PERK up! Resveratrol-Induced Stress Granules Involve PERK Pathway Activation.

A Major Qualifying Project

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Abstract

Resveratrol (Rsv) is a polyphenol produced by plants in response to cell stress and causes the formation of stress granules (SGs) at high doses. The formation of SGs often but not always results in downstream activation of the integrated stress response (ISR) and phosphorylation of the translational regulator eIF2α. Our research aimed to determine whether eIF2α phosphorylation was required for Rsv-induced SGs, and if so which eIF2α kinase (i.e., PERK, PKR, HRI, or GCN2) activates the ISR when exposed to Rsv. SG formation was visualized through fluorescence microscopy in human haploid cells (HAP1) with CRISPR-mediated deletions of each kinase, or mutation of the critical phosphorylation site of eIF2α (Ser51Ala). The Ser51Ala cell line and the ΔPERK cell line did not efficiently form SGs in response to Rsv, suggesting that PERK-mediated phosphorylation of eIF2α is required for Rsv SGs. However, a western blot showed phosphorylation of eIF2α remained in ΔPERK cells after Rsv exposure. Both experiments indicate that while the PERK-mediated phosphorylation of eIF2α may not be essential to SG formation in HAP1 cells exposed to Rsv, it is involved.

Acknowledgements

Professor Natalie Farny for her generous help and encouragement throughout the duration of our research. Her ability to be so humble and kind as we navigated through experiments made working with her a pleasure. Her consistent drive to teach us as much about the subject as she could in only a short amount of time has led us to become better biologists. We are so lucky to have gotten the opportunity to work with Natalie.

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Introduction

Resveratrol and Analogs

Resveratrol (Rsv) was first discovered in the skin of grapes and has since been found in many foods such as peanuts, dark chocolate, and blueberries (Langcake & Pryce, 1976). Rsv is a phenolic compound that has both *cis*- and *trans*- isomerizations (Figure 1) and is typically produced by plants in response to cellular injury or stressors (Frémont, 2000). Rsv has been an ongoing experimental agent in numerous clinical trials due to its anti-cancer, anti-atherogenic, anti-oxidative, anti-inflammatory, and anti-microbial properties (Piotrowska et al., 2012). The effect of this drug has been researched in both healthy individuals and those with various diseases such as type 2 diabetes, obesity, and cancer (Singh et al., 2019). Additionally, Rsv has been found to assist the human immune system and prevent cardiovascular disease (Frémont, 2000). During a stress response in humans, resveratrol is able to manage immunity by targeting specific compounds within a cell that downregulate inflammatory components (Malaguarnera, 2019). Although one of the most widely studied of its kind, Rsv is not the only compound that has these effects.

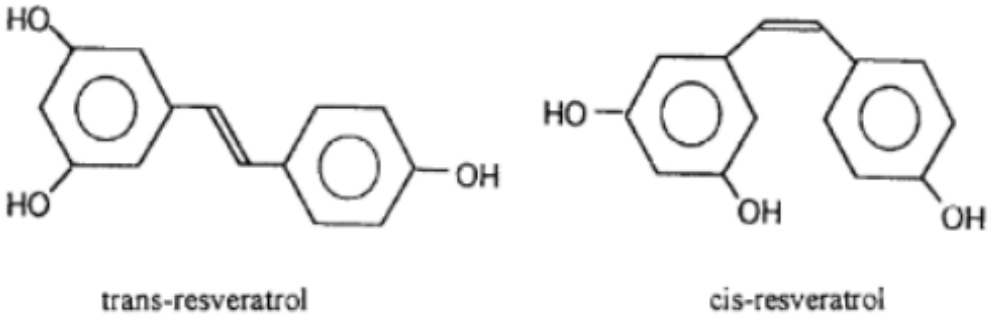

Figure 1: The *Trans*- and *cis*- Isomers of Resveratrol. Adapted from "Biological effects of resveratrol," by Fremont, 2000, *Life Sciences, 66,* 8. Copyright 2000 by ScienceDirect.

Analogs of Rsv include piceatannol, oxyresveratrol, and pinosylvin (Figure 2). All four compounds are stilbenes, or natural defense polyphenols, that have been shown to have preventative effects against chronic diseases (Reinisalo et al., 2015). Pinosylvin is a naturally occurring derivative of stilbene originally isolated from pine heartwood and found in pine leaf (Lee et al., 2005). Classified as a fungitoxin, pinosylvin is synthesized in plants in response to

fungal infections, induced stress, and physical damage (Hovelstad et al., 2006). Oxyresveratrol is a compound produced by certain types of plants in response to stress. It accumulates at infection sites and has been shown to impede growth of both fungal and viral infections (Kim & Lee, 2018). Piceatannol is produced by many fruits and herbs such as grapes, passion fruits, and blueberries. Piceatannol has been shown to inhibit ER stress by suppressing the release of reactive oxygen species (Wen et al., 2018). These compounds all appear to have significant potential health benefits, which is why resveratrol has become the focus of many recent studies.

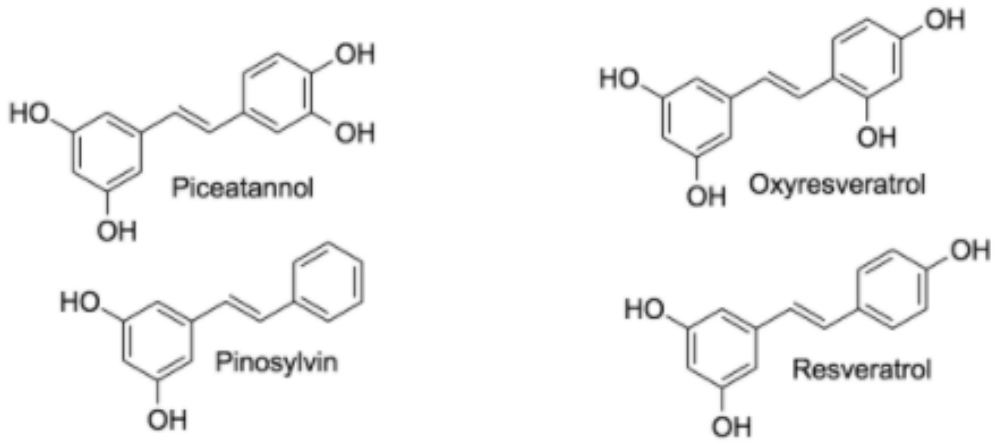

Figure 2: The Analogs of Resveratrol and Their Chemical Structures. Created by the authors on ChemDraw, 2021. Each structure differs only by where the hydroxyl groups are located in the structure.

