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Changes in Acute Stress Response in Cells of Patients Affected with Fragile X Syndrome

A Major Qualifying Project

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Abstract

Fragile X Syndrome is characterized by loss of function of the protein FMRP, which causes autism spectrum disorders and mental retardation. Stress granules are dynamic aggregations of stalled mRNA and protein formed during the cellular stress response to halt their translation and promote cell survival. FMRP reversibly halts translation in cells, and loss of function may result in upregulated translation in FXS-affected cells. Therefore, we hypothesized that FXS cells would form stress granules at a faster rate, and/or at a lower level of environmental stressor, than wild type cells. We used fluorescent microscopy to quantify stress granule formation in wild type and FXS-affected human B lymphocytes. Our results suggest that loss of FMRP does not contribute to higher stress sensitivity in FXS-affected cells.

Acknowledgements

We would like to thank everyone that helped make our project successful, including the BBT department at WPI for assisting us throughout our MQP. We especially appreciate advisors, Professors Natalie Farny and Lou Roberts, for their patience and their valuable input throughout the development of our project and report. Their guidance helped us become better scientists and students over the course of this year.

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1.0 Introduction

1.1 Cellular Stress Response

Maintenance of protein homeostasis in the cell requires regulation of transcription and translation to produce optimal protein levels. The regulation of protein synthesis is central to cell survival and growth because of the energetic requirements of cell metabolism (Advani & Ivanov, 2019). Regulation is particularly critical during periods of stress, where cell survival depends on quick and efficient mRNA translation reprogramming to conserve energy and repair damage (Advani & Ivanov, 2019). When cells encounter stress-inducing environmental conditions, a network of adaptive stress responses are activated. Stress stimuli range from abiotic factors including temperature, UV irradiation, and oxidative stress, to biotic, including viral infections and toxins. These stimuli activate the cellular integrative stress response (ISR), changing its gene expression to funnel energy towards stress adaptation and restoration of homeostasis (Advani & Ivanov, 2019).

1.1.1 Translational Control

The process of translation can be divided into four main stages: initiation, elongation, termination, and ribosome recycling (Advani & Ivanov, 2019). Translational control is used to regulate gene expression throughout a wide range of conditions (Gebauer & Hentze, 2004). Cells can regulate translation of mRNAs, most frequently in an inhibitory manner, by modifying translation initiation factors (IF). There are more than 25 polypeptides involved in translation initiation in eukaryotes, a complex process that involves many eukaryotic initiation factors (eIFs) and the small ribosomal subunit. Most regulatory events occur at this step; once elongation begins, the protein is nearly always synthesized completely (Advani & Ivanov, 2019).

During translation initiation, the small (40S) ribosomal subunit forms a 43S pre-initiation complex with other initiation factors (Gebauer & Hentze, 2004). The 43S complex contains several eIFs, which perform different functions in mRNA binding and scanning (Gebauer & Hentze, 2004). Translational control of protein synthesis often occurs through phosphorylation of these eIFs or their regulators (Gebauer & Hentze, 2004). Two translation initiation factors, eIF2 and eIF4E, are main targets of inhibitory translational control because their activities are required in most eukaryotic cells for translation initiation (Advani & Ivanov, 2019). Stress-activated kinases

regulate translation by altering the phosphorylation state of eIFs to inhibit initiation, which subsequently can either induce cell apoptosis or reduce injury to the cell (Advani & Ivanov, 2019).

The ability to relieve stress-related cell injury depends upon phosphorylation of eIF2 to reduce global translation and conserve energy. Stress-induced phosphorylation of the of the α-subunit of eIF2 occurs at serine 51. This impedes transition to the GTP-bound active form required for translation initiation (Gebauer & Hentze, 2004). This pause in translation allows the cell to adapt gene expression and rewire signaling pathways to respond effectively to the stressor. During this time, translation of a specific subset of mRNAs is upregulated to promote cell survival (Advani & Ivanov, 2019). Translation is then resumed upon withdrawal of the stressor stimulus.

1.1.2 Stress Granules

Activation of the cellular stress response can result in the formation of stress granules. Stress granules are dynamic aggregations of stalled mRNA-protein complexes (mRNPs) that quickly form in response to a variety of environmental stressors (Anderson & Kedersha, 2009). The formation of stress granules is initiated by the phosphorylation of eIF2α, as shown in Figure 1 (Kedersha et al. 1999). Phosphorylation of the alpha subunit prevents assembly of the 43S preinitiation complex required for translation. This is both necessary and sufficient for the assembly of stress granules in a cell (Kedersha et al., 1999).

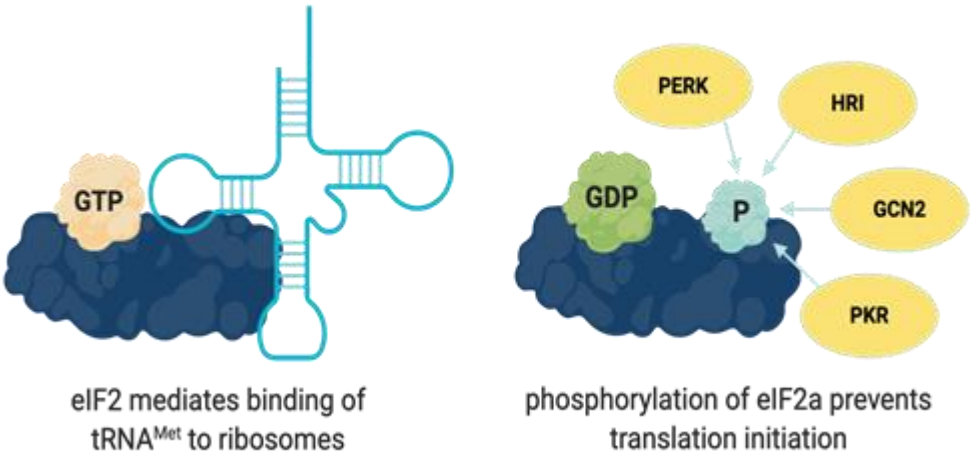


Figure 1. During normal translation, initiation factor eIF2 mediates binding of tRNA carrying methionine to the ribosome at the start location. However, phosphorylation of the eIF2α subunit prevents translation initiation, which is both necessary and sufficient for stress granule assembly.

The components of stress granules are highly dependent on RNA movement, and proteins are observed to exchange rapidly in the cytosol (Protter and Parker, 2016; Advani & Ivanov, 2019). Most parts of the 48S preinitiation complex, including small ribosomal subunits, are recruited to stress granules. However, eIF2α and large ribosomal subunits are typically not components of stress granules (Kedersha et al. 2001). Stress granules are believed to be sites of mRNA triage, where mRNAs are sorted for storage, degradation, or translation during stressor events to conserve cell resources and promote survival (Anderson and Kedersha, 2009).

