderson, P.; Kedersha, N. RNA Granules. *Journal of Cell Biology* **2006**, *172* (6), 803–808.

Dheeman, D. S.; Packianathan, C.; Pillai, J. K.; Rosen, B. P. Pathway of Human AS3MT Arsenic Methylation. *Chemical Research in Toxicology* **2014**, *27* (11), 1979–1989.

Hemond, M.; Rothstein, T. L.; Wagner, G. FAS Apoptosis Inhibitory Molecule Contains a Novel β-Sandwich in Contact with a Partially Ordered Domain. *Journal of Molecular Biology* **2009**, *386* (4), 1024–1037.

Jovanovic, B.; Eiermann, N.; Talwar, D.; Boulougouri, M.; Dick, T. P.; Stoecklin, G. Thioredoxin 1 Is Required for Stress Granule Assembly upon Arsenite-Induced Oxidative Stress. *Food and Chemical Toxicology* **2021**, *156*, 112508.

Kaku, H.; Rothstein, T. L. Faim Is a Non-Redundant Defender of Cellular Viability in the Face of Heat and Oxidative Stress and Interferes with Accumulation of Stress-Induced Protein Aggregates. *Frontiers in Molecular Biosciences* **2020**, *7*.

Kedersha, N. L.; Gupta, M.; Li, W.; Miller, I.; Anderson, P. RNA-Binding Proteins TIA-1 and Tiar Link the Phosphorylation of EIF-2α to the Assembly of Mammalian Stress Granules. *Journal of Cell Biology* **1999**, *147* (7), 1431–1442.

Kriehuber, T.; Rattei, T.; Weinmaier, T.; Bepperling, A.; Haslbeck, M.; Buchner, J. Independent Evolution of the Core Domain and Its Flanking Sequences in Small Heat Shock Proteins. *The FASEB Journal* **2010**, *24* (10), 3633–3642.

McEwen, E.; Kedersha, N.; Song, B.; Scheuner, D.; Gilks, N.; Han, A.; Chen, J.-J.; Anderson, P.; Kaufman, R. J. Heme-Regulated Inhibitor Kinase-Mediated Phosphorylation of Eukaryotic Translation Initiation Factor 2 Inhibits Translation, Induces Stress Granule Formation, and Mediates Survival upon Arsenite Exposure. *Journal of Biological Chemistry* **2005**, *280* (17), 16925–16933.

Planells-Ferrer, L.; Urresti, J.; Coccia, E.; Galenkamp, K. M.; Calleja-Yagüe, I.; López-Soriano, J.; Carriba, P.; Barneda-Zahonero, B.; Segura, M. F.; Comella, J. X. FAS Apoptosis Inhibitory Molecules: More than Death-Receptor Antagonists in the Nervous System. *Journal of Neurochemistry* **2016**, *139* (1), 11–21.

Shao, D.; Oka, S.-ichi; Liu, T.; Zhai, P.; Ago, T.; Sciarretta, S.; Li, H.; Sadoshima, J. A Redox-Dependent Mechanism for Regulation of AMPK Activation by thioredoxin1 during Energy Starvation. Cell Metabolism **2014**, 19 (2), 232–245.

Wang, Z.; Zhang, H.; Li, X.-F.; Le, X. C. Study of Interactions between Arsenicals and Thioredoxins (Human Ande. Coli) Using Mass Spectrometry. *Rapid Communications in Mass Spectrometry* **2007**, *21* (22), 3658–3666.

Wek, R. C. Role of eIF2α Kinases in Translational Control and Adaptation to Cellular Stress. *Cold Spring Harbor Perspectives in Biology* **2018**, *10* (7).

Wolozin, B.; Ivanov, P. Stress Granules and Neurodegeneration. *Nature Reviews Neuroscience* **2019**, *20* (11), 649–666.