The Integrated Stress Response and Stress Granules

A cell activates the integrated stress response (ISR) in response to environmental stressors. When the cell recognizes the stressor, the ISR is activated by one of four kinases: PERK, PKR, HRI, or GCN2. Each pathway is activated differently, either by endoplasmic reticulum stress, viral infection, oxidative stress, or by nutrient deficiencies, respectively (Anderson & Kedersha, 2009). Once activated, the kinase will then phosphorylate Eukaryotic Initiation Factor-2α (eIF2α), which inhibits translation initiation of most proteins. Phosphorylated eIF2α induces translation of activating transcription factor 4 (ATF4) and the formation of stress granules. ATF4 decides cell fate by stimulating either cell survival or cell death, depending on the stressor. PERK, HRI, and GCN2 promote cell survival, while PKR encourages apoptosis (Koromilas, 2019). All four kinases induce stress granules.

Stress granules (SGs) are cytoplasmic clusters composed of RNA-binding proteins and mRNAs, which form during molecular changes in the cell under the control of the ISR. The formation of SGs is triggered by a wide variety of stress conditions including osmotic shock, oxidative stress, ultraviolet irradiation, and ion imbalance (Legrand et al., 2020). During stress conditions in the cell, SGs play a role in preserving mRNAs and impeding their translation. This mechanism decreases protein synthesis, which conserves cellular energy that is then used for cell survival (Matsuki et al., 2012). The formation and regulation of SGs include numerous signaling pathways and post-translational modifications of SG components.

Resveratrol-Induced Stress Granule Formation

Rsv induces the ISR by increasing phosphorylation at serine 51 of eIF2α. The mechanism of phosphorylation involves blocking the exchange of eIF2-GDP to eIF2-GTP, thus reducing the energy available to the cell and resulting in translational control (Villa-Cuestra et al., 2011). This effect occurs because phosphorylation of eIF2α inhibits the ability of the eIF2 complex to deliver transfer RNA (tRNA) to the start codon which downregulates translation initiation. The halting of translation initiation is essential to the cell's stress response.

Rsv potentially induces the ISR via its binding to G3BP, a protein involved in SG assembly (Amen et al., 2021). The purpose of SGs is primarily a transient storage space for mRNAs during stress, as well as containing several RNA-binding proteins such as G3BP1, TIA-1, and eIF4E. It is theorized that stress-induced multimerization of G3BP1 is likely to initiate SG formation and that since GSBP2 is a close relative, with a similar domain architecture, it could have a similar function associated with SGs (Matsuki et al., 2013). The connection between Rsv-induced stress granules and eIF2α is not well understood.

Purpose of our Research

Resveratrol and piceatannol have been shown to induce SGs in HeLa, and U2OS human cells (Amen et al., 2021). Specifically, the authors in Amen et al determined that HEK293T cells formed SGs over time in response to treatment with Rsv (Figure 3). Interestingly, however, the same researchers tested whether Rsv induced the ISR by measuring eIF2α phosphorylation and found that it did not seem to show a significant increase. In contrast, researchers Villa-Cuestra et al., 2011 found that resveratrol did increase eIF2α phosphorylation at serine 51. In either study,

individual kinase knockouts of the pathway were not performed; therefore, the claim that Rsv does not require eIF2α phosphorylation for SG formation cannot be confirmed (Amen et al., 2021). Another recent study in 2021 showed similar results to Amen et al., 2021; the formation of stress granules was observed in U2OS cells after treatment with resveratrol, oxyresveratrol, piceatannol, and pinosylvin (Sorrento, 2021).

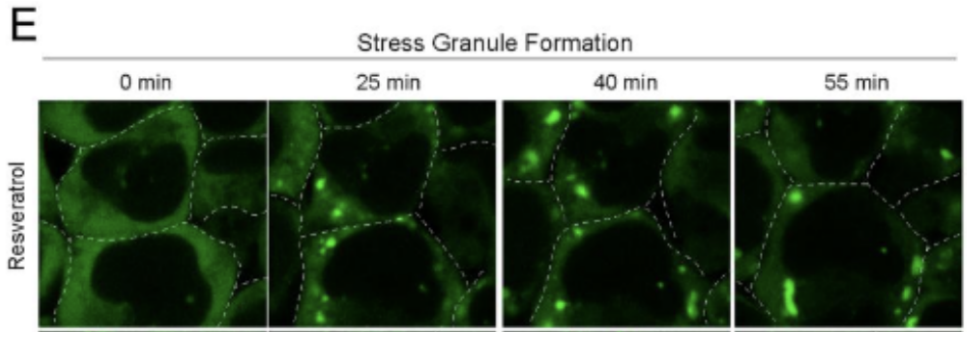

Figure 3: Rsv-Induced SG Formation. HEK293T cells forming SGs overtime when treated with Rsv. Adapted from "Stilbenes Induce Stress Granule Formation," by Amen et al, 2021, *Molecular Biology of the Cell*, mbc.E21-02-0066.

This study did not examine the role of phosphorylation kinases in resveratrol induced SGs, similar to Amen et al., 2021. These experiments were published just recently, and to our knowledge, are the only evidence of resveratrol inducing SGs. The conflicting reports of the levels of eIF2α phosphorylation after resveratrol treatment, and the lack of knowledge regarding phosphorylation kinases indicate a need for further analysis into resveratrol induced SGs.

The purpose of this project was to demonstrate whether activation of the ISR is required for resveratrol-induced stress granule formation; in other words, whether resveratrol-induced stress granule formation is eIF2α dependent or independent, followed by determining the kinase that phosphorylates eIF2α (PERK, PKR, HRI, and/or GCN2). Our research demonstrates that eIF2α is phosphorylated by protein kinase PERK to form stress granules when HAP1 cells are treated with Rsv.

