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## Activation of Nrf2 at Critical Windows of Development Alters Protein S-Glutathionylation in the Zebrafish Embryo (*Danio rerio*)

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**ACTIVATION OF NRF2 AT CRITICAL WINDOWS OF DEVELOPMENT ALTERS  
PROTEIN S-GLUTATHIONYLATION IN THE ZEBRAFISH EMBRYO (*Danio rerio*)**

A Master's Thesis Presented

by

EMILY G. SEVERANCE

Submitted to the Graduate School of the  
University of Massachusetts Amherst in partial fulfillment  
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MASTER OF SCIENCE

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Molecular and Cellular Biology

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## ABSTRACT

### ACTIVATION OF NRF2 AT CRITICAL WINDOWS OF DEVELOPMENT ALTERS PROTEIN S-GLUTATHIONYLATION IN THE ZEBRAFISH EMBRYO (*Danio rerio*)

SEPTEMBER 1, 2021

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Perturbation of cellular redox homeostasis to a more oxidized state has been linked to adverse human health effects such as diabetes and cancer. However, the impact of altering the regulation of redox homeostasis during development is not fully understood. Specifically, this project investigates the role of the Nrf2 antioxidant response pathway and its effect on glutathione (GSH; cellular redox buffer) at critical windows of development. To explore this, we used zebrafish embryos (*Danio rerio*) as a model due to the function of GSH and the Nrf2 being conserved among vertebrates. We exposed zebrafish embryos to three Nrf2 activators: two antioxidant enhancing molecules: sulforaphane (SFN; 40  $\mu$ M) and Dimethyl fumarate (DMF; 7 $\mu$ M) as well as the pro-oxidant tert-Butylhydroquinone (tBHQ; 1 $\mu$ M) for 6 hours at critical windows development: 24, 48, 72 hours post fertilization (hpf). Following exposure, we visualized Nrf2 protein levels and glutathionylation rates using immunohistochemistry and confocal imaging. We found that changes in Nrf2 expression were dependent on the tissue type with there being significant changes in Nrf2 when looking at the pancreatic beta cells. Also in the beta cells, exposure to SFN, tBHQ, and DMF were found to increase Nrf2 translocation into the nucleus. Most notably, all three Nrf2 activators significantly altered glutathionylation levels depending on the time-point the zebrafish were exposed. SFN and tBHQ were also found to significantly increase glutathionylation at 48 and 72hpf, but led to a significant decrease at 96hpf while DMF increased glutathionylation at all three time-points. Interestingly, there was little correlation between Nrf2 protein levels and glutathionylation, but zebrafish with a mutated Nrf2 did have significantly different glutathionylation rates than the wild type fish. This suggests that oxidative stress is not the sole regulator of glutathionylation and instead Nrf2 may also be regulating glutathionylation through GSH storage. My data indicate that the effects of Nrf2 activation on Nrf2 levels and glutathionylation depend on the timing of exposure to the perturbing chemicals and the tissue type. Finding these windows of development where redox homeostasis is most sensitive in humans can allow for possible preventative and/or protective measures to oxidative stress during development.

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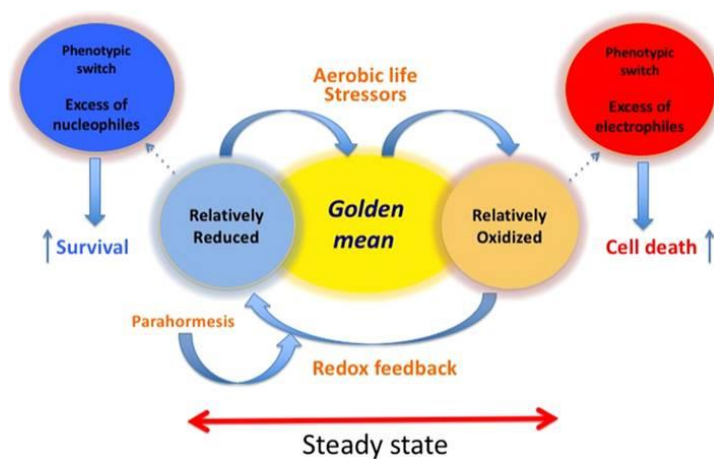
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## 1. INTRODUCTION

Redox homeostasis is the physiological steady-state between oxidants and antioxidants in the cell. Under normal physiological conditions reactive oxygen species (ROS) are produced endogenously by the mitochondria during the process of aerobic respiration and play important roles in the regulation of signal transduction pathways and cellular processes (Covarrubias, 2008). ROS production pushes the redox homeostasis to the oxidative boundary which is then brought back into balance through feedback loops consisting of cell signaling pathways and the upregulation of antioxidant defense molecules (Ursini, 2016)(Figure 1).

However, if endogenous sources of ROS are not regulated properly the cellular redox balance can be disrupted. This balance can also be disrupted by exogenous sources of ROS, such as environmental toxicants and pharmaceuticals,

challenging the redox balance and overwhelming the antioxidant defenses. When the balance of oxidants and antioxidants is shifted outside the physiological steady state, oxidative stress occurs (Covarrubias, 2008). The point in which oxidative stress occurs varies with some tissue and cellular processes being more sensitive to dysregulation than others (Kotas, 2015). Pancreatic beta cells are especially sensitive to redox modulation because of inherently less antioxidant defenses (Miki, 2018). In humans, prolonged oxidative stress has been linked to insulin resistance, glucose dysregulation, and further complications with diabetes (Asmat, 2016). Other



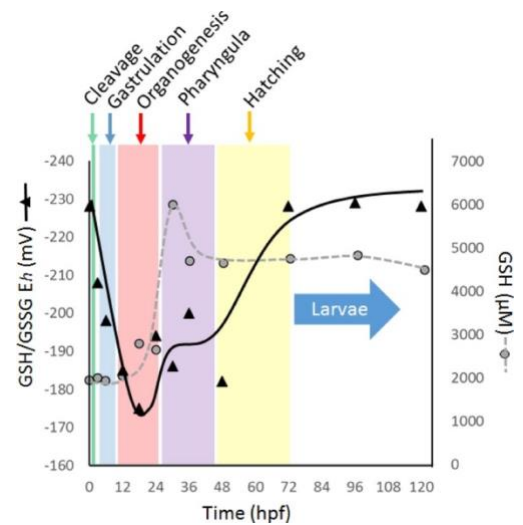
**Figure 1.** Redox Homeostasis Model; Ursini, 2016



adverse health effects in humans linked to oxidative stress include carcinogenesis, neurological diseases, and rheumatoid arthritis (Valko, 2004; Halliwell, 2001).

Redox balance is especially important during development because redox homeostasis plays an important role in cell fate decisions; A more reduced state promotes cell proliferation and survival while a more oxidized state promotes cell differentiation and apoptosis (Hansen, 2015). Perturbation of redox balance during development has been linked to teratogenesis in rats and mice as well as insulin resistance and dysregulated glucose levels in developing beta cells (Wells, 2005; Pi, 2010). Although altering redox homeostasis has been linked to adverse health effects, the impact of altering the regulation of redox homeostasis during development is not fully understood.

The primary cellular redox buffer that maintains redox homeostasis in vertebrates is glutathione (GSH). When ROS are present in the cell, the active thiol of GSH can interact with electrophiles to reduce ROS (Liu, 2013). In the process oxidized glutathione disulfide (GSSG) is formed. The glutathione redox potential (GSH  $E_h$ ), which is calculated using the Nernst equation and the ratio of reduced to oxidized GSH, acts as a redox sensor for the cell. Both the total amount of GSH and the redox potential rapidly shift during vertebrate development with different stages of growth needing different amounts of GSH (Hansen, 2014; Schafer 2001; Thompson, 2012). The zebrafish is used as a model vertebrate to study this process due to its fast development time and ease of exposure. In the zebrafish embryo, the redox environment rapidly changes from a relatively reduced to a more oxidized



**Figure 2.** Measured changes in GSH  $E_h$  during zebrafish development; Timme-Laragy, 2013

state (Figure 2). The redox environment then continues to stay relatively oxidized from 18-48hpf. These fluctuations in the redox environment coincide with a period of rapid differentiation during the processes of gastrulation and organogenesis. Followed by finer differentiation and morphogenesis. After 48hpf, the redox environment slowly returns to a reduced state until it stabilizes at around -230 mV (Timme-Laragy, 2013). The total amount of cellular GSH also fluctuates during this time, rapidly increasing from 0 to 48hpf and then stabilizing (Timme-Laragy, 2013). Due to the rapid changes in the redox environment during development, embryos are inherently more susceptible to exogenous sources of oxidative stress. The susceptibility or altering redox regulation during this time of rapid change is not fully known and makes researching the critical windows of development between 24 and 72hpf particularly important.