1.1.3 Types of Stress

In mammals, there are four protein kinases that phosphorylate eIF2α during a stress response. They consist of general control nonderepressible-2 kinase (GCN2), PKR-like ER kinase (PERK), protein kinase RNA (PKR), and heme-regulated inhibitor kinase (HRI) (Gebauer & Hentze, 2004). Each kinase is activated in response to a specific type of environmental stressor as shown in Figure 2. GCN2 is induced when the cell experiences a lack of nutrients (e.g., amino acid deprivation), as well as UV irradiation, and inhibition of the proteasome. PERK responds to misfolded proteins in the endoplasmic reticulum (ER stress). In response to double-stranded viral RNA, PKR participates in antiviral responses mediated by interferon. HRI is activated by the lack of heme, oxidative stress, and heat stress in tissues with high blood content (Wek et al. 2006).

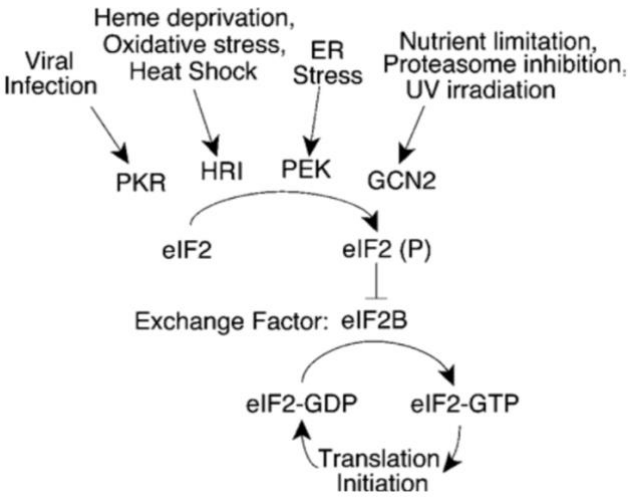


Figure 2. Types of stress that activate each kinase-mediated stress response in mammalian cells. These pathways result in the phosphorylation of eIF2α. The stress response generally results in lowered protein levels due to translation restriction (Wek et al. 2006).

1.2 Fragile X Syndrome

Fragile X syndrome (FXS) is a hereditary disease that causes autism spectrum disorders and mental retardation. The disease is caused by inactivation or loss of function of the *FMR1* gene on the X chromosome that encodes the translational repressor fragile X mental retardation protein (FMRP) (Sabaratnam, 2006). FMRP is an RNA-binding protein found in neuronal soma and synaptodendrites, specifically targeting mRNAs in their coding region to repress translation (Darnell et al. 2011). FMRP interacts with its target mRNA transcripts to reversibly halt ribosome translocation (Darnell et al. 2011). FMRP is critical for regulation of local dendritic protein synthesis, which enables synapses to autonomously alter structure and function. This was shown with *Fmr1* knockout studies in mice, when a missense mutation introduced in the RNA binding domain of FMRP resulted in an FXS phenotype (Darnell et al. 2011). Loss of FMRP also causes deficits in synaptic architecture and plasticity, which are most likely responsible for behavioral and cognitive dysfunctions found in FXS patients and animal models (Jacquemont et al. 2018; Udagawa et al. 2013). However, it is unknown to which extent FXS may be due to broad translational dysregulation as opposed to abnormal translation regulation of a few transcripts (Jacquemont et al. 2018).

FMRP loss of function results in upregulation of mRNA translation due to loss of ribosome stalling regulation. Several studies in *Fmr1* knockout mice found a ~20% increase in protein synthesis and increased levels of FMRP target mRNAs (Richter et al. 2015, Udagawa et al. 2013). In wild type cells, FMRP may slow translocation of ribosomes across the mRNA during elongation, repress translation, and reduce total amount of protein in the cell. These processes are disrupted in FXS cells, resulting in more polypeptide translation from FMRP target mRNAs (Darnell et al. 2011, Udagawa et al. 2013).

1.3 Linking Fragile X Syndrome and the Cellular Stress Response

Stress granules are aggregates of mRNAs that have been arrested from translation. Phosphorylation of eIF2α halts translation, causing other elongating ribosomes to run off the mRNA transcript and its recruitment to a stress granule (Kedersha et al. 2001). FMRP is known to bind to and slow translation of its target mRNAs by halting ribosome translocation. FMRP loss of function causes ribosomes to move faster along mRNAs and produce more polypeptides. When

eIF2α phosphorylation halts translation initiation in FXS cells, their associated ribosomes should run off the mRNA more quickly. This may result in stress granule formation at earlier time points or at lower levels of environmental stress compared to wild type cells (Anderson and Kedersha, 2009).

Sodium arsenite ($NaAsO_2$), a stressor that acts through the HRI pathway (McEwen et al. 2005), has been found to be sufficient for stress granule formation at high concentrations (Kedersha et al. 1999). Previous work in this lab had observed a greater sensitivity of FXS lymphocytes to high-dose (500μM) sodium arsenite stress as demonstrated by an increase in SG formation (Merrill et al 2019). This data indicates that FXS cells may be more sensitive to acute levels of stress (Merrill et al., 2019). In this study we further investigate the time points and threshold required for sodium arsenite to trigger stress granule formation in wild type and FXS human B lymphocytes. We hypothesize that FXS cells will form stress granules at a faster rate, and/or at a lower level of environmental stressor, than wild type cells. We predict that increased translation in cells of some FXS patients will result in quicker aggregation of mRNAs into stress granules in FXS cells. Our research will also expand the specificity of earlier findings by analyzing a broader range of stress conditions.

2.0 Materials and Methods

2.1 Cell Line Maintenance

Wild type and FXS human B lymphocytes from siblings were a generous gift from Dr. Joel Richter (University of Massachusetts Medical School, Worcester, MA). The cells were maintained in 1x RPMI (Sigma Life Science, Catalog No: R8758) with 15% fetal bovine serum, and 1% penicillin/streptomycin. Human B lymphocytes were subcultured in suspension every 3-4 days. To subculture, cells were resuspended in cell medium and counted, then reseeded at 200,000 cells/mL. New RPMI medium was added to bring the total volume to 25 mL. A new T75 flask was used every three passages. Cells were incubated at 37°C with 5% CO_2.

2.2 Antibodies and Reagents

Coating solutions from commercial sources include poly-L-lysine (Sigma Life Science, Catalog No: P4707), poly-L-ornithine solution (Sigma Aldrich Catalog No: P4957), and collagen (Sigma Aldrich, Catalog No. 125-50). Primary antibody used was anti-G3BP (Abcam, Catalog No: #181150(EPR13986(B))). Anti-rabbit IgG secondary antibody with Alexa 594 fluorescent marker (Cell Signaling Technology, Catalog No: 8889S) and Hoechst nucleusar stain (Life Technologies, Catalog No: 333342) were used in immunofluorescence staining.

2.3 Cell Plating

Cells were plated on 11 mm coverslips in 12-well plates. Plates and coverslips were irradiated with UV light for several minutes to sterilize. For plating, a range of coating methods were tested to determine the optimal procedure for adherence of suspended human B lymphocytes to coverslips. Initial experimental protocol utilized 0.5 mL/well of poly-L-lysine incubated at 37°C for 30 minutes. Collagen and poly-L-ornithine were also trialed to examine cell adherence. Poly-L-ornithine was found to be most effective and was utilized for all subsequent experiments. Coating was incubated for 30 minutes at 37°C, then removed. Wells were rinsed three times with ultrapure filtered water and air dried in the hood.