Materials and Methods

Cell Line Maintenance

Six chronic myelogenous leukemia derived HAP1 cell lines were maintained: wildtype, S51A mutant, ΔPERK, ΔPKR, ΔHRI, and ΔGCN2. These haploid cells are derived from KBM7, a cell line initiated from a patient with chronic myelogenous leukemia (Beigl et al., 2020). Complete Dulbecco's Modified Eagle Medium (DMEM) was used for maintenance of the HAP1 cell lines (DMEM with 10% Fetal Bovine Serum, 1% Penicillin/Streptomycin, and 1% glutamine). These cell lines were split 1:3 or 1:6 depending on when the cells reached 90% confluency. After the experiments were finished, the cells were counted under 630x magnification.

eIF2α Dependence Experiment

HAP1 Wild type cells and HAP1 S51A mutant cells were plated in a 12 well plate with 18mm round glass coverslips at a concentration of 3.0 X 10^5 cells per well. 1 mL of cell solution was pipetted into each of the 12 wells on both the wild type and mutant plate. The plates were incubated at 37℃ for 24 hours to allow the cells to grow on the coverslips. After incubation, the cells were briefly visualized under the microscope, to determine cell health and density, and then treatment began.

To begin treatment, 500 µL of medium was removed from each of the 24 wells and transferred into six different conical tubes. Each conical tube contained 2 mL of medium total and were labeled for each of the treatments: ethanol (negative control), resveratrol (Rsv) 100 µM, Rsv 200 µM, Rsv 300 µM, Rsv 400 µM and Rsv 500 µM. Figure 4A and 4B show the set up for each of the plates and indicate what concentration of treatment was put in each well. Ethanol was added at the highest volume of Rsv. 500 µL of each drug treatment was pipetted into each of the labeled wells according to Figures 4A and 4B below. Once the treatment was placed in all wells, the plates were incubated at 37℃ for 1 hour.

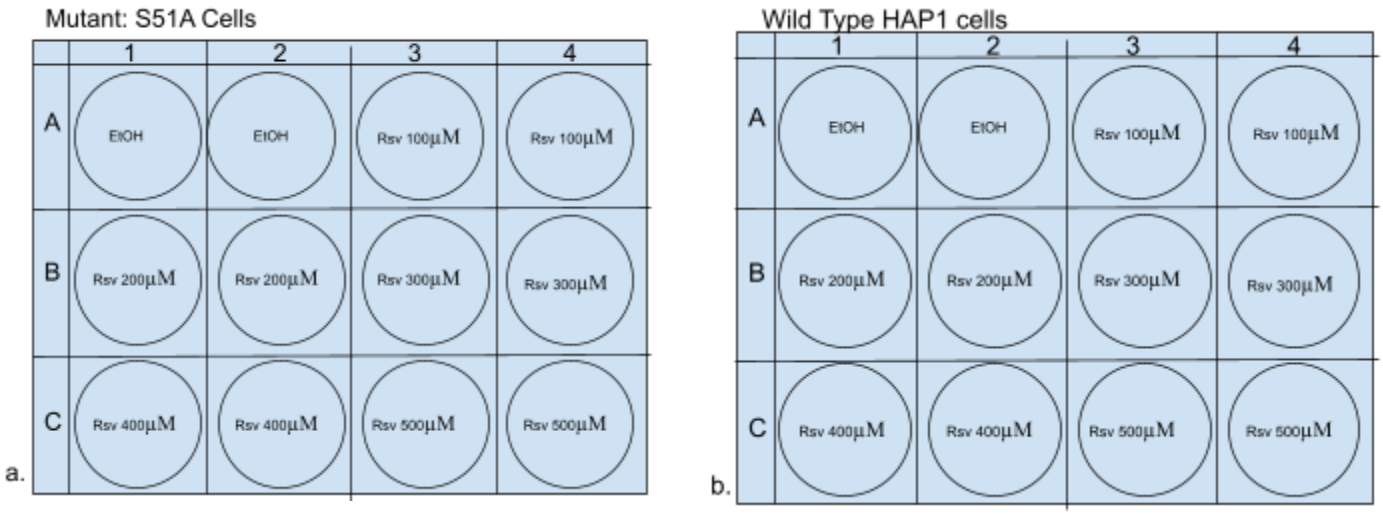

Figure 4: Experimental setup for the Resveratrol Concentration Experiment. (a) S51A mutant cells and (b) wild type HAP1 cells were treated for one hour at the concentration of EtOH, and Rsv indicated.

The drug treatments were removed and the 24 wells were washed with phosphate-buffered saline (PBS). 500 µL of 4% paraformaldehyde in PBS was added to each well and the plates were shaken for 10 minutes in order to create strong covalent cross-links between molecules and keep cells in place. The fixation solution was removed and 500 µL of methanol was added to each well with rotation for 10 minutes in order to dehydrate the cells. The plates were washed with PBS once more.

<u>eIF2α Pathway Experiment</u>

HAP1 Wild type cells, HAP1 ΔHRI, HAP1 ΔPERK, HAP1 ΔPKR, and HAP1 ΔGCN2 mutant cells were plated in a 12 well plate with 18mm round glass coverslips at a concentration of 3.0 X 10^5 cells per well. 1 mL of cell solution was pipetted into each of the 15 wells used. The plates were incubated at 37℃ for 24 hours to allow the cells to grow on the coverslips. After incubation, the cells were briefly visualized under the microscope, to determine cell health and density, and then treatment began.

To begin treatment, 500 µL of medium was removed from each of the 24 wells and transferred into three different conical tubes. Each conical tube contained 2.5 mL of medium

total and were labeled for each of the treatments: ethanol, arsenite 500 µM, and Rsv 300 µM. Figure 5 shows the set up for each of the plates and indicates what concentration of treatment was put in each well. Ethanol was used as the negative control, added at the maximum volume of Rsv. 500 µL of each drug treatment was pipetted into each of the labeled wells according to Figures 5. Once the treatment was placed in all wells, the plates were incubated at 37℃ for 1 hour.