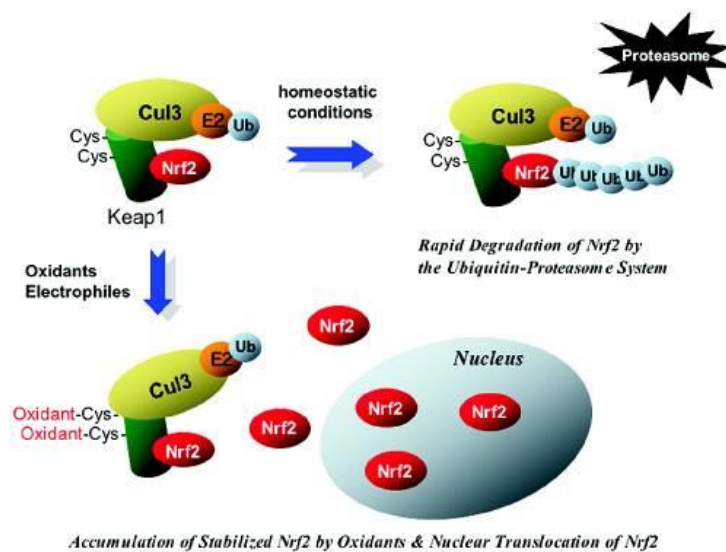
Another regulative role of GSH is S-glutathionylation. Glutathionylation is a post-translational modification where GSH is attached to a protein (Checconi, 2019). Glutathionylation is typically used to alter or disrupt protein function during times of oxidative stress when the protein can potentially be damaged by free radicals. Glutathionylation is also used to store and transport GSH as the process is reversible (Hansen, 2014). The enzyme Gstp can perform glutathionylation in order to store and deliver GSH to hydrophobic regions of protein that is not readily available to it (Tew, 2011). During development glutathionylation levels fluctuate, an important role in regulating signal transduction pathways such as NfκB (Hansen, 2014).

The main redox feedback involved in redox homeostasis is the Nrf2 antioxidant response pathway. Nuclear Factor-(erythroid-derived-) like factor 2 (Nrf2) is a transcription factor that regulates the cellular response to redox disruptions (Lin, 2016). The zebrafish Nrf2 co-ortholog to human Nrf2 is Nrf2a. Under homeostatic conditions, Nrf2 is bound to Keap1 and repressed in the cytosol (Figure 3). Keap1 then recruits the Cul E3 ubiquitin ligase complex to Nrf2 which results in the ubiquitination and subsequent

proteasomal degradation of Nrf2 (Kobayashi, 2004). This results in a half-life of under 20 minutes under homeostatic conditions (Kobayashi, 2004). Upon redox perturbation, Keap1 acts as a stress sensor protein. Pro-oxidants suppress Keap1 activity by modifying the cysteine residues needed to bring over the E3 ubiquitin ligase (Kobayashi, 2006). This stabilizes Nrf2 and allows it to translocate to the nucleus; in

the nucleus, Nrf2 binds to a DNA sequence known as the antioxidant response element (ARE). The ARE is located in the promoter region of genes encoding for Glutathione (GSH) synthesis, ROS elimination, phase 2 detoxification enzymes, and more. These gene products work to combat any xenobiotic induced redox disruptions (Lin, 2016).

Some compounds known to interact with Nrf2 that were used in the project include tert-butyl-hydroquinone (tBHQ), sulforaphane (SFN), and dimethyl fumarate (DMF). tBHQ is a pro-oxidant that generates ROS over time by entering a redox cycle. Redox cycling involves coupled reduction and oxidation reactions that can produce free radical species which then react with

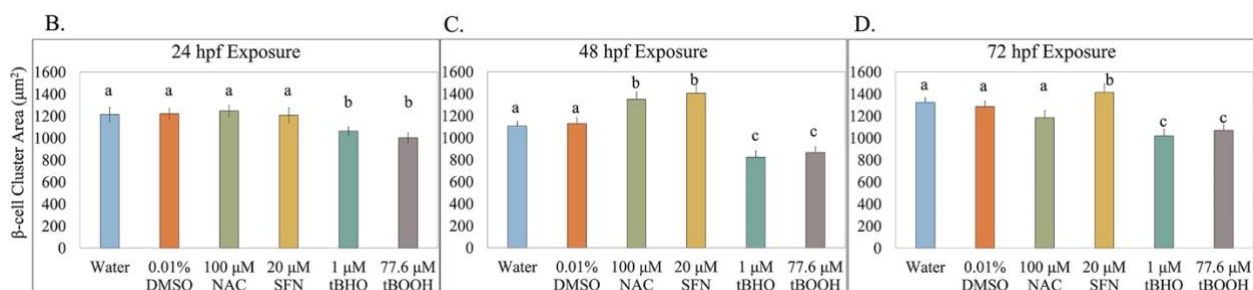


**Figure 3.** Model of Nrf2 activation; Kobayashi, 2006.

oxygen to form active oxygen species and eventual oxidative stress (Cohen, 1987). This upregulates the antioxidant response through the Nrf2 pathway. It can also directly interact with cysteine151 of Keap1 to activate Nrf2 (Robledinos-Anton, 2019). SFN is an antioxidant promoting compound found in vegetables like broccoli and brussels sprouts. SFN also directly interacts with the cysteine151 residue of Keap1 to activate Nrf2 (Kubo, 2017). SFN has also been shown to regulate Nrf2 genes through the PI3K pathway as well as reducing inflammation through the inhibition of NfκB (Juge, 2007). DMF is another antioxidant promoting compound that interacts with the cysteine151 residue of Keap1 to activate Nrf2 (Robledinos-Anton, 2019). DMF is also able to interact with GSH directly. When in the cell, DMF rapidly interacts with the free cysteine residues of GSH. This forms a stable conjugate that leads to a rapid depletion of free GSH in the cell. However, after 10 hours GSH returns to stable levels and by 24 hours the amount of free GSH in the cell is two-fold higher (Brennan, 2015). DMF can also upregulate the enzyme glutathione reductase (GSR) to increase GSH recycling from GSSG back to the reduced GSH form (Hoffman, 2017). DMF is currently an FDA approved drug for the treatment of multiple sclerosis under the name Tecfidera ®. Although there has been extensive research of the role and effectiveness of DMF as a Nrf2 activator, DMF is a relatively novel chemical in regard to zebrafish research (Satoh, 2017).

Previous research in the Timme-Laragy lab looked at the critical windows of redox modulation during pancreas development. They found that tBHQ significantly lowered cellular GSH levels and caused a more oxidized redox potential at 96hpf (Sant, 2017). tBHQ also decreased endocrine pancreas area and altered islet morphology at 96hpf following exposures at 24, 48, and 72 hpf. SFN exposure had no affect at 24hpf, but significantly increased endocrine pancreas area at 96hpf with the 48 and 72 hpf exposures (Rastogi, 2021)(Figure 4). The

endocrine pancreas was found to be most sensitive at the 48 hpf exposure. When looking at Nrf2 expression, untreated zebrafish had very low Nrf2 expression in the endocrine pancreas, but there was expression in other organs including the exocrine pancreas. tBHQ and SFN exposure at 48hpf led to no increase in Nrf2 expression in the endocrine and exocrine pancreas at 96 hpf. Both the untreated and treated fish had similar levels of protein glutathionylation levels in the endocrine pancreas at 96hpf (Rastogi, 2021).



**Figure 4.** Changes in Beta Cell area at 96hpf after exposure to redox modulators at 24, 48, or 72hpf; Rastogi, 2021

For my project I will be studying the effects of activating Nrf2 with both the pro-oxidant tBHQ and antioxidant enhancing molecules SFN and DMF at the critical windows of zebrafish development previously studied. By visualizing changes in Nrf2 protein levels immediately after exposure followed by changes in glutathionylation, we can better understand the role of Nrf2 activation during development and the relationship between Nrf2 and GSH. This project also serves as a way to investigate the changes in Nrf2 and the GSH redox state within the critical windows to better understand the phenotypic changes the lab has previously seen at 96hpf and beyond.

### ***1.1 Research Objective***

The aims of this project were to (1) Measure Nrf2 protein expression and expression patterns after activation at different critical windows zebrafish development (24 vs. 48 vs. 72 hours post fertilization) and (2) Measure the effect of Nrf2 activation on GSH redox conditions via post-translational modification of proteins. To meet these aims zebrafish were exposed to Nrf2 activators: 40 $\mu$ M SFN, 1 $\mu$ M tBHQ, 7 $\mu$ M DMF and then IHC with confocal imaging was used to measure Nrf2 expression and glutathionylation.