Cells were plated in 0.5 mL at 2 x 10_6 cells per mL. To increase cell concentration as needed, cells were centrifuged at 1000 rpm for 5 minutes. Removed medium was saved for use in drug treatments. 0.5 mL of each cell type was added to wells for the negative control and three

treatments. The plate was incubated overnight at 37°C to allow for cell growth and adherence to coated coverslips.

2.4 Stress Treatments and Immunofluorescence

Cells were subjected to acute environmental stress by incubating the plate with varying concentrations of arsenite at 37°C for different time periods. A dilute stock of sodium arsenite was created using ultrapure water and the stock solution of 500mM sodium arsenite was then further diluted using medium saved from cell plating. Treatments were administered using 0.5 mL of specified arsenite concentrations. The negative control (0 µM) well contained only used medium and no arsenite. Figure 3 shows an example of how the 12-well plates were set up for treatment.

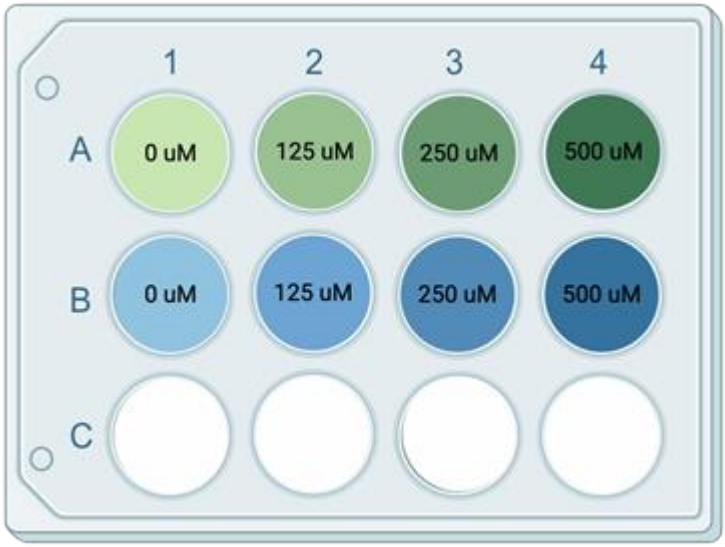


Figure 3. Acute exposure assay treatments at varying arsenite concentrations. The 12-well plate is labeled with corresponding arsenite concentrations used, between 0 µM (negative control for stress) and 500 µM.

Treatment medium was removed, and cells were fixed using 0.5 mL of 4% paraformaldehyde to examine stress granule formation at that time. Plates were placed on the shaker at slow speed. After ten minutes, 0.5 mL of methanol was added to each well and the plates were replaced on the shaker. Wells were rinsed three times with PBS, then treated with 0.5 mL of 5% bovine serum albumin (BSA), a nonspecific blocking solution. Blocking solution was applied for either 1 hour at room temperature or overnight at 4˚C.

The blocking solution was removed, 0.5mL of a solution containing primary antibody rbαG3BP at a 1:1000 ratio in blocking solution (1mL BSA for 1μL 1˚ antibody) was used for cytoplasmic protein G3BP. The primary antibody solution was removed after either 1 hour on the benchtop shaker or overnight at 4˚C. The wells were rinsed three times with PBS for 5 minutes each on the shaker. The secondary antibody solution of anti-rabbit IgG (Fab2) with Alexa 594 fluorescent marker was then added at 1:2000 (1mL BSA for 0.5μL 2˚ antibody, 0.5mL per well) to mark the bound G3BP and fluoresce red, indicating if stress granules had formed. Hoechst dye (1mL BSA for 0.4μL dye) was used to stain nuclei. Secondary antibody-stained plates were covered with foil for 45 minutes to prevent photons from interfering with fluorescence before being rinsed again with PBS three times. Coverslips were mounted using polyvinyl mounting medium.

2.5 Stress Granule Data Collection

Blinded slides were created using opaque laboratory tape and observed under the fluorescent microscope (Zeiss, Vert.A1, AXIO) at 20X objective (200x total magnification) and the red fluorescence filter (blue for observing nuclei). A two-channel counter was used to record the number of cells that contained stress granules and the ones that did not. At least three fields of view and over 100 cells were counted per coverslip. The percent of cells with stress granules present was calculated and recorded. As the adherence method was improved, a larger number of cells were available for counting (150-300), closer to a representative sample of the population of that well's coverslip. Once all coverslips were scored, they were unblinded and the data was entered into the master spreadsheet for statistical analysis. Each coverslip was counted twice, once by each group member, and the average was used.

2.6 Protein Composition and Translation Rate

To confirm the identity of each cell line, an FMRP western blot was performed using an anti-FMRP antibody and frozen samples of FXS-affected and WT cells. In FXS patients, the Fmr1 gene encoding FMRP is not expressed through transcriptional silencing, resulting in lack of FMRP protein. Therefore, there should be no FMRP detected by western blot in the FXS-affected cells (Jacquemont et al. 2018). FMRP is present in WT cells and a band at 75-80 kDa should be seen.

To investigate the rate of translation in each cell line, a puromycin runoff experiment was conducted. 25 million total cells from each cell line were suspended in 1 mL of medium and placed on ice to slow ribosomes to create a true zero time point for translation speed. At t=0, 1µL of puromycin (10mg/mL stock) was added to all intact cells in each tube to a final concentration of 10µg/mL. At each time point 100 µL of cell suspension (that has been incorporating the puromycin into nascent proteins) received 100 µL of 2x SDS sample buffer to halt the reaction. A heat block at 37 ˚C was used to bring the cells back to full translation speed, and an ice bucket was used to keep the zero time points inactive. All samples were boiled, and the DNA was sheered with a small gauge needle in preparation of running an SDS-PAGE gel. 15 µL of each time point sample was loaded into corresponding lanes. A PVDF membrane was used to blot the gel and then probed twice, once with anti-puromycin antibody and once with anti-GAPDH antibody.

2.7 Data Analysis

Series of T tests were run to identify instances of significant difference in data sets. A two-tailed T test was used to determine significance in either direction and it was run based on precedent that the two data sets are similar in variance and were collected independently. T tests were run comparing percent of stressed cells of FXS cell line to the WT cell line at each arsenite concentration used. In addition, a T test was used to determine significance between the two time points (30 and 60 minutes) to infer if the duration of exposure was responsible for variation in percent stressed cells for that concentration of arsenite. A T test was also used to confirm that within each time duration of experiment the increase in percent stress was the result of the increase in arsenite concentration to which they were exposed.

The puromycin runoff blots were quantified using ImageJ software. Signal intensity was measured by the software within an area box. The intensity of the signal was calculated by subtracting the background value and calculating intensity per unit area above the background value. The individual lane values were taken for above (high molecular weight) and below (low molecular weight) the GAPDH signal. GAPDH and its antibody were applied to each lane as a load control to indicate the amount of total protein present in each lane. The initial time zero was used as the background value for all signals. The longest time duration of the experiment was set as the 100% intensity value and a ratio of sample signal/GAPDH signal was calculated for each lane on each blot to determine signal intensity average in relation to the opposing cell type.