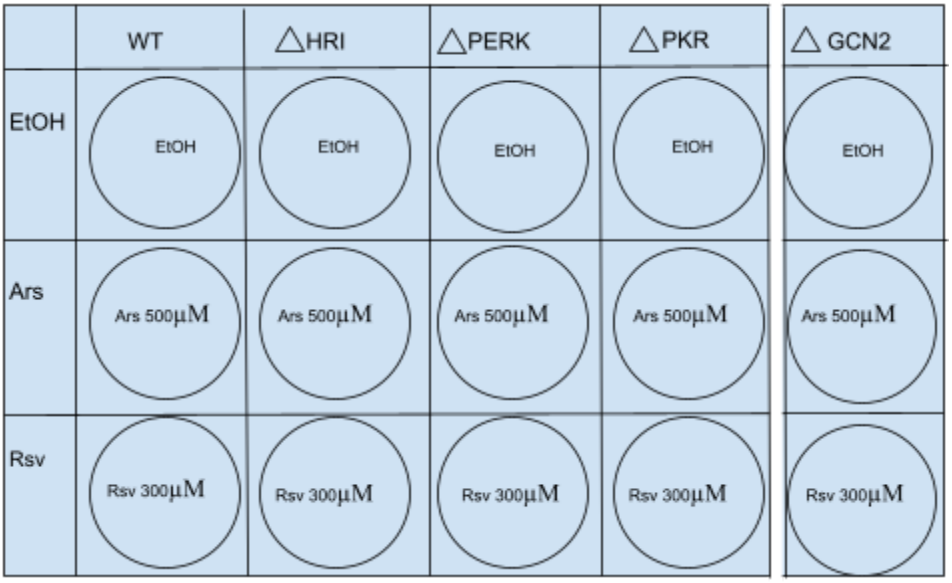

Figure 5: Experimental setup for the eIF2α Pathway Experiment. WT, ΔHRI, ΔPERK, ΔPKR, and ΔGCN2 were treated for one hour at the concentration of EtOH, Ars and Rsv indicated. EtOH was the negative control and Ars was the positive control.

The drug treatments were removed and the 15 wells were washed with phosphate-buffered saline (PBS). 500 µL of 4% paraformaldehyde in PBS was added to each well and the plates were shaken for 10 minutes in order to create strong covalent cross-links between molecules, essentially gluing the cells together. The fixation solution was removed and 500 µL of methanol was added to each well with rotation for 10 minutes in order to dehydrate the cells. The plates were washed with PBS once more.

Staining Cells

After fixation, each well was treated with 500 µL of blocking solution, 5% bovine serum albumin (BSA) in PBS, and placed on the rotator for 1-4 hours at room temperature. After the blocking solution was removed, the wells were treated with the primary antibody solution, diluted as shown on Table 1. The primary antibody solution detects and binds to the stress granule-resident protein G3BP. After sitting for 1 hour, the primary antibody solution was removed and the plates were washed three times for 5 minutes with 1X PBS. The last wash was removed and 500 µL of the secondary antibody was added to each well (Table 1). The secondary antibody, conjugated with Alexa Fluor dye molecules, binds to the primary antibody allowing for fluorescent microscopy. The plates were left at room temperature for 1 hour. The secondary antibody was removed and plates were washed again three times with 1X PBS. On the last wash, wells were left with 1 mL of 1X PBS and the coverslips were mounted onto glass slides using warmed polyvinyl mounting media. Slides were counted after setting for 24 hours in a dark drawer to avoid degradation by light.

Table 1: Antibodies used in staining cells for Fluorescence Microscopy

Antibody	Experiment	Dilution	Company	Catalog #
G3BP Polyclonal antibody	Immunofluorescence primary stain	1:2000 in 5% BSA	Proteintech	13057-2-AP
anti-mouse IgG Alexa Fluor 488 (green) stain	Immunofluorescence secondary stain	1:1000 in 5% BSA	Jackson ImmunoResearch Labs	AB_2313584
Hoechst 33342 (blue) nuclear stain	Immunofluorescence secondary stain	1:1000 in 5% BSA	Thermofisher	62249
Rabbit anti-phospho-eIF2α	Western blot primary stain	1:500 in 5% milk in 1X PBS + 0.5% Tween-20	Cell Signaling Technology	3597
Goat anti-rabbit HRP	Western blot secondary stain	1:5000 in 5% milk in 1X PBS + 0.5% Tween-20	Cell Signaling Technology	7074S

Rabbit anti-eIF2α	Western blot primary stain	1:500 in 5% milk in 1X PBS + 0.5% Tween-20	Cell Signaling Technology	5324

Western Blot

An acute exposure assay was performed on HAP1 WT, HAP1 ΔHRI, HAP1 ΔPERK, HAP1 ΔPKR, and HAP1 ΔGCN2 cells. The 12-well plates were arranged as shown in Figure 5.

Plates were incubated for 1 hour with treatments. Media was aspirated and all wells were rinsed once with 1X PBS. 150 µL of SDS sample buffer + DTT was added to each well. The contents of each well were then added to labeled 1.5 mL tubes. All tubes were briefly centrifuged to eliminate bubbles. The contents of each tube was then sheared with a syringe for 20 strokes. All tubes were placed into a heat block at 95℃ for 10 minutes and then centrifuged again at maximum speed for 5 minutes.

5 µL of Sigma BLUEye ladder (product no. 94964) was added to well 1 of two gels. The well arrangement of the gels can be seen in Table 2. 20 µL of each sample was added to each well. The gel was run at 117 mV for 80 minutes. Gels were removed and put into transfer buffer to equilibrate. Proteins from the gel were transferred to PVDF membrane by electroblotting at 110 mV for 60 minutes. The success of electroblotting was visualized using Ponceau S stain. Ponceau S stain was removed with wash buffer (1X PBS + 0.5% Tween-20). Both membranes were blocked on a rotator for 1 hour in 5% milk in wash buffer (WB).

A 1:500 dilution of primary antibody, Table 1, was mixed in 5% milk in WB. 7.5 mL of this mixture was added to the membranes in a sealed plastic packet and rocked overnight at 4℃. The dilution mixture was returned to the tube and stored in the fridge. Both membranes were washed with WB three times for five minutes each. 20 mL of a 1:5000 dilution of secondary antibody, Table 1, in 5% milk in WB was added to each membrane to sit for 60 minutes. The secondary antibody was removed and saved in the fridge. The membranes were washed in WB vigorously by hand three times for 30 seconds each. The membranes were then washed in WB three times for five minutes on the rotator. Membranes were removed and 1 mL of developing solution was added to each membrane. The membranes were wrapped in plastic wrap and

imaged in BioRad ChemiDoc XRS+ System. The primary antibody and secondary antibody procedure was repeated once more on the following day. Membranes were imaged again.