For aim 1 I hypothesize that exposure to Nrf2 activators increases Nrf2 expression. SFN, DMF, and tBHQ are known to prevent degradation and stabilize Nrf2 in the cell (Robledinos-Anton, 2019; Kubo 2017). The degree in which Nrf2 expression increases will depend on the sensitivity of the tissue being measured. For aim 2 I hypothesize that exposure to SFN and DMF decrease glutathionylation while tBHQ increases it due to a more reduced and a more oxidized redox state, respectively. Glutathionylation is also dependent on the exposure time-point because the amount of cellular GSH and the redox potential fluctuates throughout development and is also known to affect glutathionylation rates (Hansen, 2015). I also hypothesize that DMF alters glutathionylation the most since it is known to interact with GSH directly. To test these hypotheses zebrafish will be exposed to known Nrf2 activators SFN, tBHQ, and DMF at time points previously used in the redox modulation studies. Then, Nrf2 expression levels and expression patterns will be measured immediately after exposure. GSH expression levels and patterns will also be measured 24 hours after Nrf2 activation to investigate the downstream impact of Nrf2 activation. I will also look at the effect of knocking down Nrf2 using a Nrf2 mutant zebrafish line with a mutated DNA binding domain that has regular Nrf2 expression but decreased transcriptional activity. I hypothesize that mutating the DNA binding domain has no

effect on Nrf2 expression but does increase glutathionylation due to the inability to upregulate antioxidant defense molecules.

### ***1.2 Significance***

Investigating critical windows of Nrf2 activation is important to further elucidate the impact of Nrf2 expression during development and better understand the relationship between redox modulation, Nrf2, and glutathione during key points in development. Previous studies looking at redox modulation in the developing zebrafish found that redox disruption lead to altered pancreas development. However, the exact mechanism causing these changes is not fully known. Finding the mechanisms leading to phenotypic changes as well as the most sensitive tissue and time-points to redox modulation in the vertebrate zebrafish model will help better understand the sensitive windows and tissue in human development. With the current research continuing to find links between oxidative stress and adverse human health outcomes such as diabetes, cancer, and neurodegenerative disorders as well as the prevalence of environmental toxicants linked to oxidative stress in humans, it is important to better understand the effect of Nrf2 activation during development and how that impacts GSH in the cell. Finding these windows of development where redox homeostasis is most sensitive in humans can allow for possible preventative and/or protective measures to oxidative stress during development.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals and Reagents

Dimethyl Sulfoxide (DMSO; Catalog #BP231-1), tert-Butylhydroquinone (tBHQ; Catalog #50-196-7735), Paraformaldehyde (PFA; Catalog # AAJ19943K2), and Methanol (Catalog #A412-4) were purchased from Fisher Scientific (Pittsburgh, PA, USA). dl-Sulforaphane (SFN; Catalog #S4441) and Dimethyl Fumarate (DMF; Catalog #242926) were purchased from Millipore-Sigma (Burlington, MA, USA). Vectashield Antifade Mounting Medium with DAPI (Catalog #H-1200) was purchased from Vector Laboratories (Burlingame, CA, USA). Chicken Anti-Rabbit IgG AlexaFluor 594 (Catalog # A-21442), AlexaFluor 568 tagged Streptavidin (Catalog #S11226), RNAlater (Catalog #AM7020), and Biotinylated Glutathione Ethyl Ester (BioGEE; Catalog #G36000) were purchased from Invitrogen (Carlsbad, CA, USA).

### 2.2 Fish Husbandry and Embryo Sampling

Homozygous wild type ( $nrf2a^{+/+}$ ) and  $nrf2a^{fh318/fh318}$  zebrafish (*Danio rerio*) embryos on an AB strain background were used to perform qPCR experiments (Mukaigasa, 2012). Homozygous wild type ( $nrf2a^{+/+}$ ) and  $nrf2a^{fh318/fh318}$  crossed with *Tg(insa:eGFP)* zebrafish embryos on an AB strain background were used to visualize Nrf2 and glutathionylation in the immunohistochemistry and BioGEE experiments. The  $nrf2a^{fh318/fh318}$  genotype is a loss of function mutation caused from a point mutation in the DNA binding domain of Nrf2, resulting in impaired transcriptional activity (Mukaigasa, 2012).



All breeding adults were maintained on an automated Aquaneering (San Diego, CA, USA) system in accordance with the Guide for the Care and the Use of Laboratory Animals of the National Institutes of Health and with approval from the University of Massachusetts Amherst Institutional Animal Care and Use Committee (Animal Welfare Assurance Number A3551-01). The fish were housed at 28.5 °C on a 14 h light, 10 h dark cycle, and fed GEMMA Micro 300 (Skretting, Westbrook, ME, USA) twice daily. Large breeding tanks were setup with approximately 2:1 female to male ratio. Embryos were collected 1 h post fertilization, washed, and screened for fertilization status. The embryos were dechorionated at 24 hours post fertilization (hpf).

### ***2.3 Exposures***

To investigate critical windows of Nrf2 activation, zebrafish embryos were exposed to the following chemicals at either 24, 48, or 72 hpf: sulforaphane (SFN; 40  $\mu$ M), tert-Butylhydroquinone (tBHQ; 1  $\mu$ M), Dimethyl fumarate (DFM; 7  $\mu$ M). 0.01% DMSO, the solvent for all three chemicals, served as a control. All embryos were exposed for 6 hours in borosilicate glass scintillation vials with 1 ml 0.3x Danieau's per embryo. Following the 6-hour exposure, embryos were immediately fixed in 4% PFA for immunohistochemistry (IHC) or archived in RNAlater for quantitative real-time PCR (qRT-PCR). For the BioGEE exposures, embryos were exposed for 6 hours and then washed and put into clean Danieau's. For 2 hours prior to fixation, the embryos were exposed to 100  $\mu$ M BioGEE and then fixed in 4% PFA, 24 hours after the initial exposure to the Nrf2 activators.

## ***2.4 Immunohistochemistry***

Embryos were fixed in 4% PFA overnight at 4 °C with a ratio of 15 embryos/ mL PFA. Embryos were then rinsed in 0.1% PBS-Tween-20 (PBST) and 100% methanol before being stored in 1 mL of 100% methanol at -20 °C overnight. Samples were rehydrated by adding increasing levels of PBST. Heat antigen retrieval was performed by heating the samples at 70 °C for 20 minutes. Immediately after the heat antigen retrieval, the embryos were permeabilized using ice cold acetone for 8 minutes. Samples were then washed with PBST and blocked using 5% sheep serum at room temperature for 2 hours. After the 2 hours, the anti-Nrf2 antibody was added to the samples at a ratio of 1:1000 for 48 hours at 4 °C. Then, samples were washed with block and PBST and placed in Alexa-594 tagged anti-rabbit antibody at a ratio of 1:1000 overnight at 4 °C. Samples were then washed in block and PBST and then stored in Vectashield containing DAPI at 4 °C until imaging. A group of embryos (n=5) also went through this procedure without the addition of the anti-Nrf2 antibody to control for non-specific binding of the secondary antibody.

## ***2.5 BioGEE***

Embryos were exposed to 100 µM BioGEE for 2 hours and then fixed in 4% PFA overnight at 4 °C. After fixation, all of the steps were identical to the IHC procedure up until the addition of the primary antibody. The samples were blocked in 5% sheep serum at room temperature for two hours and then the samples were placed in AlexaFlour 568 tagged Streptavidin at a ratio of 1:5000 for 1 hour at room temperature. Then, samples were washed in block and PBST and stored in Vectashield containing DAPI at 4 °C until imaging.

## ***2.6 Microscopy***

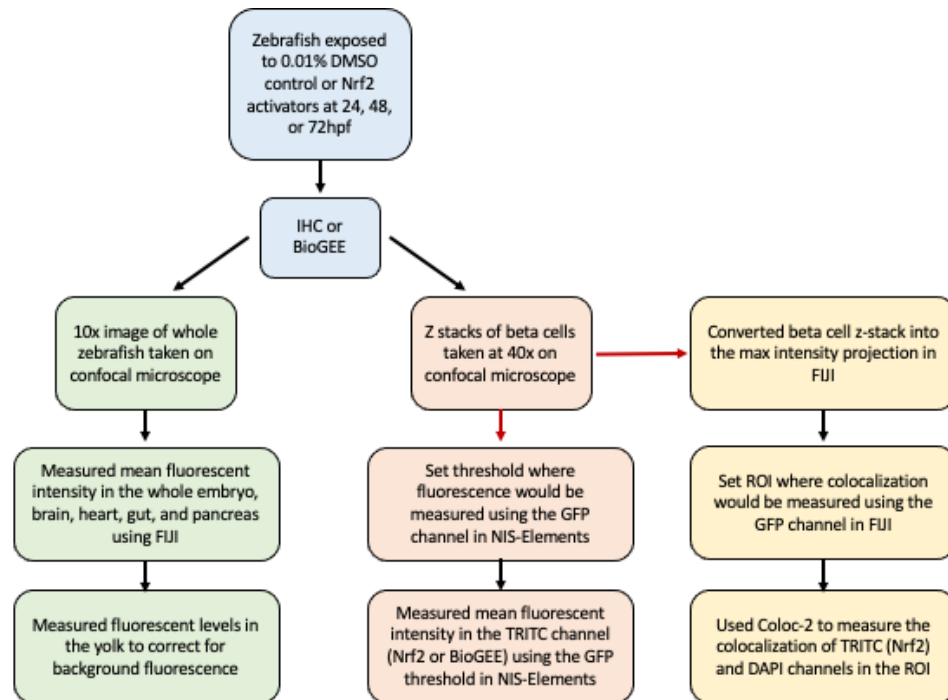
Embryos that went through the immunohistochemistry or BioGEE process were imaged using the Nikon A1R-SIME: Nikon TiE stand with A1 Resonant Scanning Confocal and N-SIM Structured Illumination Super-Resolution equipped with 405 nm, 488 nm, 561 nm and 640 nm laser lines. Images were taken on the TRITC (Nrf2 or BioGEE), FITC (Insulin), and DAPI (nuclei) channels using the same laser intensity and gain for each image. Single layer full body images of the fish focused on the endocrine pancreas were taken using the 10x objective. Z-stacks were taken of the entire endocrine pancreas using the 40x objective. Beta cell images presented here are confocal sections cropped to the area containing the endocrine pancreas. A group of embryos with only secondary antibody were also imaged and found to have consistently low levels of non-specific binding.