Appendix

Summary Data	Within each box: Item 1 = N Item 2 = ΣX Item 3 = Mean Item 4 = ΣX² Item 5 = Variance Item 6 = Std. Dev. Item 7 = Std. Err.				
	C1	**C2**	**C3**	**C4**	**Tot.**
R1	3 149.19 49.73 7962.965899999999 271.87 16.49 9.52	3 204.76999999999998 68.2567 15181.3441 602.21 24.54 14.17	3 276.58000000000004 92.1933 25556.103600000002 28.64 5.35 3.09	3 283.16999999999996 94.39 26737.238900000004 4.41 2.1 1.21	12 913.71 76.1425 75437.6525 533.23 23.09 6.67
R2	4 148.57999999999998 37.145 5815.7778 98.92 9.95 4.97	4 236.16000000000003 59.04 15389.0822 482.07 21.96 10.98	4 356.23 89.0575 31819.163500000002 31.4 5.6 2.8	4 360.71000000000004 90.1775 32573.242100000003 15.11 3.89 1.94	16 1101.68 68.855 85597.2656 649.41 25.48 6.37
R3	3 86.9 28.9667 2602.8147999999997 42.81 6.54 3.78	3 200.04 66.68 15653.2194 1157.28 34.02 19.64	3 254.57999999999998 84.86 21715.439 55.89 7.48 4.32	3 252.24 84.08 21380.4488 86.05 9.28 5.36	12 793.76 66.1467 61351.922 804.3 28.36 8.19
R4	---	---	---	---	---
Tot.	10 384.67 38.467 16381.5585 176.05 13.27 4.2	10 640.97 64.097 46223.6457 571.04 23.9 7.56	10 887.39 88.739 79090.7061 38.29 6.19 1.96	10 896.12 89.612 80690.9298 43.09 6.56 2.08	40 2809.15 70.2288 222386.8401 643.69 25.37 4.01

ANOVA Summary					
Source	**SS**	**df**	**MS**	**F**	**P**
Rows	649.83	2	324.92	1.43	0.2563
Columns	17647.47	3	5882.49	25.81	<.0001
r x c	425.63	6	70.94	0.31	0.9264
Error	6380.82	28	227.89		
Total	25103.75	39			

Critical Values for the Tukey HSD Test		
	HSD[.05]	**HSD[.01]**
Rows [3]	14.61	18.71
Columns [4]	18.45	23.08
Cells [12]	42.02	49.97

HSD=the absolute [unsigned] difference between any two means (row means, column means

Appendix 1, 24 hour hold data.

Summary Data	Within each box: Item 1 = N Item 2 = ΣX Item 3 = Mean Item 4 = ΣX^2 Item 5 = Variance Item 6 = Std. Dev. Item 7 = Std. Err.				
	C1	**C2**	**C3**	**C4**	**Tot.**
R1	3 122.75 40.9167 5425.3499 201.41 14.19 8.19	3 267.6 89.2 23912.0946 21.09 4.59 2.65	3 275.58000000000004 91.86 25340.3462 12.78 3.58 2.06	3 281.21999999999997 93.74 26374.800199999998 6.62 2.57 1.49	12 947.15 78.9292 81052.5909 572.26 23.92 6.91
R2	3 117.66 39.22 4637.0466 11.21 3.35 1.93	3 274.65 91.55 25180.1091 17.95 4.24 2.45	3 275.59000000000003 91.8633 25360.4003 21.89 4.68 2.7	3 273.49 91.1633 24955.223299999998 11.48 3.39 1.96	12 941.39 78.4492 80132.7793 571.05 23.9 6.9
R3	3 76.78 25.5933 2023.4078 29.18 5.4 3.12	3 251.24 83.7467 21145.779799999997 52.63 7.25 4.19	3 246.70999999999998 82.2367 20338.356099999997 24.87 4.99 2.88	3 261.40999999999997 87.1367 22863.194900000002 42.4 6.51 3.76	12 836.14 69.6783 66370.7386 737.26 27.15 7.84
R4	---	---	---	---	---
Tot.	9 317.19 35.2433 12085.8043 113.37 10.65 3.55	9 793.49 88.1656 70237.9835 34.94 5.91 1.97	9 797.88 88.6533 71039.1026 38.05 6.17 2.06	9 816.12 90.68 74193.2184 23.43 4.84 1.61	36 2724.68 75.6856 227556.1088 609.63 24.69 4.12

ANOVA Summary					
Source	**SS**	**df**	**MS**	**F**	**P**
Rows	650.94	2	325.47	8.61	0.0015
Columns	19658.88	3	6552.96	173.39	<.0001
r x c	120.33	6	20.06	0.53	0.78
Error	907.04	24	37.79		
Total	21337.19	35			

Critical Values for the Tukey HSD Test			
	HSD[.05]	**HSD[.01]**	
Rows [3]	6.27	8.08	
Columns [4]	8	10.07	
Cells [12]	18.11	21.71	
			HSD=the absolute [unsigned] difference between any two means (row means, co means, only if the column effect is significant; and between cell means, only if th

Appendix 2, 48 hour hold data.