Table 2: SDS-PAGE Gel Well Setup

Gel 1:

1	2	3	4	5	6	7	8	9	10
ladder	WT eth	WT ars	WT rsv	HRI eth	HRI ars	HRI rsv	GCN2 eth	GCN2 ars	GCN2 rsv

Gel 2:

1	2	3	4	5	6	7	8	9	10
ladder	WT eth	WT ars	WT rsv	PKR eth	PKR ars	PKR rsv	PERK eth	PERK ars	PERK rsv

Results

Resveratrol-induced stress granules are eIF2α dependent

An acute exposure assay was performed on HAP1 WT and HAP1 S51A mutant cells to evaluate the percentage of cells producing stress granules in response to treatment with resveratrol. Ethanol was used as the negative control. Cells were viewed under the fluorescence microscope to determine which had stress granules and which did not. Cells that were positive for stress granules showed small, bright, green dots in the cytoplasm. Cells that were negative for stress granules did not show any dots and were diffusely green throughout the cytoplasm. The difference of positive and negative cells can be seen in Figure 8, where the WT ethanol cell image does not show stress granules but the arsenite and resveratrol images do.

Five different concentrations of Rsv were tested to determine the optimal concentration for producing stress granules without killing cells. The percent positive for stress granules in the HAP1 WT cells at each Rsv concentration is shown in Figure 6. ANOVA and paired t-tests were run to analyze the data and it was found that only 300 μM and 500 μM concentrations were significant from the control, with p values of 0.04 and 0.0002 respectively. 300 μM was determined to be the minimum effective concentration, because at 500 μM there was significant toxicity.

To determine whether Rsv stress granules require the eIF2α pathway, we measured stress granule formation in HAP1 eIF2α serine 51 to alanine (S51A) non-phosphorylatable mutant cells at each Rsv concentration, as shown in Figure 6. Very few S51A cells were SG-positive compared to the negative control, indicating that Rsv-induced stress granules are dependent on eIF2α phosphorylation. In Figure 6 it can be seen that at a concentration of 500μM there was a 10% stress granule formation in the mutant cells while at a concentration of 300μM, only 1% of cells were positive for stress granule formation. Stress granule formation was closest to significance at 500 μM, the strongest and most toxic concentration, with a p value of 0.07.

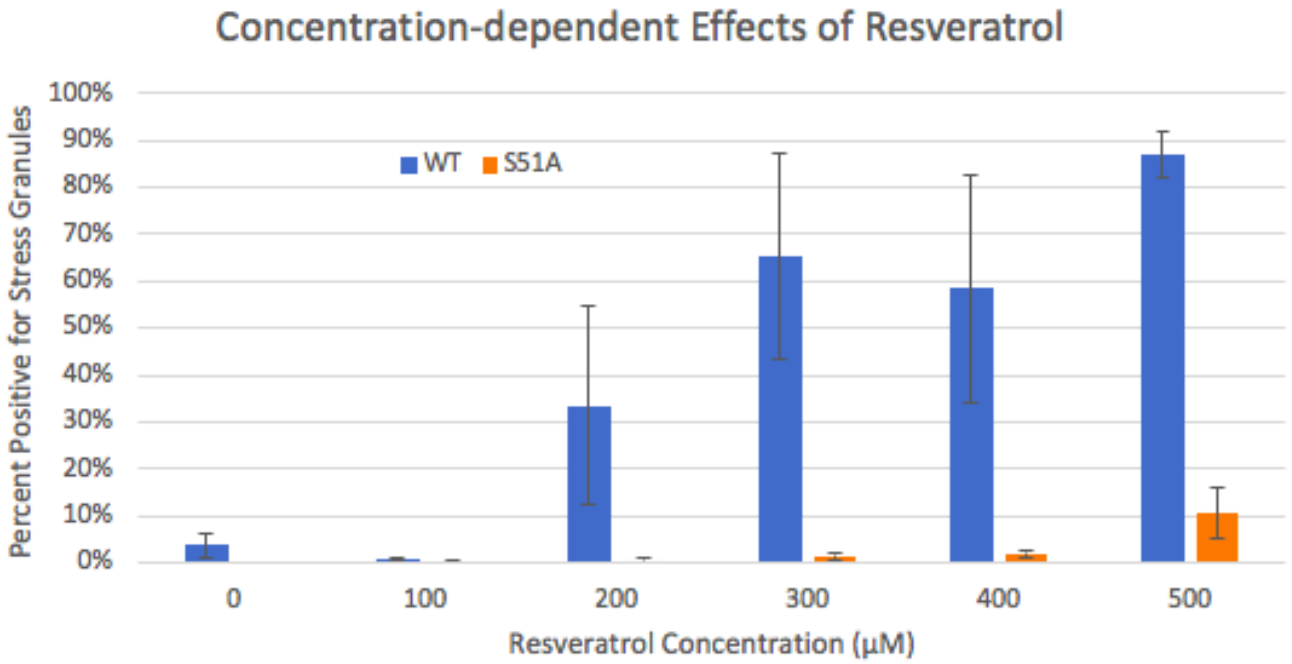

Figure 6: Stress granule formation in HAP1 WT and S51A mutant cell lines. WT or S51A cells were treated for one hour at the concentration of Rsv indicated. Cells were counted by fluorescence microscopy and percent of total cells forming stress granules was calculated. Error bars represent the standard error of the mean. N=4.

Resveratrol-induced stress granules are formed via the eIF2α PERK pathway

Our previous result indicated that Rsv SGs require eIF2α phosphorylation. eIF2α can only be phosphorylated in mammalian cells by one of four kinases: HRI, PKR, PERK or GCN2. To identify the specific kinase responsible for activating the ISR in response to Rsv, an acute exposure assay was performed on HAP1 knockout cell lines: ΔPKR, ΔPERK, ΔHRI, ΔGCN2 and the WT control. Each cell line except WT has a frameshift mutation resulting in a knockout of one of the four kinases responsible for the phosphorylation of elF2α. The knockout cell line which exhibits the lowest rate of stress granules formation when exposed to Rsv may potentially be responsible for the activation of the ISR.

The results of the acute exposure assay are shown in Figure 7. Ethanol was used as a negative control, and showed a low rate of stress granules formation in all cell lines. The ΔHRI cell line was used as a positive control under the treatment of arsenite due to pre-existing literature which demonstrates limited SG formation after arsenite exposure (McEwen et al., 2005) . As seen in Figure 7, ΔHRI was the only cell line that showed a low average percentage of stress granules formation of 1.14% when exposed to arsenite. The other remaining cell lines

formed on average more than 50% stress granules when exposed to arsenite, supporting prior research that arsenite is HRI dependent for the activation of the ISR pathway.