## ***2.7 Colocalization***

The colocalization analysis was performed using the Coloc-2 plug-in for Fiji (Costes, 2004, Schindelin, 2012). The analysis was performed on the beta cell images converted to max intensity projections. The FITC channel was used to select the endocrine pancreas as the region of interest (ROI). Then, the TRITC (Nrf2) and DAPI(nuclei) channels were run through the program to analyze colocalization at the ROI (Figure 1). All groups were compared to the wildtype DMSO control of their respective time-point which, as expected, had low levels of colocalization. The Pearson's R coefficients given from the Coloc-2 analysis were then put through the cocor R package to generate Z scores and significance.

## 2.8 Statistics

Fiji was used to measure mean Nrf2 and BioGEE fluorescent intensity in the body, brain, heart, gut, and pancreas using the 10x images (Schindelin, 2012). For the body measurements all parts of the fish were traced apart from the eyes, swim bladder, and yolk. The yolk was measured and subtracted out from the images to correct for background fluorescence. Beta cell area was also measured in Fiji using the same slice in the middle of the z-stack for each image. Images were blinded before analysis. A batch analysis work-flow was created on NIS elements to measure Nrf2 and BioGEE fluorescent intensity in the beta cells (Figure 1). First, a threshold was set using the FITC channel to just measure just the beta cells. Then, using the region defined by the FITC channel, the mean fluorescent intensity of the TRITC channel (Nrf2 or BioGEE) was measured. To assess statistical significance an Analysis of Variance (One-way ANOVA) followed by a Tukey-Kramer post-hoc test was used. All statistics were performed in JMP Pro 14. All experiments had an n of 8-10 embryos per exposure group.



**Figure 5.** Workflow of Methods

## RESULTS

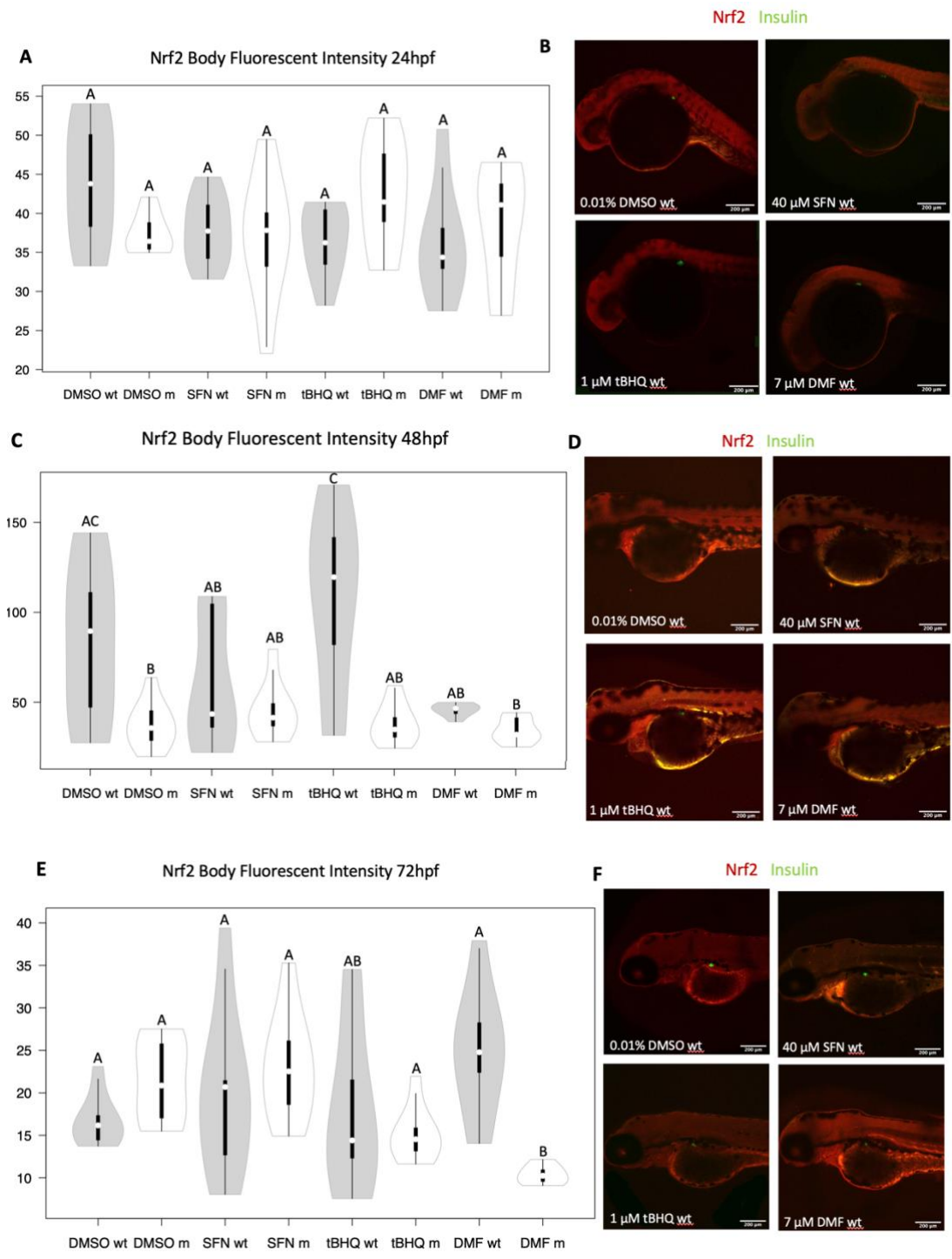
**3.1 AIM 1:** Measure Nrf2 expression and expression patterns after activation at different critical windows (24 vs. 48 vs. 72 hours post fertilization)

**Hypothesis:** Exposure to Nrf2 activators increases Nrf2 expression.

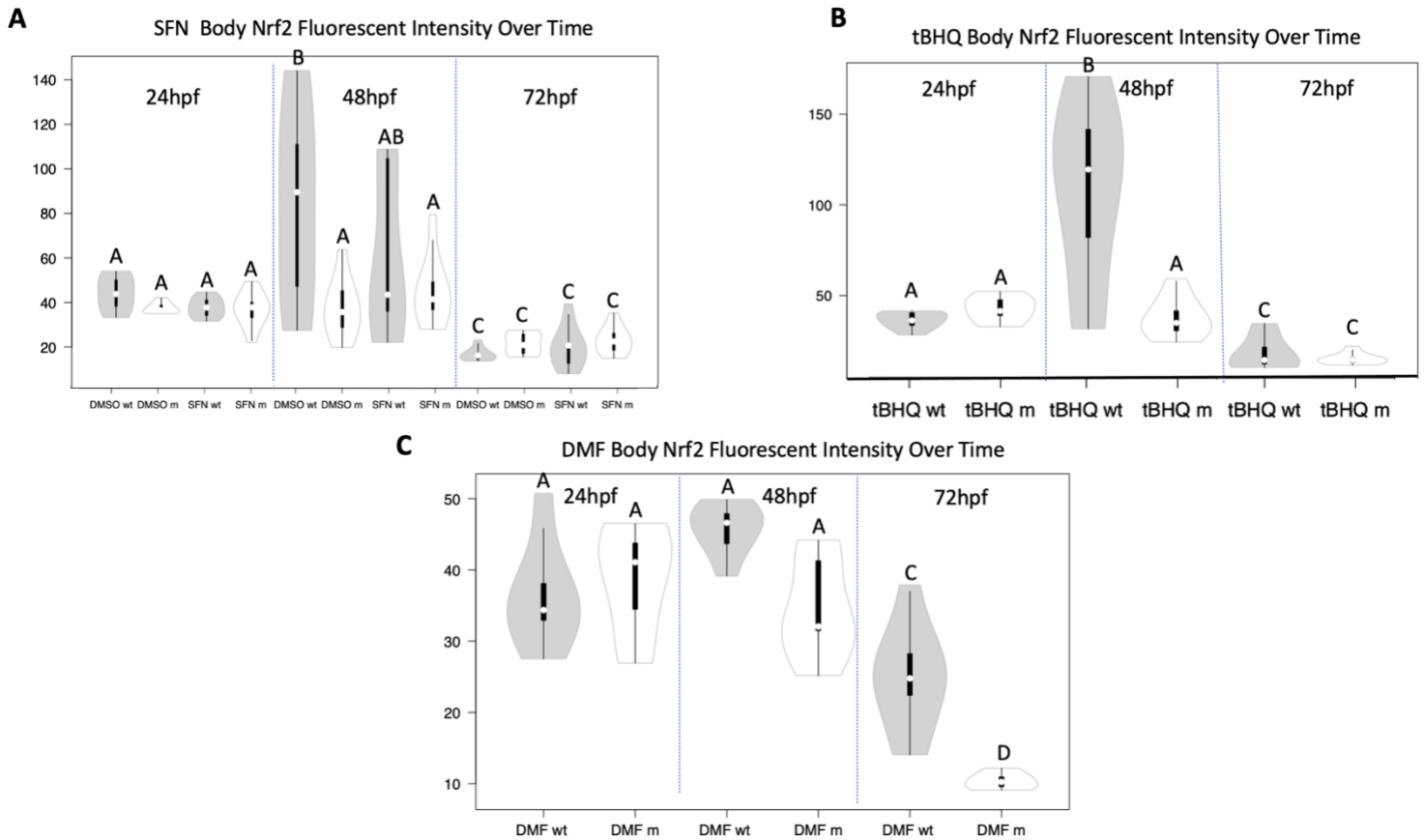
### ***3.1.1 Nrf2 activators do not significantly alter Nrf2 expression in the developing zebrafish body***