3.0 Results

To confirm the identity of the cell lines used in these experiments, FMRP levels were examined via western blot analysis. The genotype of FXS-affected individuals involves loss of function of the *FMR1* gene encoding FMRP. Wild type cells have the native FMRP protein, and the FXS-affected cell line does not express FMRP (Jacquemont et al., 2018). Consistent with this knowledge, FMRP protein (between 70-80kDa) was detected in the WT cell sample after a 15-minute exposure. No FMRP was observed in the FXS-affected cell sample at either time point. Figure 4 shows the results of this experiment done in duplicate where the WT lanes show a band the size of FMRP while the FXS lanes show no band of that size, confirming that the cell lines were not mixed or swapped during the course of the experiments.

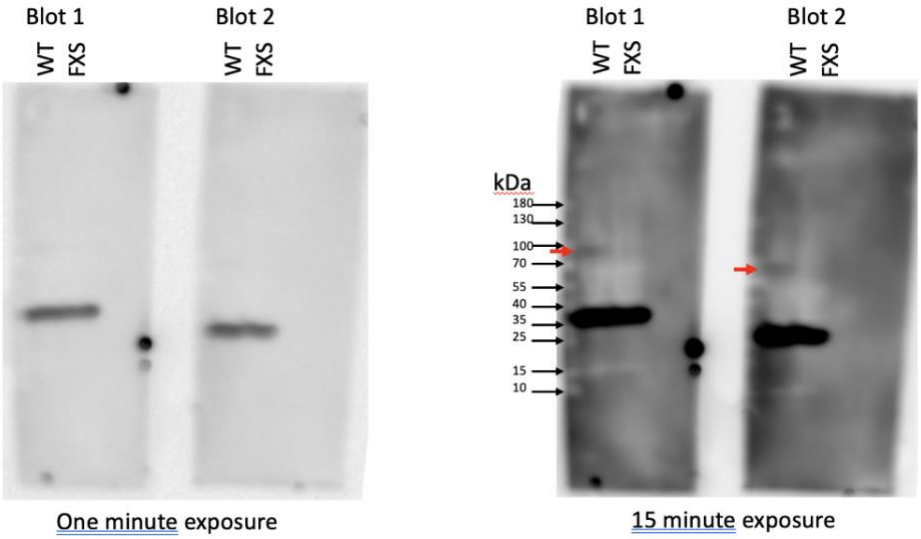


Figure 4. FMRP Western blot at 1 and 15-minute exposure times. No bands detected in 75-80kDa range at 1 minute. Distinct bands in WT samples between 100 and 75 kDa ladder on both blots at 15 minutes of exposure (red arrows in right hand panel). The dark band at approximately 35 kDa represents a non-specific band known to be detected by this antibody and consistent with manufacturer's reports.

Previous research has shown that cells form stress granules in response to a variety of environmental stress conditions (Advani & Ivanov, 2019). Studies have also found some FXS-affected cells to have a higher rate of protein translation compared to wild type cells (Richter et al. 2015, Udagawa et al. 2013). To examine the differences, if any, in the stress response and stress granule formation of the WT and FXS cell lines, we treated each cell line with arsenite and counted the number of cells that contained stress granules after 60 minutes treatment followed by staining (Figure 5).

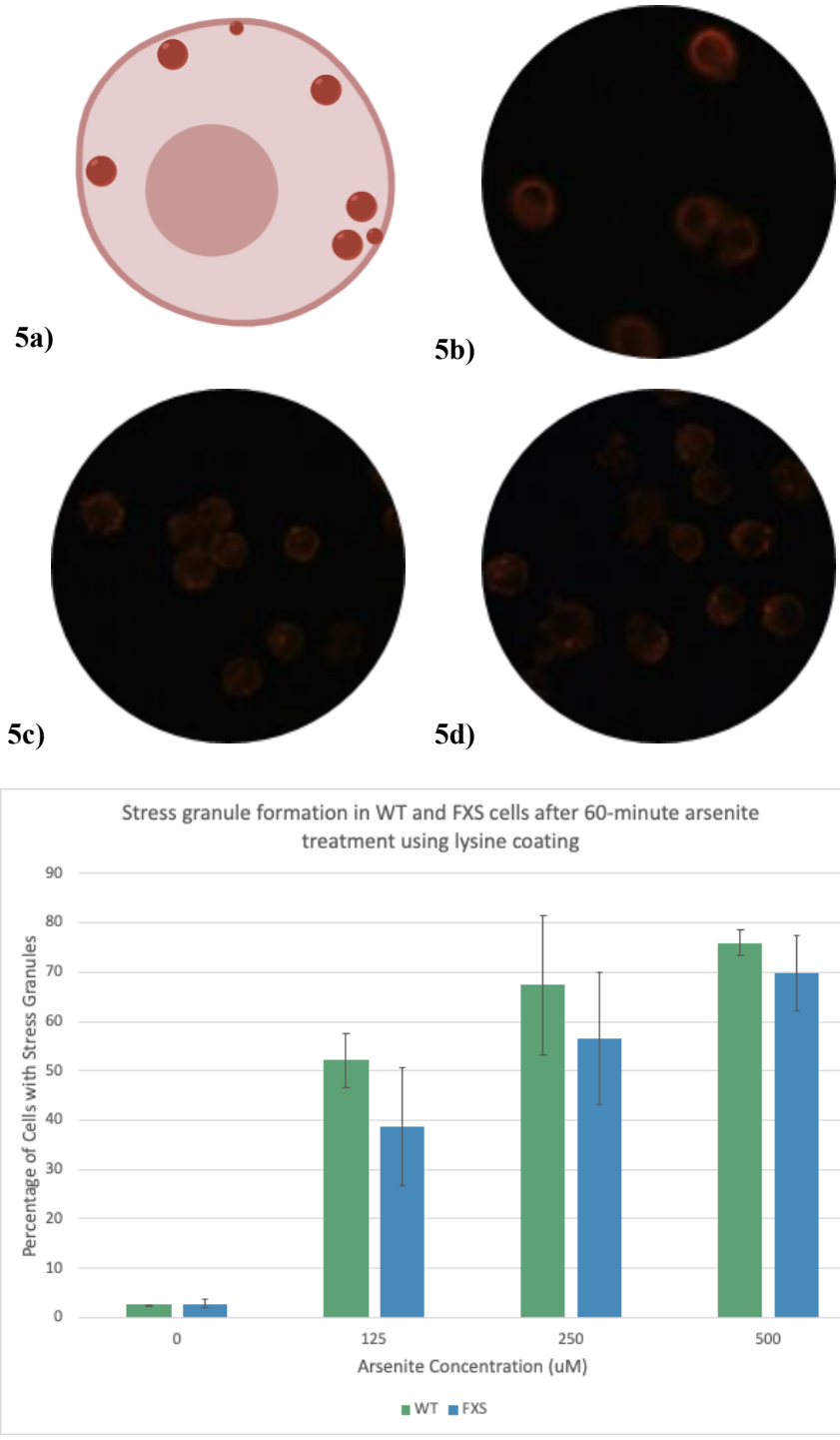


Figure 5. (a) Depiction of stress granules; cells with stress granules present as large glowing dots. (b) FXS cells treated with 0 µM arsenite for 60 minutes. No stress granules are present in this cell. (c) FXS cells treated with 250 µM arsenite for 60 minutes. (d) FXS cells treated with 500 µM arsenite for 60 minutes. Most cells in this image contain stress granules, which appear as glowing dots in cell cytoplasm. (e) Percentage of stress granule formation in FXS and WT cells with poly-L-lysine coated slides after 60-minute arsenite treatments at four different concentrations. Three replicates were performed and counted

once by each team member to obtain an average value. Error bars represent standard error of the mean (+/- SEM).