The ΔPERK cell line showed the least amount of SG formation when exposed to Rsv, with an average percent positive of 7.55%. This indicates that the PERK kinase of the eIF2α pathway may be necessary for SG formation in HAP1 cells when exposed to Rsv. The other cell lines when exposed to Rsv formed a range of 79% to 90% positive for SGs over the 4 replicates, a difference of over 10 fold more SG formation compared to ΔPERK. This decrease in SG formation in the ΔPERK cell line is also supported by statistical analysis. Using paired-t tests, the ΔPERK cell line with Rsv treatment was shown to have the only significant response compared to WT, with a p value of 0.000023.

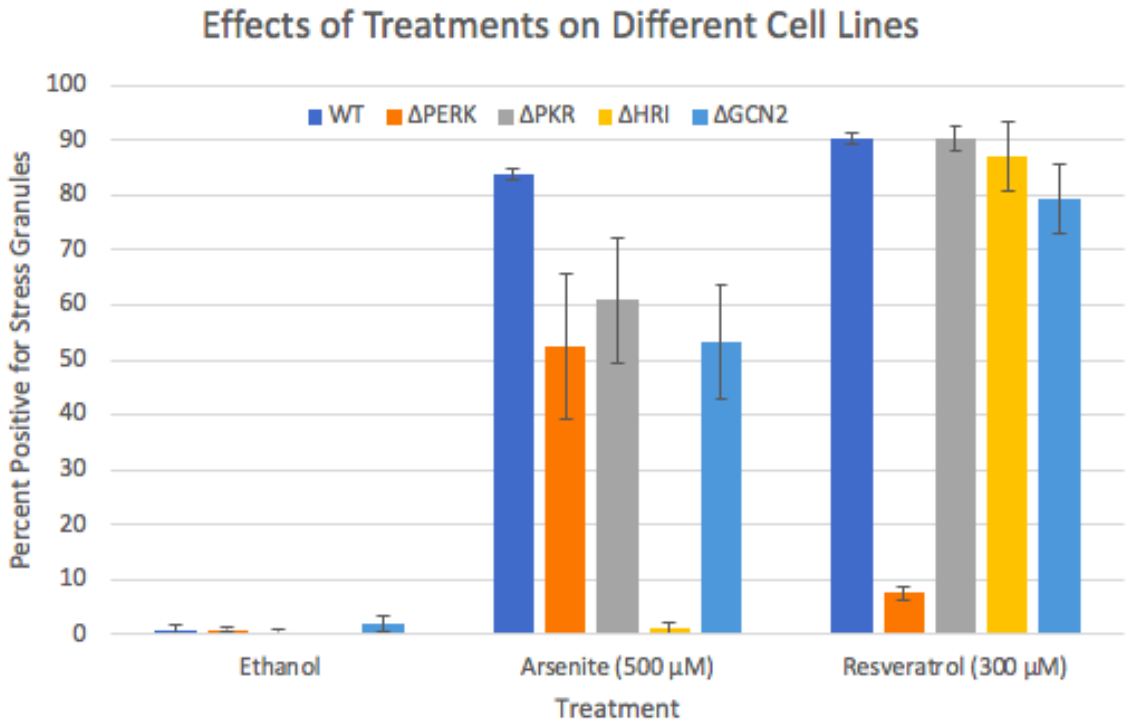

Figure 7: Stress granules formation in HAP1 WT, ΔPERK, ΔPKR, ΔHRI and ΔGCN2 cell lines. All cell lines were treated for one hour with ethanol, arsenite and resveratrol at the concentration indicated. Cells were counted by fluorescence microscopy and percent of total cells forming stress granules was calculated. Error bars represent the standard error of the mean. N=4.

Images of the WT, ΔHRI, and ΔPERK cell lines under each condition were collected to compare the presence of SG formation (Figure 8). All ethanol treatments showed no presence of SG formation, as was expected. Treatment with arsenite formed SGs in the WT and ΔPERK lines; as mentioned previously, HRI is known to be responsible for arsenite-induced SG

formation and therefore the ΔHRI line does not show SGs. The ΔPERK cell line also does not show SG formation when treated with Rsv, signifying that PERK is likely responsible for Rsv-induced SG formation.

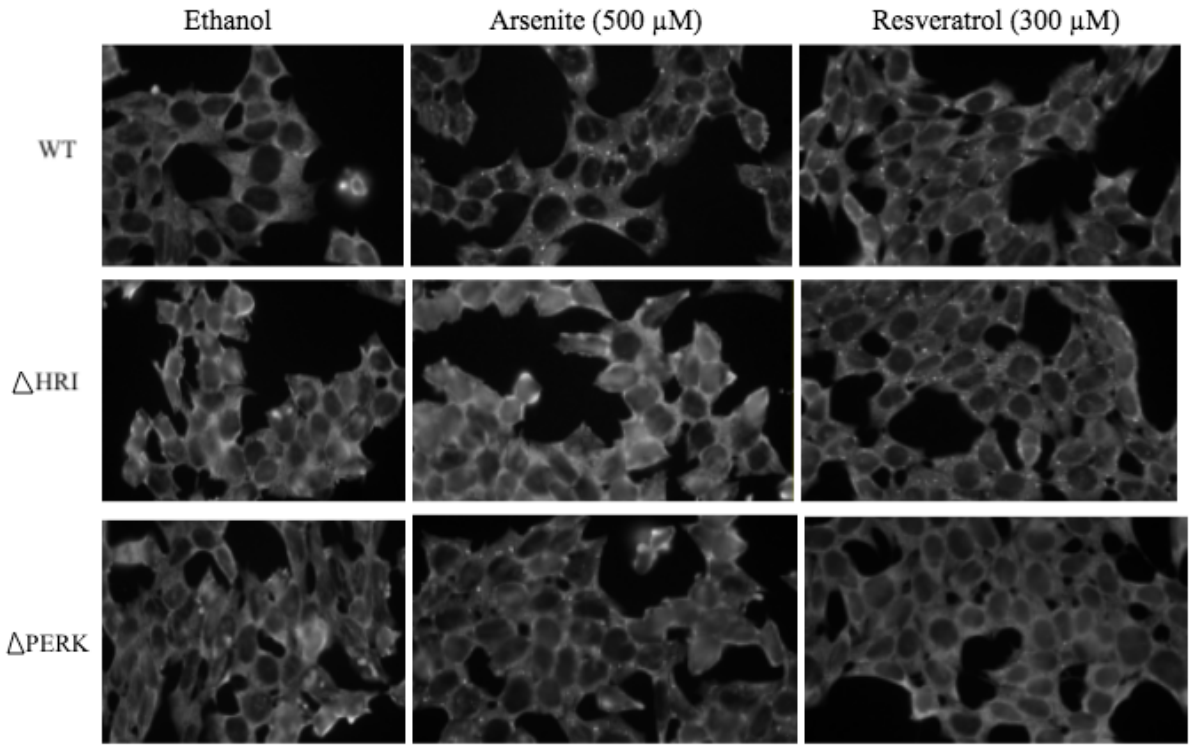

Figure 8: Images of HAP1 Cells From the Fluorescence Microscope. HAP1 WT, ΔPERK, and ΔHRI cell lines were treated with ethanol, arsenite (500 μM) and resveratrol (300 μM) and counted by fluorescence microscopy. Stress granules are shown as bright white dots on the cell cytoplasm.