To study how Nrf2 activators affect Nrf2 expression across the body of the zebrafish embryo, zebrafish were exposed to either 40  $\mu$ M SFN, 1  $\mu$ M tBHQ, or 7  $\mu$ M DMF and then immunohistochemistry was performed using an anti-Nrf2 antibody. With the 24-hour exposures all three Nrf2 activators trended towards a decrease in Nrf2 fluorescence when compared to the wildtype DMSO control (Figure 6a). The antioxidant enhancing molecules, SFN and DMF, showed no changes in fluorescence at both 48hpf and 72hpf while the pro-oxidant tBHQ increased Nrf2 expression at 48hpf and showed no change at 72hpf(Figure 6c,e).

When comparing the Nrf2 wildtype fish and mutants, DMSO and tBHQ mutants significantly reduced Nrf2 fluorescence at 48 hours while the DMF mutants significantly decreased Nrf2 at 72 hours ( $p < 0.05$ ; Figure 6c,e). This supports that Nrf2 has some level of self-regulation in the developing zebrafish embryo. When comparing total levels of Nrf2 fluorescence in the embryo at the different time points, the embryos at 72hpf had significantly less Nrf2 than the other two time-points. The 24- and 48-hour exposure time-points had mostly equal levels of Nrf2 expression aside from the wildtype DMSO and tBHQ groups having significantly more Nrf2 levels at 48 hours (Figure 7).



**Figure 6.** Nrf2a activators do not significantly alter Nrf2 expression in the body of the zebrafish embryo(A,C,E) Nrf2a fluorescent intensity in the body after a 24, 48, or 72hpf exposure to Nrf2 activators (40 $\mu$ M SFN, 1 $\mu$ M tBHQ, 7 $\mu$ M DMF), respectively. (B,D,F) Representative images of the homozygous wild type (*nrf2a*<sup>+/+</sup>) x Tg(*insa*:eGFP) embryos at 24, 48, or 72hpf, respectively. The TRITC channel depicts Nrf2 and the FITC channel depicts the pancreatic beta cells. n=10 fish; Different letters indicate significant differences ( $p < 0.05$ ) from the wildtype DMSO control as determined by a ONE-WAY ANOVA followed by a Fisher's LSD Post-Hoc Test.



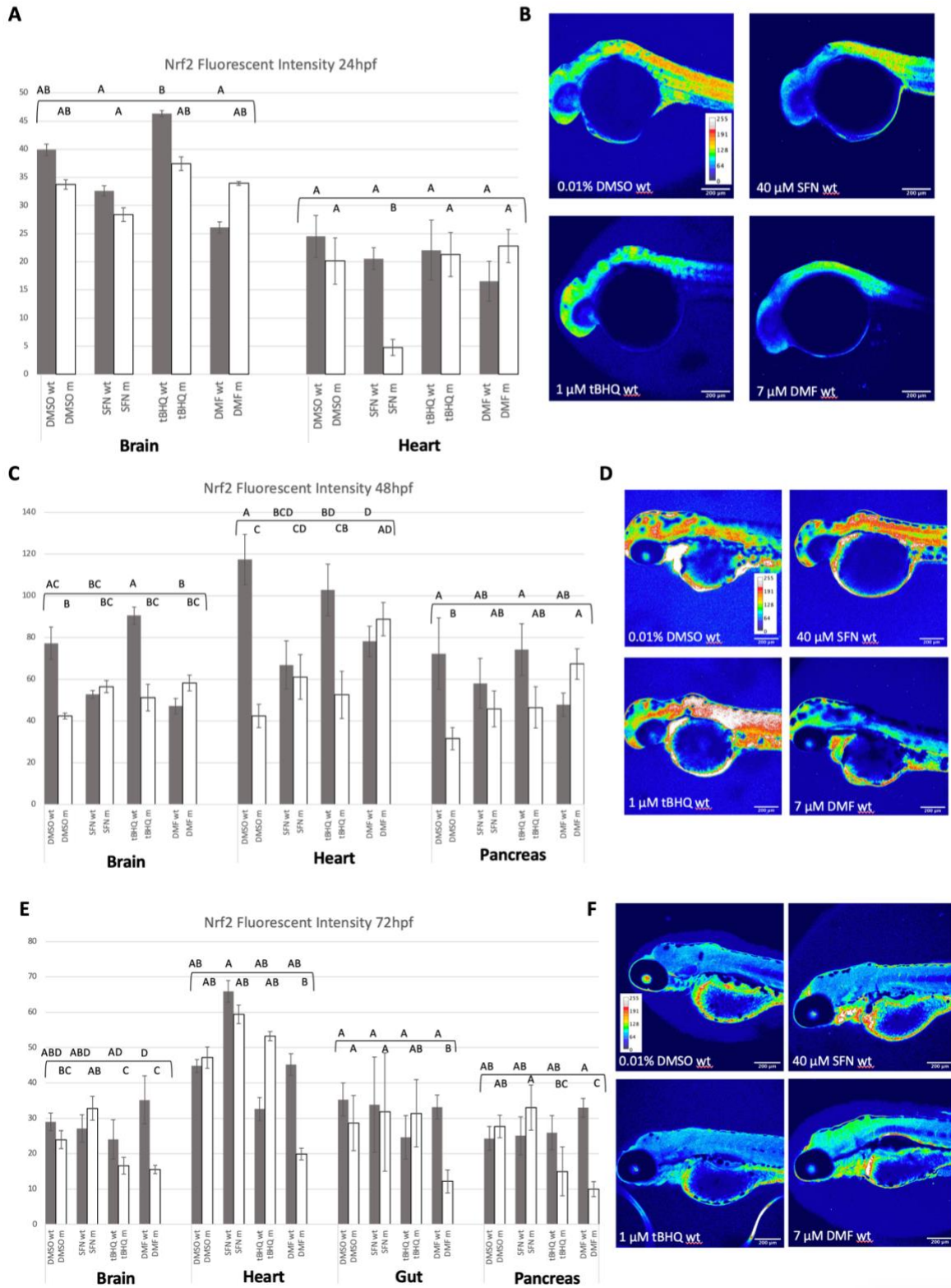
**Figure 7.** Nrf2 expression decreases at 72hpf. (A) Nrf2 fluorescent intensity in the body after exposure to 40  $\mu$ M SFN or 0.01% DMSO control at 24, 48, and 72hpf. (B) Nrf2 fluorescent intensity in the body after exposure to 1  $\mu$ M tBHQ at 24, 48, and 72hpf.(C) Nrf2 fluorescent intensity in the body after exposure to 7  $\mu$ M DMF at 24, 48, and 72hpf n=10 fish; Different letters indicate significant differences ( $p < 0.05$ ) from the wildtype DMSO control as determined by a ONE-WAY ANOVA followed by a Fisher's LSD Post-Hoc Test.