Our results showed no significant difference between the response of FXS-affected cells and WT cells to the same arsenite concentration. There was also no clear dose-dependent stress response when comparing the 125, 250, and 500 μM arsenite treatments of both wild type and FXS-affected cells.

To improve our sampling methods, coating reagents were compared. Poly-L-lysine was used in beginning trials as a coating agent to increase cell adherence when plating. This is one of a few common coating solutions used for nonadherent cell lines. Due to a low number of cells per slide when counting, the efficacies of two additional coating agents were examined (collagen and poly-L-ornithine). An experiment was performed using poly-L-lysine as one coating material and poly-L-ornithine as an alternative. Collagen (not shown here) had fewer cells than poly-L-lysine and was not pursued any further.

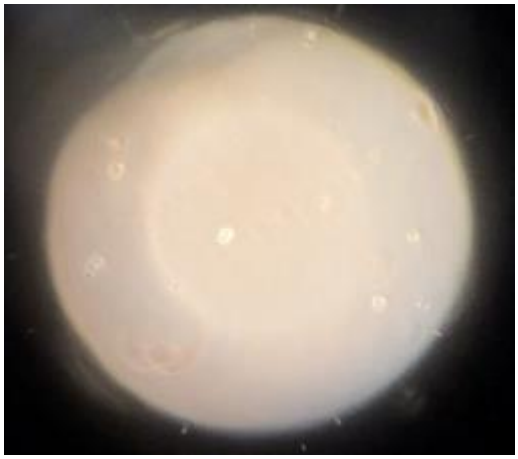
6a)  **6b)** 

Figure 6. Photos taken through microscope under 100X magnification; cells are visible as small, bright, circular forms. (a) Coverslip using poly-L-ornithine coating agent. (b) Coverslip using poly-L-lysine as a coating agent.

As seen in Figures 6a and 6b, a significantly higher number of cells were observed on the poly-L-ornithine-coated coverslip than the poly-L-lysine-coated coverslip. From this point we then used poly-L-ornithine as a coating agent, increasing the sample number of cells per coverslip for each experimental replicate. Increased cell counts give a greater probability that the sample is representative of the population and increases statistical validity.

To determine if stress granule formation was different in the cell lines over time, we conducted arsenite treatments at 30 and 60 minutes using the poly-L-ornithine coating. Four replicates of each arsenite concentration at both 30 minutes (Figure 7) and 60 minutes (Figure 8) were performed with ornithine coating.

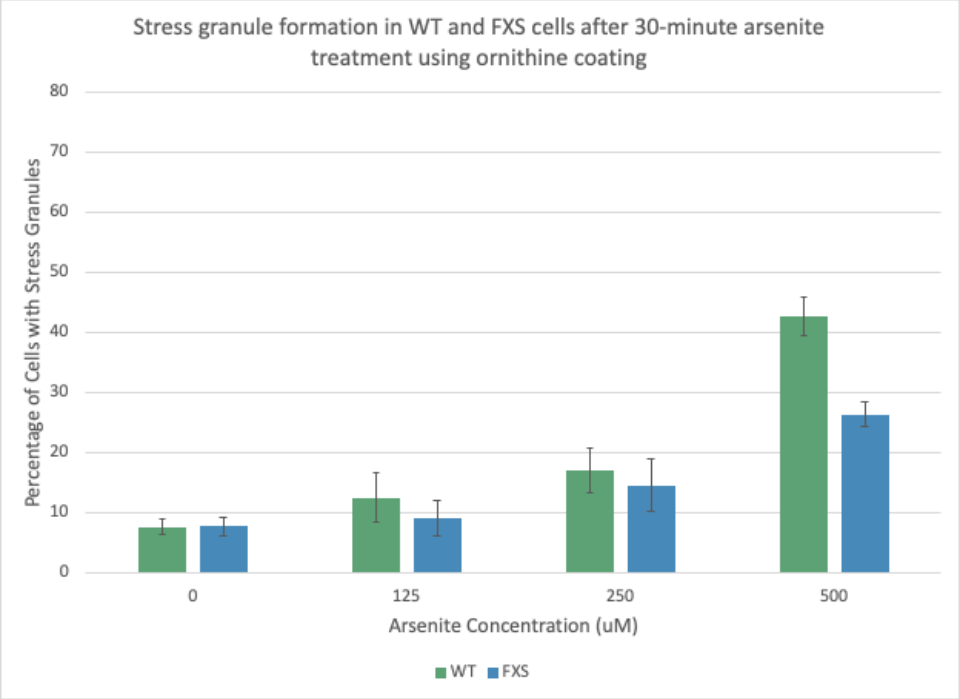


Figure 7. Stress granule formation in FXS and WT cells after 30-minute arsenite treatment. Coverslips were coated with poly-L-ornithine prior to cell plating to increase cell adherence. Cells were treated for 30 minutes with varying concentrations of arsenite. Four replicates were performed. Three of the replicates were counted twice, once by each group member. The last replicate was only counted once because of COVID-19 limitations. Error bars represent +/- SEM.

Upon treatment with 500 µM arsenite, the percentage of stressed WT cells was significantly higher than that of FXS cells after 30 minutes (Figure 7, $p=0.005$). There was no significant difference seen at any of the other concentrations of arsenite at this time point.