Western Blot

The five HAP1 cell lines were treated with ethanol, arsenite, and Rsv to perform a Western blot to quantify phosphorylation of eIF2α. The presence of phosphorylated eIF2α in each cell line are shown in Figure 9.

Lanes 1, 4, and 7 represent the cell lines treated with ethanol (Figure 9). Ethanol was used as a negative control, and no phosphorylation was seen in these lanes. Lanes 2, 5, and 8 represent the cell lines treated with arsenite (Figure 9). ΔHRI treated with arsenite was also used as a negative control for our experiment. As expected lane number 5 (top) shows a faint band,

indicating the absence of phosphorylation. Lanes 3, 6, and 9 represent the cell lines treated with Rsv (Figure 9). The ΔPERK cell line treated with Rsv (lane 9 bottom) showed the same level of eIF2α phosphorylation as WT treated with Rsv. It was expected that if PERK was required for Rsv-induced SGs, then no phosphorylation of eIF2α would be seen. The total eIF2α is below the phosphorylated eIF2α in Figure 9, showing the total distribution of eIF2α in all five cell lines in all of the wells. The bands for total eIF2α are of fairly equal intensity across all cell lines and treatments, indicating an equal protein load.

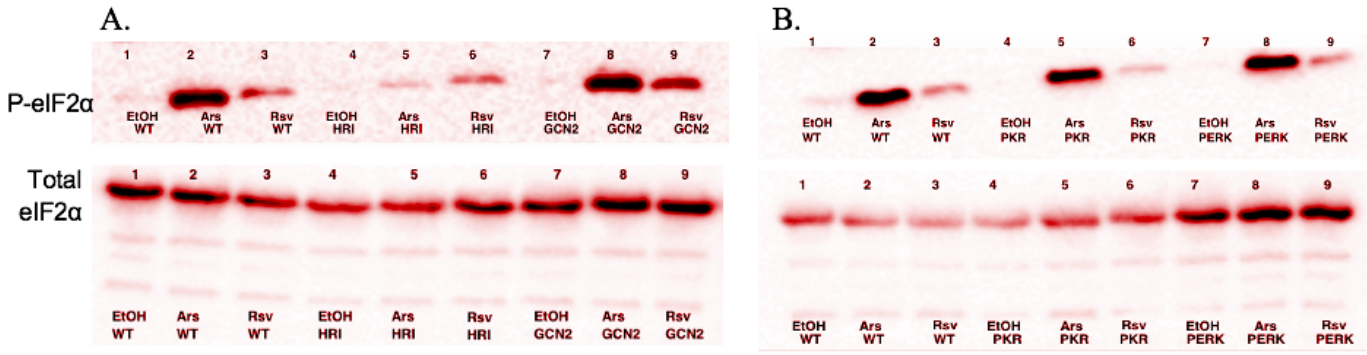

Figure 9A and B: Western Blot. Phosphorylated eIF2α is shown in the top row of A and B with the corresponding total eIF2α in the bottom row. Photos were taken on the BioRad ChemiDoc XRS+ System at 3 second exposure.

Discussion

HAP1 Ser51Ala and WT Exposure Assay

The first assay investigated two main questions: the minimum optimal concentration of Rsv for treatment of HAP1 cells, and whether Rsv-induced SGs were eIF2α dependent. The WT HAP1 cell line was the focus for determining the concentration-dependent effects of Rsv. Five concentrations, ranging from 100 μM to 500 μM were investigated. WT HAP1 cells showed minimal SG formation in the 100 μM and 200 μM concentrations; interestingly, 400 μM was also statistically insignificant from the negative control. Only 300 μM and 500 μM proved to be statistically significant from the control. At a 500 μM concentration, although SGs were consistently formed, cell loss from the glass coverslips was so significant that often we couldn't find 250 cells to count (the accepted minimum for accurate scoring). Additionally, the cells at 500 μM appeared significantly stressed, with abnormal morphology. The optimal concentration of Rsv was determined to be 300 μM, as there was less cell death, less cell loss and the concentration was high enough to cause SG formation.

To investigate the second question of this experiment, an eIF2α knockout cell line (S51A) was used alongside the WT. This cell line was also treated with the five concentrations of Rsv. At all concentrations except 500 μM, the rate of SG formation was insignificant in comparison to the ethanol treatment; this indicated that eIF2α was likely necessary for Rsv-induced SG formation. At 500 μM, though statistically significant in SG formation, there was high toxicity as was seen in WT cells. This observation led to the conclusion that 500 μM was such a high dose of Rsv for HAP1 cells that they would be forced to produce SGs due to the amount of stress the cells were put under.

HAP1 Kinase Knockout Exposure Assay

The eIF2α kinase involved in the Rsv SG pathway was investigated next. The Rsv acute exposure assay showed that the PERK knockout cell line exhibited the lowest percentage of stress granule formation out of the 5 HAP1 cell lines. A percent positive of 7.55% showed a significant reduction when compared with the other knockout cells which varied from 79% to 90%. Additionally, observations made under fluorescent microscopy of the ΔPERK cell line exposed to Rsv indicate the cells inability to handle extracellular stress. This resulted in an inability to accurately count cells positive for SG formation in 2 out of the 4 replicates

completed. For example, some ΔPERK cells had lysed while others had abnormal morphology, where green fluorescent polyps were seen protruding from the cell. These morphological changes indicate that the ΔPERK cells were under a great deal of stress when Rsv was introduced and the cells were treated. From the results it can be suggested that the PERK kinase pathway plays a major role in the phosphorylation of eIF2α when treated with resveratrol.