### ***3.1.2 Nrf2 activators alter Nrf2 expression in multiple organs at 48hpf***

To further elucidate that impact of Nrf2 activators on the embryo, the same images used for the full body analysis were further analyzed to measure Nrf2 fluorescence levels in the following parts of the embryo: brain, heart, gut, and pancreas. For the 24-hour fish only the brain, and heart were measured and at 48hpf all but the gut was measured. This is due to the fact that the exocrine pancreas bud does not begin to form until 30hpf and the organs associated with the gut are not present until 72hpf (Ruzicka L, 2019). When exposed at 24 hours, there were no significant changes between the wildtype DMSO controls and the Nrf2 activators (Figure 8 a,b). However, tBHQ did show an increase in Nrf2 expression in the brain while having lower Nrf2 levels when looking at the full body. With the 48-hour exposures, all three Nrf2 activators had decreased Nrf2 fluorescence across all organ types (Figure 8 c,d). Most notably, the wildtype DMSO fish had significantly more Nrf2 in the heart and brain ( $p < 0.05$ ). This is a different expression pattern than what was seen when looking at the whole body where tBHQ had higher Nrf2 expression. When exposed at 72 hours, there were no significant changes in Nrf2 stabilization (Figure 8 e,f). However, DMF did show a trend towards an increase in Nrf2 expression in the yolk, brain, and pancreas that was not seen when looking at the whole embryo.

When comparing the Nrf2a wildtype and mutant fish, mutant fish showed a marked decrease in Nrf2 at all time-points and for each organ. As with the full embryo results, the 48-hour DMSO and tBHQ mutant fish and the 72-hour DMF mutant fish had significantly lower Nrf2 fluorescence ( $p < 0.05$ ). There were also significant changes in Nrf2 mutants not seen in the full embryos including the 24 hpf SFN hearts and the 72hpf tBHQ brains ( $p < 0.05$ ).



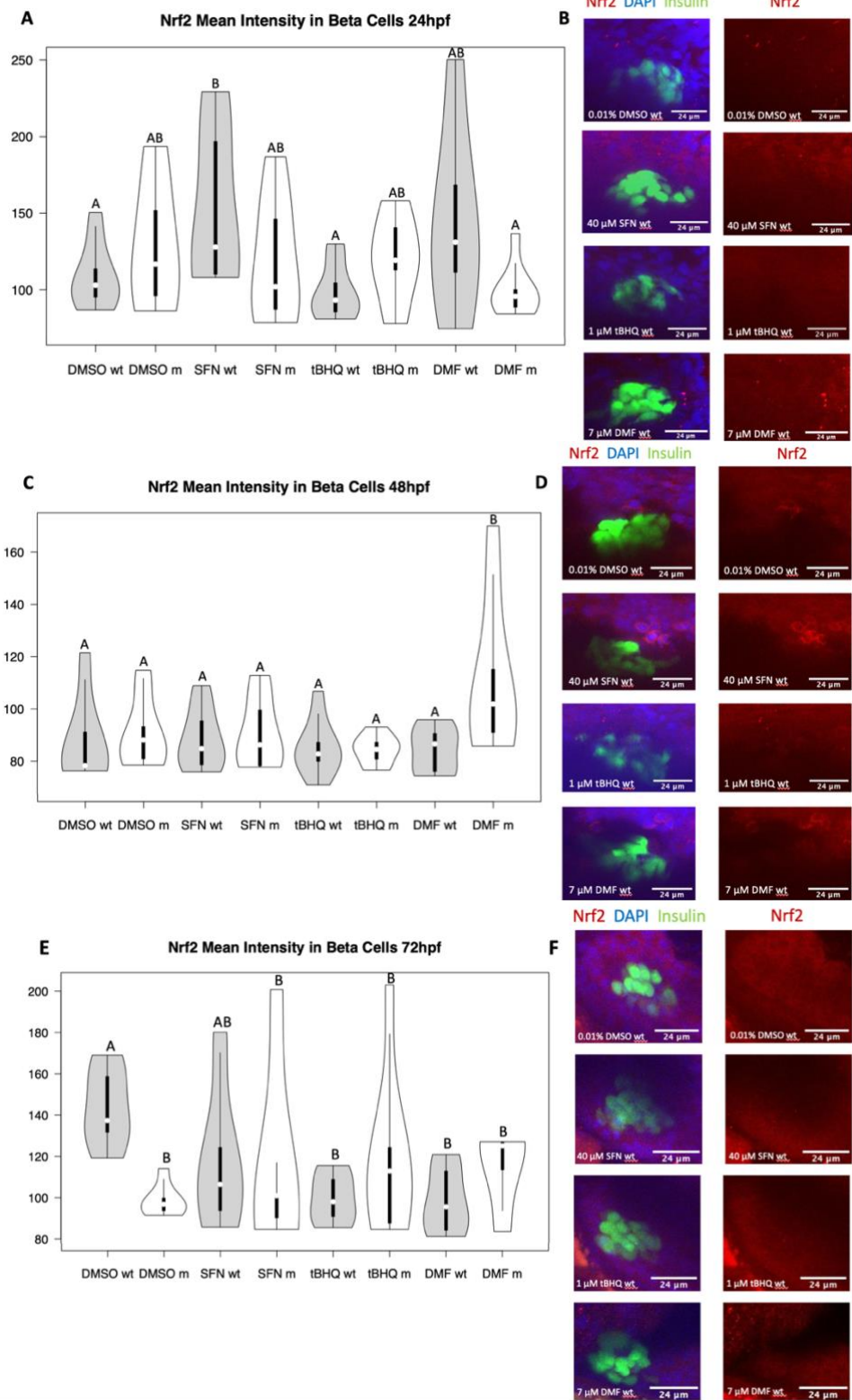


**Figure 8.** Nrf2 activators alter Nrf2 expression in multiple organs at 48hpf. (A,C,E) Nrf2 fluorescent intensity in the brain, heart, gut and pancreas at 24, 48, and 72hpf, respectively. (B,D,F) Representative images of the homozygous wild type (*nrf2a*<sup>+/+</sup>) embryos at 24, 48, or 72hpf, respectively. All fish are displayed using a heatmap where blue is the least Nrf2 expression and white is the most. n=10 fish; Lines represent the standard error mean. Different letters indicate significant differences ( $p < 0.05$ ) from the wildtype DMSO control as determined by a ONE-WAY ANOVA followed by a Fisher's LSD Post-Hoc Test.

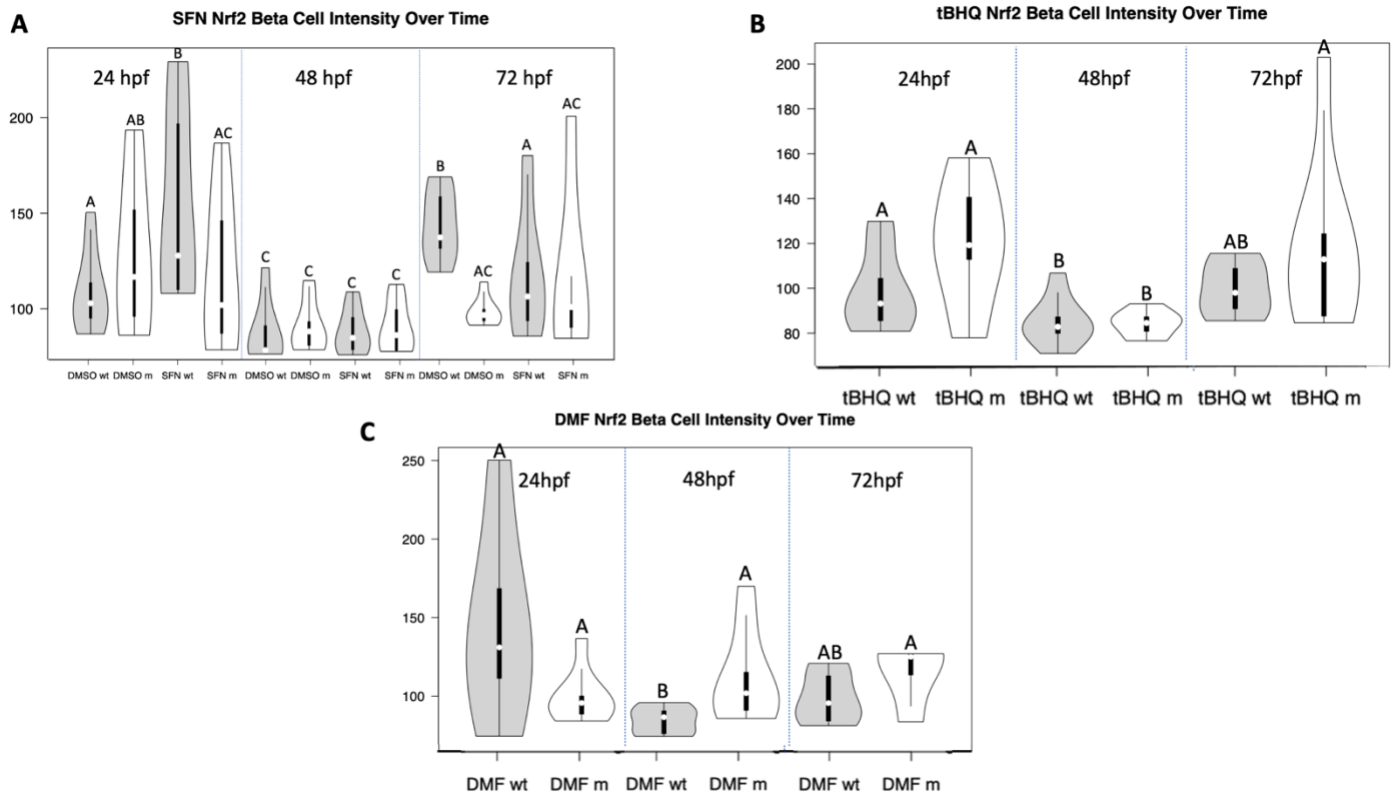
### ***3.1.3 Nrf2 activators alter Nrf2 protein expression in pancreatic beta cells***