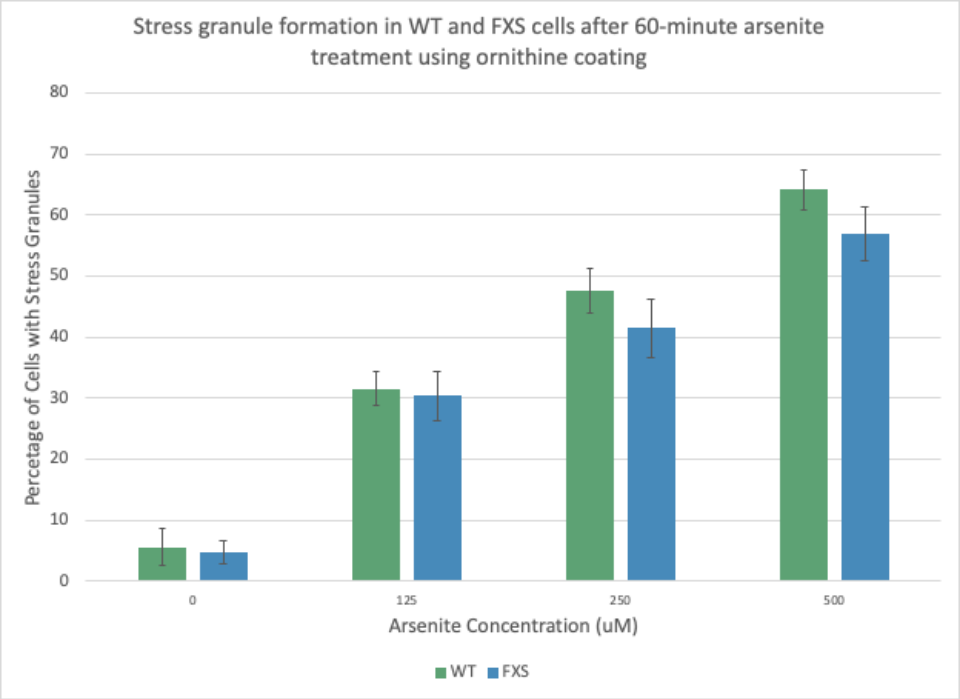


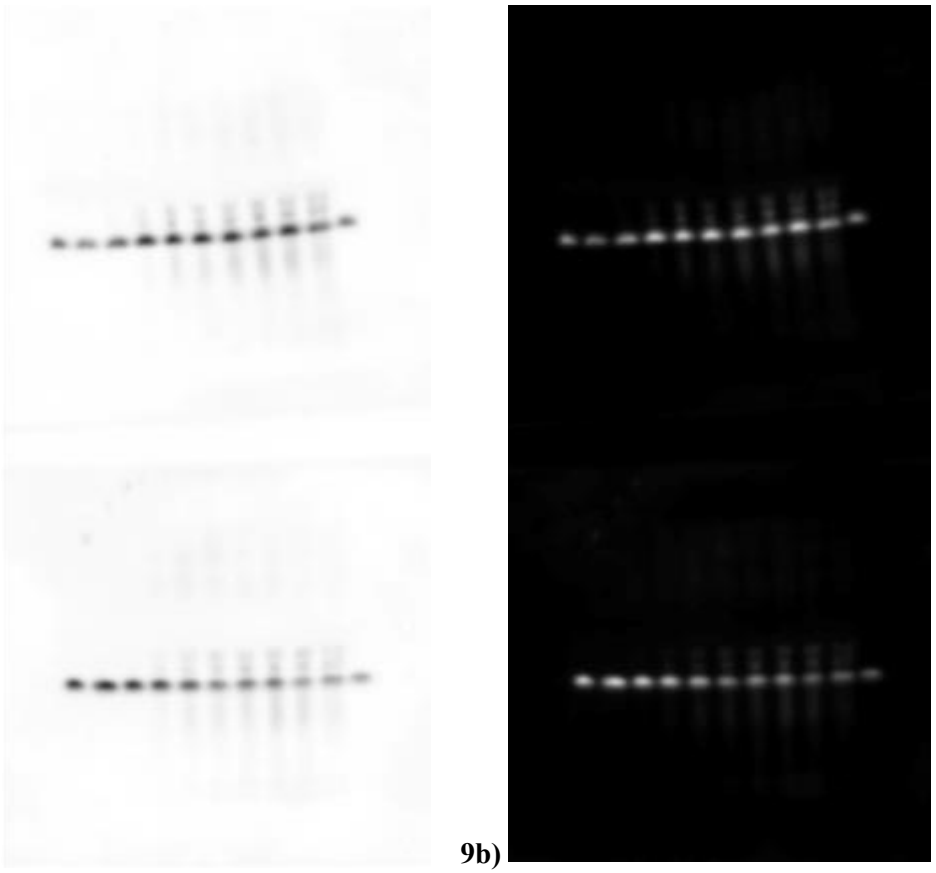
Figure 8. Stress granule formation in FXS and WT cells with ornithine-coated slides after 60-minute arsenite treatment. Coverslips were coated with poly-L-ornithine prior to cell plating to increase cell adherence. Cells were treated for 60 minutes with varying concentrations of arsenite. Four replicates were performed. Two of the replicates were counted twice, once by each group member. The last two replicates were only counted once because of COVID-19 limitations, which prevented access to the lab. Error bars represent +/- SEM.

At 60 minutes (Figure 8), there was an overall higher percentage of cells with stress granules for both the wild type and FXS cell lines than at 30 minutes, most likely due to the longer period of exposure to arsenite. This demonstrates a time-sensitive component of a stress response, as well as dose dependency.

Within the 60-minute treatment, no significant difference was found in stress granule formation between FXS-affected and WT cells. While not statistically significant, FXS-affected cells had a consistently lower stress response compared to WT cells at each arsenite concentration.

Previous research has indicated that some FXS-affected patients demonstrate an increased rate of protein translation due to lack of FMRP function as a repressor. To determine if differential stress granule formation was due to differences in protein synthesis rate, a puromycin-incorporating experiment was completed, and data points were collected at time intervals over 18 minutes.

Puromycin is a useful molecule for observing translation rates within cultured cells because it is essentially an analog of the stop codons' aminoacyl-adenosine terminus of the tRNA that accepts the growing polypeptide chain (Petska, 1971). The puromycin forms a peptide bond with the C terminal end of the growing peptide chain and terminates it prematurely by effectively blocking the site for any additional peptide bonds to form (Petska, 1971). The shape of puromycin facilitates the ribosome to eject the entire peptide with puromycin bound to it as if it was a termination factor (Petska, 1971). This ensures that all ribosomes actively translating when the puromycin is introduced into the cell can incorporate it into the nascent protein, thus tagging them as newly formed (Petska, 1971). As the cells are warmed back up and more protein is being translated, more puromycin is being incorporated, proportional to the protein synthesis rate. An antibody against puromycin is then used to detect the amount of protein synthesized after a given time and a synthesis rate can be compared between two cell lines, as seen in Figure 9.



9a) **9b)**

Figure 9. The puromycin runoff experiment blot with WT cells on the top, and FXS cells on the bottom. (a) Original blot image. (b) Inverted blot image, which was used to better observe signal intensity. The bold bands in the center of both blots are the GAPDH signals used as a control. The signal above and

below the GAPDH signals indicate high (above) and low (below) molecular weight proteins translated and tagged by puromycin. Signal intensity for all areas was calculated using ImageJ software.

ImageJ software was used to determine signal intensity of the puromycin antibody blot after also staining it with GAPDH antibody. At each time point, the signal intensities for GAPDH, high molecular weight proteins, and low molecular weight proteins were analyzed and recorded. Signal intensity was then normalized relative to GAPDH signals at each time point. The results were graphed over time separately for high and low molecular weight proteins (Figures 10 and 11).