Western Blots

While the results from the exposure assay appear conclusive, the phosphorylated eIF2α Western blot call those results into question. The Western blot shows the presence of phosphorylated eIF2α in the ΔPERK cell line, which would not be possible if PERK was solely responsible for Rsv-induced activation of eIF2α. Previous studies show that Rsv does not induce eIF2α phosphorylation as much as arsenite does (Amen et al., 2021). Results from this study indicated that Rsv-induced SGs may be formed by a quicker, auxiliary pathway than arsenite-induced SGs (Amen et al., 2021). Our exposure assay results do not correlate with this conclusion, but it is possible that the phosphorylation of eIF2α is responsible for other cellular functions. The focus of the Amen et al. study was to understand SG clearance, or the way that SGs are removed from the cell after the stressor is over. These researchers believe that the quick SG clearance they saw with Rsv treatment may be the result of minimal eIF2α phosphorylation. They hypothesize that SG clearance may play a large role in SG biology and the effect SGs have on cell response. Based on the results from our p-eIF2α Western blot and Amen et al., the clearance of SGs in correlation with SG formation pathways requires further study to understand these results.

Future Directions and Sources of Error

The results obtained from this project can most certainly help direct and inspire future experimentation towards understanding the relationship between Rsv, PERK, and eIF2α. In the future, this project could be continued and expanded by using an eIF2α inhibitor in combination with the ΔPERK cell line. Continuing the experiment in this way would potentially provide answers to the conflicting results we obtained with our kinase knockout exposure assay and western blot.

Although we were careful to avoid errors we did encounter a few throughout our project. A potential source of error was simply human error and specifically measurement errors. The majority of the data collected was based on the presence or absence of SGs. In order to determine this the cells were viewed on a microscope slide. To ensure all cells that were positive or negative for SGs were scored accordingly specific criteria were discussed at the beginning of our experimentation and periodically throughout replicates. However, having four student researchers counting SGs, not all scores were the same and measurement variability was possible. To account for this potential error, some slides had to be recounted, but some variability still remained. Additionally, the quality of the microscope used could have affected our results as the imaging of the small SGs was not as clear as it could have been with a higher objective lens. HAP1 cells are very small so trying to pick out even smaller SGs was difficult some of the time. A final source of error was the maintenance of sterile techniques. Since our work was completed in a communal lab space and incubator, we encountered many issues with contamination to our cells. The sterility of the cell cultures was difficult to maintain due to them being stored in a communal lab space. The cultures had to be restarted once due to fungal spores found in other team's cultures. Low level contamination may have affected the stress tolerance of our cells as fungal contamination is a source of stress.

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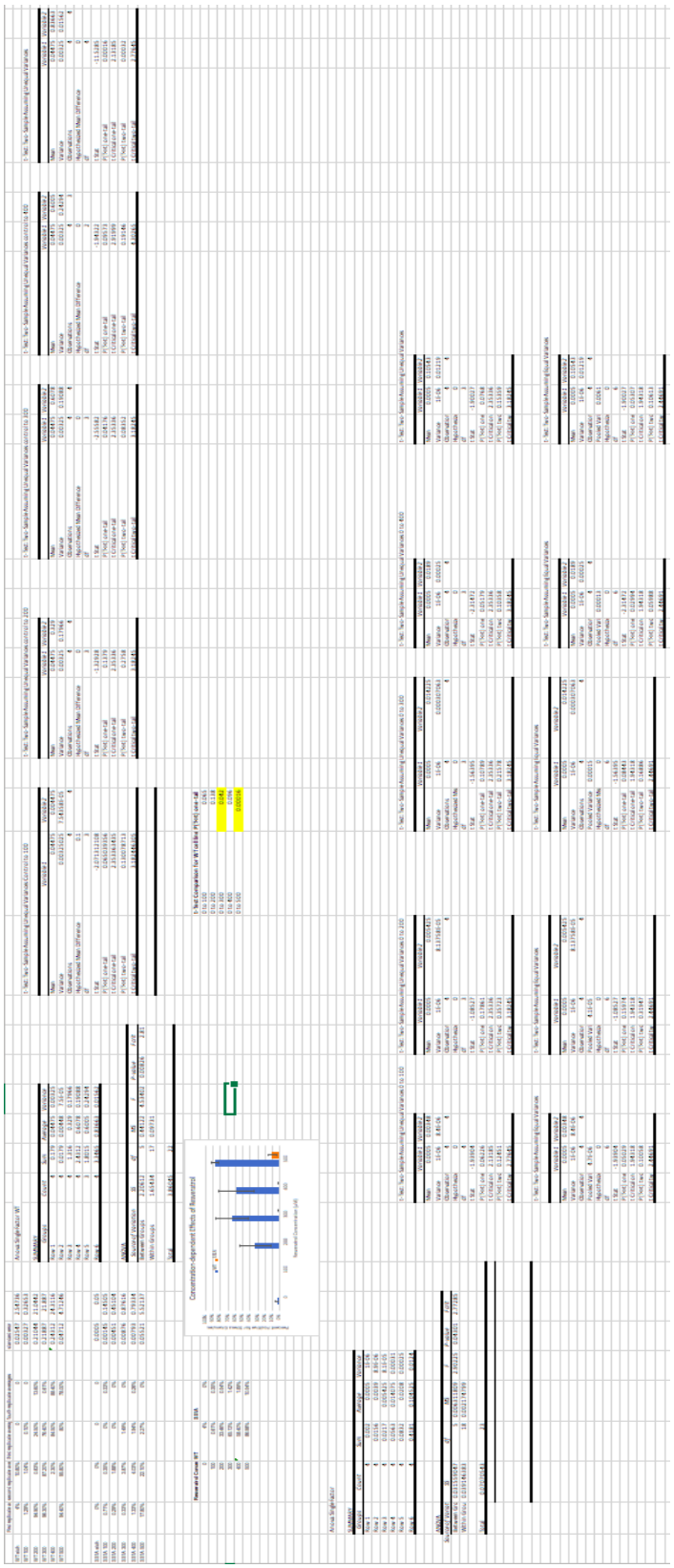
Appendix A: Raw Data for HAP1 WT and S51A Acute Exposure Assay



Appendix B: Raw Data for HAP1 WT, ΔPERK, ΔPKR, ΔGCN2, and ΔHRI Acute Exposure Assay