To study how Nrf2 activators affect Nrf2 expression in the pancreatic beta cells of developing zebrafish, zebrafish were exposed to either 40  $\mu$ M SFN, 1  $\mu$ M tBHQ, or 7  $\mu$ M DMF and then z-stacks were taken of the endocrine pancreas stained with an anti-Nrf2 antibody. When exposed at 24 hours, the antioxidant enhancing molecule SFN significantly increased Nrf2 expression ( $p < 0.05$ ) and DMF also showed an upward trend in Nrf2 (Figure 9a,b). On the other hand, exposure to all three Nrf2 activators at 72hpf had a significant decrease in Nrf2 with the change in tBHQ and DMF being significant ( $p < 0.05$ ; Figure 9e,f). There was no change in Nrf2 expression when exposed at the 48-hour time-point (Figure 9c,d).

When looking at differences between the wildtype and mutant fish, there were both increases and decreases in Nrf2 expression across all exposure time-points (Figure 9). DMF mutant embryos had significantly increased Nrf2 in their beta cells at 48 hours while DMSO mutants had significantly decreased Nrf2 at 72 hours ( $p < 0.05$ ). As with the full embryo experiments, there does seem to be some level of Nrf2 self-regulation. However, with both increases and decreases being seen across all three time-points, no discernable relationship can be determined at this time. When comparing Nrf2 expression in the beta cells at different time-points, the embryos exposed at 48hpf had significantly less Nrf2 than the 24- and 72-hour time-points. The 24- and 72-hour time-points had similar levels of Nrf2 aside from the DMSO controls having significantly more Nrf2 at 72hpf than most of the other groups (Figure 10).



**Figure 9.** Nrf2 activators alter Nrf2 stabilization in beta cells. (A,C,E) Nrf2a fluorescent intensity in the pancreatic beta cells after a 24, 48, or 72hpf exposure to Nrf2 activators ((40μM SFN, 1μM tBHQ, 7μM DMF), respectively. (B,D,F) Representative images of the homozygous wild type (*nrf2a<sup>+/+</sup>*) x Tg(*ins:aeGFP*) zebrafish at 24, 48, or 72hpf, respectively. Images are the max intensity projection of the beta cell z stack where FITC represents the beta cells, TRITC represents Nrf2, and DAPI represents nuclei. n= 8-10 fish; Different letters indicate significant differences ( $p < 0.05$ ) from the wildtype DMSO control as determined by a ONE-WAY ANOVA followed by a Fisher's LSD Post-Hoc Test.

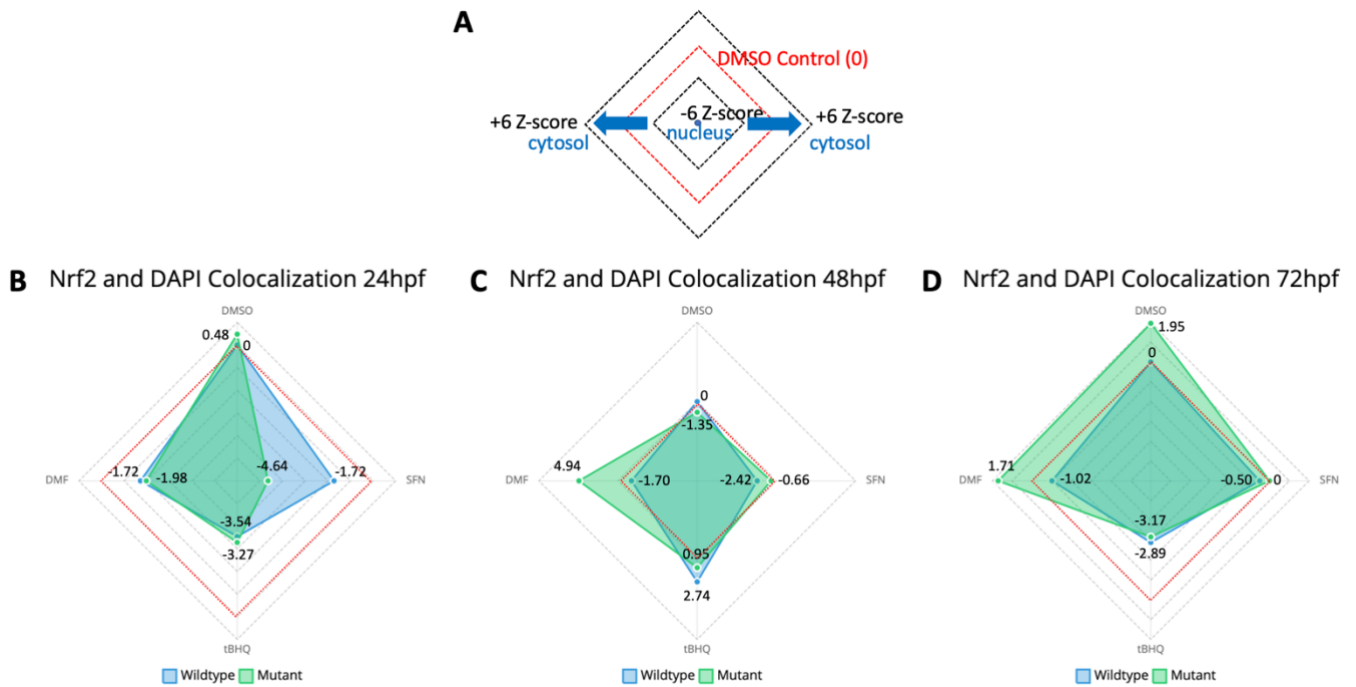


**Figure 10.** Nrf2 expression decreases at 48hpf in the beta cells. (A) Nrf2 fluorescent intensity in the pancreatic beta cells after exposure to 40  $\mu$ M SFN at 24, 48, and 72hpf. (B) Nrf2 fluorescent intensity in the beta cells after exposure to 1  $\mu$ M tBHQ at 24, 48, and 72hpf. (C) Nrf2 fluorescent intensity in the beta cells after exposure to 7  $\mu$ M DMF at 24, 48, and 72hpf n=10 fish; Different letters indicate significant differences ( $p < 0.05$ ) from the wildtype DMSO control as determined by a ONE-WAY ANOVA followed by a Fisher's LSD Post-Hoc Test.

### 3.1.4 Nrf2 activators increase Nrf2 translocation into the nucleus of beta cells

To further investigate how exposure Nrf2 activators impacted Nrf2, colocalization between anti-Nrf2 antibody staining and DAPI staining was used to measure relative amounts of Nrf2 in the nucleus. As previously stated, Nrf2 translocates into the nucleus when activated in order to regulate the antioxidant response element. At the 24-hour time-point, all three exposures greatly increased Nrf2 translocation into the nucleus when compared to the wildtype DMSO

control (Figure 11b). tBHQ had the greatest increase in Nrf2 translocation ( $p < 0.001$ ), but SFN and DMF also trended towards a significant increase ( $p < 0.08$ ). At the 48-hour time-point SFN and DMF had a significant increase in Nrf2 translocation ( $p < 0.08$ ) while tBHQ had significantly more Nrf2 in the cytosol than the control (Figure 11c). At 48 hours there was also an increase in Nrf2 across all exposure groups and the control when compared to the other time-points. Both the 24 and 72-hour time-points had a Pearson's R score averaging 0.35 while the average R score at the 48-hour time-point was 0.55. When exposed at 72 hours, only tBHQ had an increase in Nrf2 translocation in comparison with the control (Figure 11d). There were no differences in colocalization between the Nrf2 wildtype and mutant fish aside from the following exposure groups: SFN 24hpf, DMF 48hpf, DMSO 72hpf, and DMF 72hpf. Interestingly, all of these groups also had different Nrf2 levels in their pancreatic beta cells.



**Figure 11.** Nrf2 activators increased Nrf2 translocation into the nucleus. (A) Legend for correctly reading the radar charts where the red line represents the wildtype DMSO control. Radar charts of embryos exposed to Nrf2 activators (40 $\mu$ M SFN, 1 $\mu$ M tBHQ, 7 $\mu$ M DMF) at 24(B), 48(C), and 72hpf(D). Points represent the z-score of the colocalization in comparison to the wildtype control where a lower z-score means greater colocalization. n=8-10 fish.