Trial 1-24	SG -	SG +
GFP 0	142	63
GFP 100	126	88
GFP 250	28	180
GFP 500	12	193
AS3MT 0	165	51
AS3MT 100	131	76
AS3MT 250	38	174
AS3MT 500	30	174
FL-TRX1 0	165	46
FL-TRX1 100	150	58
FL-TRX1 250	48	155
FL-TRX1 500	53	147

Trial 2- 24	SG -	SG +
GFP 0	83	126
GFP 100	52	154
GFP 250	15	195
GFP 500	16	195
AS3MT 0	128	82
AS3MT 100	106	100
AS3MT 250	22	187
AS3MT 500	19	184

Trial 3-24	SG -	SG +
GFP 0	87	121
GFP 100	23	184
GFP 250	6	207
GFP 500	7	199
AS3MT 0	118	107
AS3MT 100	75	129
AS3MT 250	9	205
AS3MT 500	12	218
TRX1 0	136	72
TRX1 100	40	168
TRX1 250	25	181
TRX1 500	19	188

Trial 4-24	SG-	SG+
AS3MT 0	135	84
AS3MT 100	27	192
AS3MT 250	23	184
AS3MT 500	21	189
TRX1 0	146	64
TRX1 100	18	191
TRX1 250	21	197
TRX1 500	25	182

Appendix 3, Raw data for the initial 24 Hr hold experiment

Trial 2-48	SG -	SG +
GFP 0	93	112
GFP 100	32	177
GFP 250	26	186
GFP 500	17	204
AS3MT 0	121	90
AS3MT 100	28	182
AS3MT 250	26	169
AS3MT 500	26	179
TRX1 0	152	54
TRX1 100	50	153
TRX1 250	49	165
TRX1 500	44	172

Trial 3-48	SG -	SG +
GFP 0	121	87
GFP 100	24	195
GFP 250	14	208
GFP 500	16	189
AS3MT 0	130	73
AS3MT 100	12	198
AS3MT 250	9	203
AS3MT 500	16	196
TRX1 0	147	65
TRX1 100	26	188
TRX1 250	26	175
TRX1 500	18	188

Trial 4-48	SG -	SG +
GFP 0	157	56
GFP 100	13	199
GFP 250	12	193
GFP 500	7	206
AS3MT 0	128	82
AS3MT 100	13	193
AS3MT 250	14	191
AS3MT 500	13	194
TRX1 0	169	42
TRX1 100	26	191
TRX1 250	37	175
TRX1 500	20	191

Appendix 4, Raw data for the initial 48 Hr hold experiment

Plasmid/Condition	Cells Counted	Transfected		% Transfected
GFP 1x 24 HR	232	63		27.15517
AS3MT 2X 48 HR	274	42		15.32847
AS3MT 2X 24 HR	143	92		64.33566
TRX1 2X 24 HR	373	81		21.71582
AS3MT 1X 24 HR	218	67		30.73394
GFP 2X 24 HR	147	54		36.73469
TRX1 1X 24 HR	176	66		37.5
AS3MT 1X 48 HR	234	47		20.08547
GFP 2X 48 HR	185	86		46.48649

TRX1 1X 48 HR	212	16		7.54717
TRX1 2X 48 HR	225	17		7.555556
GFP 1X 48 HR	211	78		36.96682

Appendix 5, Raw data for the transfection refinement experiment

Condition	1 (Trial B)				2 (Trial B)				3 (Trial A)		
	No SG	Yes SG	% SG		No SG	Yes SG	% SG		No SG	Yes SG	% SG
GFP Control	203	11	5.14		56	2	3.45		207	9	4.166667
GFP Ars	22	5	18.52		6	1	14.29		79	127	61.65049
AS3MT Control	212	4	1.85		189	13	6.44		209	1	0.47619
AS3MT Ars	60	149	71.29		187	22	10.53		143	63	30.58252
Trx1 Cont	194	18	8.49		178	24	11.88		194	13	6.280193
Trx1 Ars	134	74	35.58		154	54	25.96		89	112	55.72139
FAIM Control	216	5	2.26		197	9	4.37		217	9	3.982301
FAIM Ars	135	67	33.17		193	15	7.21		102	62	37.80488

Appendix 6, Raw data for Trial A and Trial B of the refined experiment