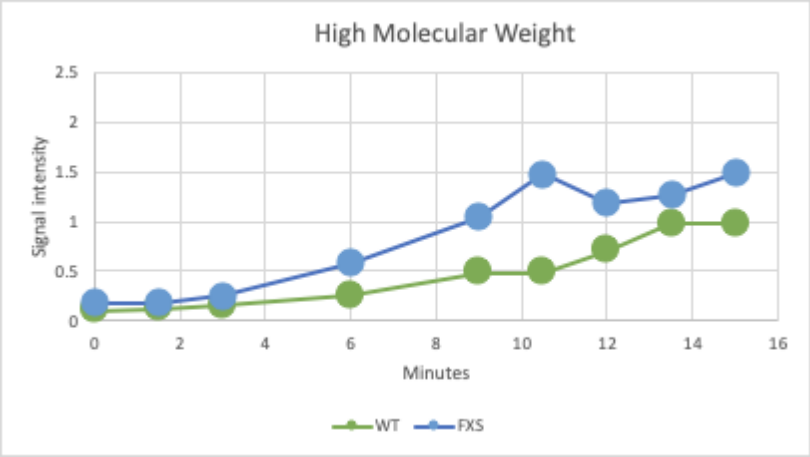


Figure 10. The puromycin run off blot quantification for wild type and FXS-affected cells for high molecular weight proteins over 18 minutes of puromycin treatment.

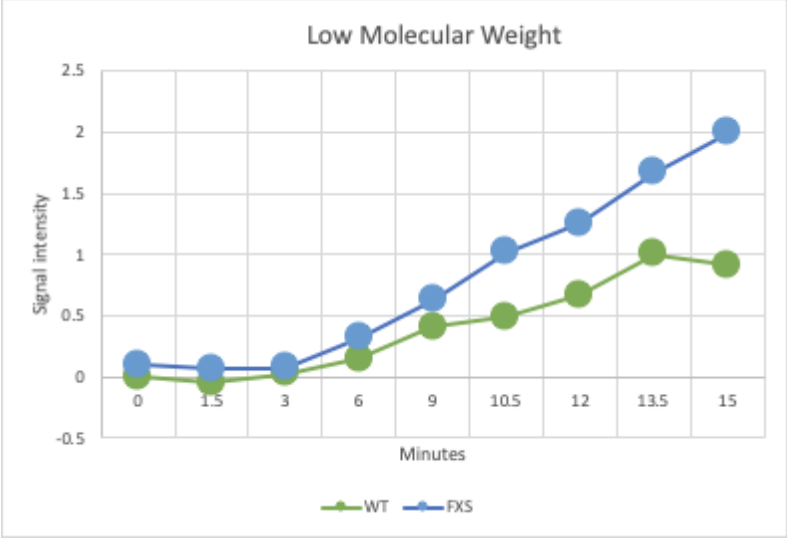


Figure 11. The puromycin run off blot quantification for Wild type (WT) and FXS-affected cells for low molecular weight proteins over 18 minutes of puromycin treatment.

When plotting signal intensity over time, the data revealed that FXS cells have a higher translation rate compared to WT cells (Figures 10 and 11). In both graphs and for both cell lines, the signal intensity increases over time as more protein is actively translated and puromycin is incorporated. Higher protein translation by FXS cells was seen at all time points.

4.0 Discussion

Numerous studies have focused on FMRP translational regulation and its impacts on protein synthesis in both WT and FXS-affected cells, as well as general translational responses to stress in WT cells. However, the impacts of loss of FMRP on translational regulation and protein synthesis under conditions of stress have not been fully explored. FMRP loss of function results in upregulation of translation in the cells of some patients, but it is not yet understood how this may impact the cellular stress response of FXS patients. Previous work in this lab observed a significant increase in stress granule formation in FXS-affected B lymphocytes in response to arsenite, indicating higher sensitivity to environmental stress (Merrill et al. 2019). However, no significant differences were observed in other cell lines or while using BPA as a cellular stressor. In this study, we investigated the threshold requirements, if any, to trigger stress granule formation in wild type and FXS human B lymphocytes.

Our observations show significantly increased levels of stress granule formation in WT cells after a 30-minute exposure to 500 μM arsenite. Contrary to our original hypothesis, this showed that more WT cells formed stress granules than FXS-affected cells. This hypothesis was based on the idea that FXS-affected cells lack FMRP, which is involved in downregulating translation of proteins. Without FMRP, FXS-affected cells should translate proteins at a faster rate. In addition, phosphorylation of eIF2α triggered by the cellular stress response may prevent further ribosome binding. Once translated, the mRNA should be free of ribosomes and be sequestered into a stress granule. We predicted this process would happen faster or at lower concentrations of stress in FXS cells, as ribosomes would more quickly run-off of mRNAs and therefore these mRNAs would be available to localize to the stress granule.

However, our stress granule results suggest that this was not the case. Our stress granule results could mean that either FXS-affected cells did not have a higher baseline rate of protein synthesis, or that despite increased protein synthesis rates the mRNA was still bound by ribosomes after stress initiation and therefore could not be sequestered into stress granules. We then confirmed using the puromycin incorporation assay that our FXS-affected cells did indeed have a higher baseline translation rate compared to WT cells.

FMRP is involved in repressing translation of certain ASD-linked mRNAs (Darnell et al. 2011). It has been demonstrated that lack FMRP results in excessive translation of those mRNAs

(Jacquemont et al 2016). Regulation of FMRP activity involves a cascade pathway from extracellular signal-regulated kinase (ERK) that stimulates cap-dependent translation by phosphorylation of initiation factors such as eukaryotic initiation factor 4E (eIF4E). Deletion of the repressor for eIF4E or overexpression of eIF4E induces similar synaptic plasticity deficits as *Fmr1 KO* mice (Gkogkas et al. 2014). Overactivity of a translation initiation factor producing the same behavior deficits as an FXS-affected model mouse provides evidence that increased translation initiation of mRNAs might explain the reduced or delayed stress response observed in FXS-affected cells.

If the rate of initiation was increased in FXS-affected cells due to overactivity of eIF4E, new ribosomes may be able to access the target mRNA due to absence of the FMRP repressor. As a result, the translating mRNA would not be free to enter a stress granule. This may result in fewer FXS-affected cells forming stress granules than WT cells and might explain our results regarding differential formation of stress granules.

Overall, our results refute our initial hypothesis and provide significant evidence that FXS-affected cells form stress granules more slowly than WT cells. This finding is rather striking, particularly considering the significantly higher protein translation rate found in FXS cells. Additional puromycin incorporating experimental repeats would reinforce data demonstrating that FXS cells have a higher translation than WT cells (Darnel et al 2011). If this holds true, then the initiation rate might be the primary factor that determines if an mRNA is ribosome free and available for a stress granule. Next steps in this investigation should examine and compare initiation rate in FXS and WT cells. A recently-developed methodology for measuring and comparing translation initiation and elongation rates using a mathematical model based on codon occupancy and ribosome density (Sharma et al. 2019) could be applied by a team of students in the future. Due to the complex nature of protein synthesis modulation in cells, we now predict that lack of FMRP causes increased translation initiation in FXS cells and delays mRNA sequestration into stress granules.

Bibliography

Advani, V.M. and Ivanov, P. (2019). Translational Control under Stress: Reshaping the Translatome. *BioEssays, 41*(5). doi: 10.1002/bies.201900009

Anderson, P. and Kedersha, N. (2009). Stress Granules. *Current Biology, 19*(10), R397-398. https://doi.org/10.1016/j.cub.2009.03.013

Darnell, J. et al. (2011). FMRP Stalls Ribosomal Translocation on mRNAs Linked to Synaptic Function and Autism. *Cell, 146*(2): 247-261. https://doi.org/10.1016/j.cell.2011.06.013

Gebauer, F. and Hentze, M.W. (2004). Molecular Mechanisms of Translational Control. *Nature Reviews Molecular Cell Biology, 5*(10):827-835. doi: 10.1038/nrm1488

Gkogkas C.G. et al. (2014). Pharmacogenetic Inhibition of eIF4E-Dependent Mmp9 mRNA Translation Reverses Fragile X Syndrome-like Phenotypes. *Cell Reports, 9(5):*1742-1755. Doi: https://doi.org/10.1016/j.celrep.2014.10.064

Jacquemont, et al. (2018). Protein synthesis levels are increased in a subset of individuals with fragile X syndrome. *Human Molecular Genetics, 27*(12): 2039-2051.

Kedersha, N., et al. (1999). RNA-Binding Proteins TIA-1 and TIAR link the phosphorylation of Eif-2α to the assembly of mammalian stress granules. *J Cell Bio, 147*(7): 1431-1441. 10.1083/jcb.147.7.1431

Kedersha, N. et al. (2001). Evidence that ternary complex (eIF2-GTP-tRNAiMet)-deficient preinitiation complexes are core constituents of mammalian stress granules. *Mol Bio Cell, 13*(1): 195-210. 10.1091/mbc.01-05-0221

Mazroui R, Huot M, Tremblay S, Filion C, Labelle Y, Khandjian EW. 2002. Trapping of messenger RNA by fragile X mental retardation protein into cytoplasmic granules induces translation repression. Human Molecular Genetics. 11(24):3007-17.

McEwen, E. et al. (2005). 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*. 280(17) 16925–16933. DOI: 10.1074/jbc.M412882200

Merrill, L. J., Leclaire, R. D., & de Ayreflor Reyes, S. R. (2019). *Investigating the Impact of Fragile X Syndrome on Stress Granule Formation.* Retrieved from https://digitalcommons.wpi.edu/mqp-all/7055

Pestka, S. (1971) Inhibitors of ribosome functions. *Annual Review Microbiol*. 25, 487-562 https://www.annualreviews.org/doi/pdf/10.1146/annurev.mi.25.100171.002415

Protter, D. and Parker, R. (2016). Principles and Properties of Stress Granules. *Trends in Cell Biology, 26*(9):668-679. https://doi.org/10.1016/j.tcb.2016.05.004.

Richter, J., Bassell, G. & Klann, E. (2015). Dysregulation and restoration of translational homeostasis in fragile X syndrome. *Nat Rev Neurosci* 16, 595–605. https://doi.org/10.1038/nrn4001

Sabaratnam, M. (2006). Fragile-X syndrome. Psychiatry, 5(9): 325-330.

Sharma AK, Sormanni P, Ahmed N, Ciryam P, Friedrich UA, Kramer G, et al. (2019) A chemical kinetic basis for measuring translation initiation and elongation rates from ribosome profiling data. PLoS Comput Biol 15(5): e1007070. https://doi.org/10.1371/journal.pcbi.1007070

Sigma Aldrich. Poly-L-Ornithine Solution (0.1%). Product number A-004-M. Properties and description. https://www.sigmaaldrich.com/catalog/product/mm/a004m?lang=en&region=US

Udagawa, T. et al. (2013). Genetic and acute CPEB1 depletion ameliorate fragile X pathophysiology. *Nature medicine*, *19*(11), 1473–1477. doi:10.1038/nm.3353

Wek, R.C., Jiang, H-Y., Anthony, T.G. (2006). Coping with stress: eIF2 kinases and translational control. *Biochem Soc Trans, 34*(1):7–11. doi: https://doi.org/10.1042/BST0340007

Appendix A: Raw Data from Percent Stress Granule Containing Counts

		WT							
		30 minutes				60 minutes			
Arsenite concentration (uM)		0	125	250	500	0	125	250	500
Lysine	Trial 1	0	41	59.605	74.205	2.2	60.35	82.15	76.5
	Trial 2	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	2.2	41.75	65.3	76.45
	Trial 3	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	2.3	52.6	33.97	68.79
Average		0	41	59.61	74.21	2.58	52.15	67.41	75.97
Standard Deviation						0.06	9.34	24.45	4.44
Standard Error						0.03	5.39	14.12	2.56

		FXS							
		30 minutes				60 minutes			
Arsenite concentration (uM)		0	125	250	500	0	125	250	500
Lysine	Trial 1	9.295	13.66	40.42	53.745	1.7	35.8	62.6	75.85
	Trial 2	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	2.5	51.15	63.3	71.15
	Trial 3	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	4.44	10.5	22.7	51
Average		9.30	13.66	40.42	53.75	2.840	38.73	56.57	69.83
Standard Deviation						1.41	20.53	23.24	13.20
Standard Error						0.81	11.85	13.42	7.62

Percent stressed cells at Arsenite concentration (uM)		WT							
		30 Minutes				60 minutes			
		0	125	250	500	0	125	250	500
Ornithine	Trial 1	6.06	7.26	19.40	34.07	14.68	27.28	51.3	60.65
	Trial 2	7.525	24.5	25.54	49.92	3.13	39.655	47.22	70.06
	Trial 3	11.155	11.075	15.64	43.77	2.79	28.7	37.55	56.5
	Trial 4	5.9	7.24	7.6	43.3	1.8	30.5	54.4	69.4
Average		7.66	12.52	17.04	42.77	5.60	31.53	47.62	64.15
Standard Deviation		2.44	8.19	7.50	6.53	6.08	5.57	7.33	6.66
Standard Error		1.22	4.09	3.75	3.27	3.04	2.79	3.66	3.33

Percent stressed cells at Arsenite concentration (uM)		FXS							
		30 minutes				60 minutes			
		0	125	250	500	0	125	250	500
Ornithine	Trial 1	5.355	11.47	14.195	29.07	9.985	30.67	38.975	69.4
	Trial 2	10.95	16.095	26.725	22.495	1.225	29.125	55.59	52.06
	Trial 3	9.585	5.67	6.955	23.575	5.13	40.79	34.58	49.37
	Trial 4	4.9	3.14	10.4	30.4	3.1	20.8	36.6	56.9
Average		7.70	9.09	14.57	26.39	4.86	30.35	41.44	56.93
Standard Deviation		3.03	5.83	8.63	3.93	3.77	8.20	9.61	8.88
Standard Error		1.51	2.91	4.31	1.97	1.89	4.10	4.80	4.44