**3.2 AIM 2:** Measure the effect of Nrf2 activation on GSH redox via post-translational modification of proteins at critical windows (24, 48, 72hpf)

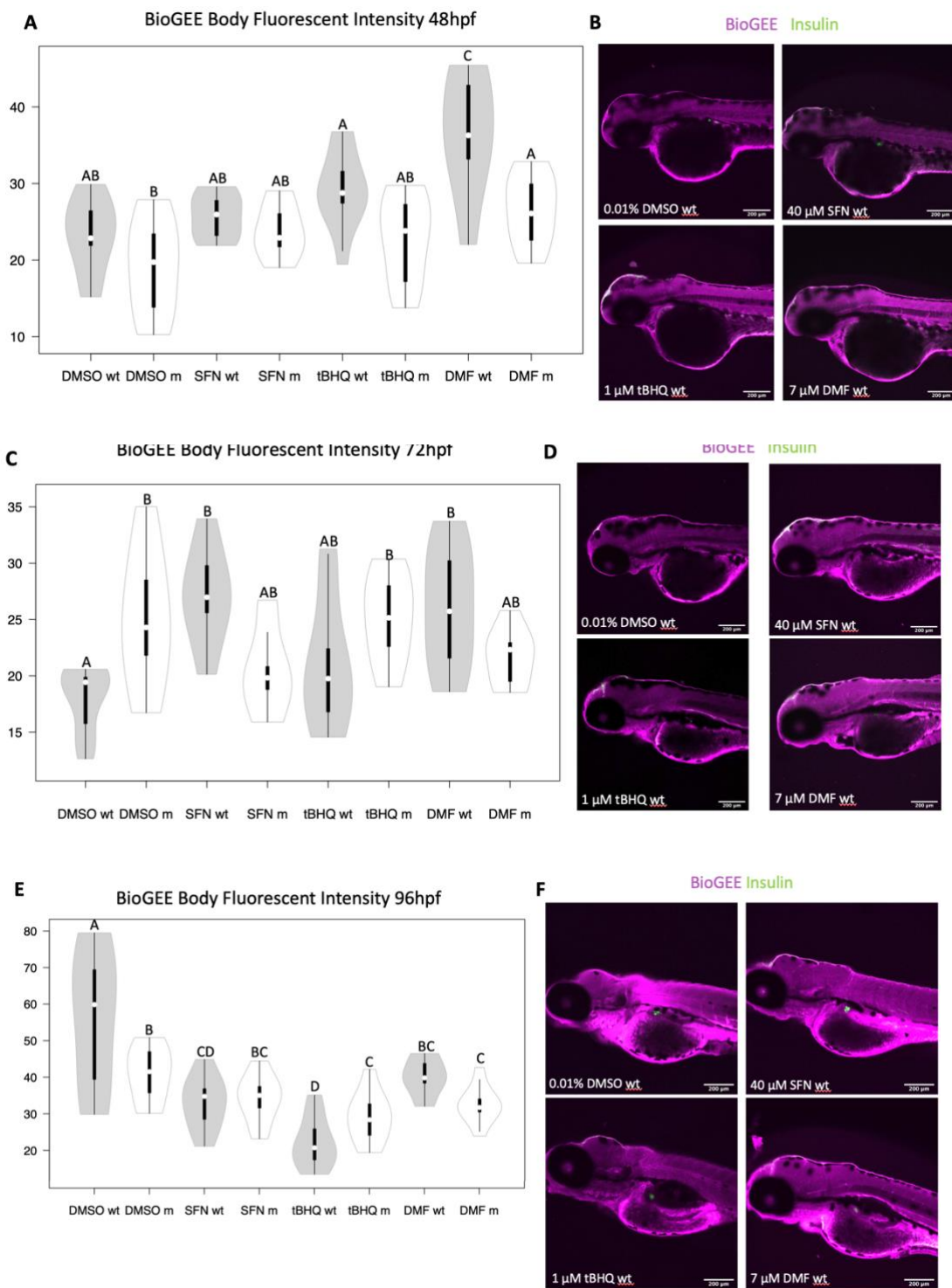
**Hypothesis:** Exposure to SFN and DMF decreases glutathionylation while tBHQ increases it. Glutathionylation is also dependent on the exposure time-point.

### ***3.2.1 Nrf2 activators alter glutathionylation levels in the zebrafish body***

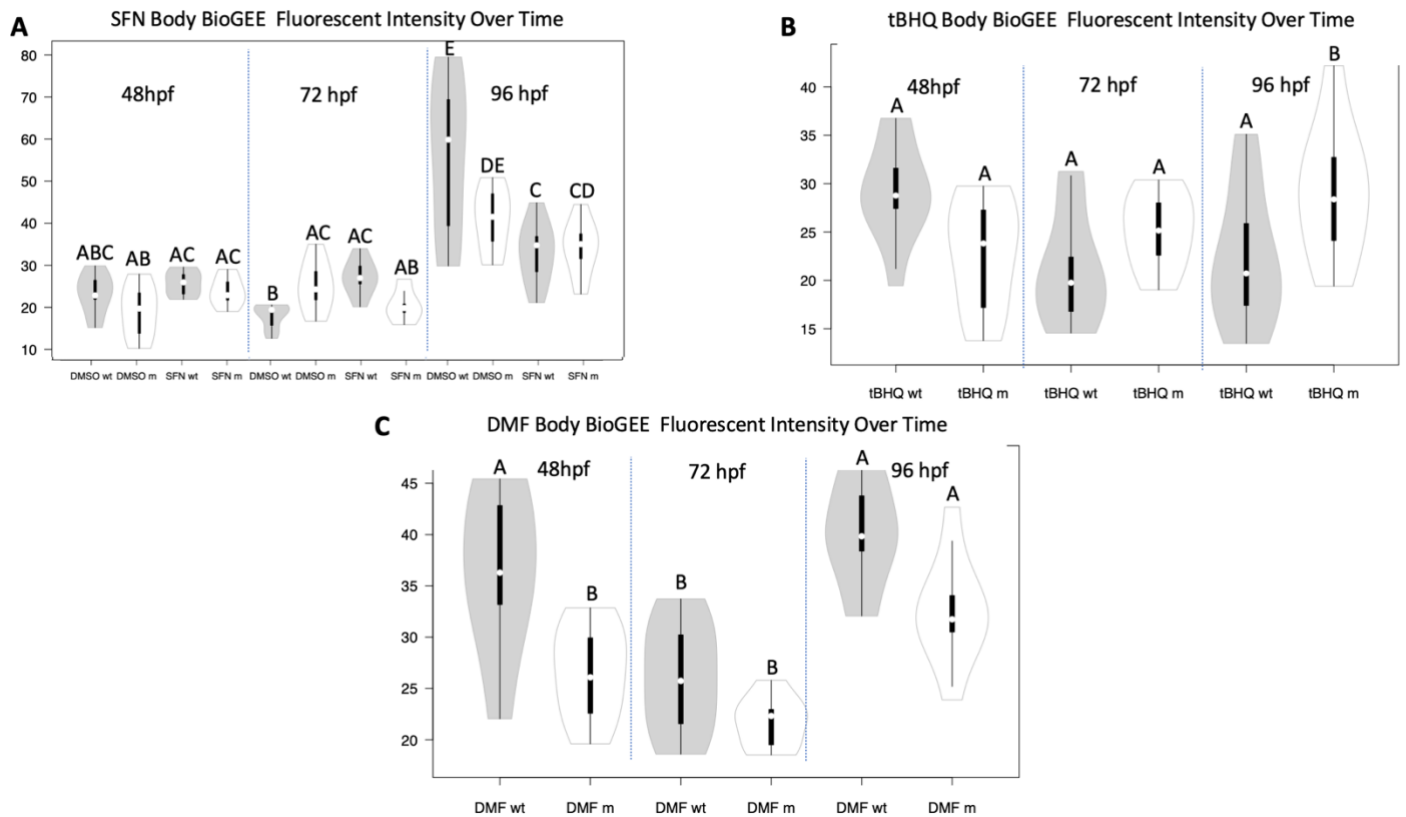
To investigate how Nrf2 activators affect glutathionylation across the zebrafish body, zebrafish were exposed to either 40  $\mu$ M SFN, 1  $\mu$ M tBHQ, or 7  $\mu$ M DMF and then exposed to 100  $\mu$ M BioGEE 24 hours later. With the 48hpf embryos (exposed at 24hpf), tBHQ and DMF increased glutathionylation, with DMF being a significant increase ( $p < 0.05$ ; Figure 12a,b). With the 72hpf embryos, both antioxidant enhancing molecules, SFN and DMF, significantly increased glutathionylation while the 96 hour fish showed a significant decrease in glutathionylation for all three Nrf2 activators ( $p < 0.05$ ; Figure 12c-f).

When looking at differences between the wildtype and mutant fish, there were changes in glutathionylation at all three time-points and for every exposure group. For the 48 hours fish in particular, all four groups had some level of variation with the mutant fish and the DMSO mutant controls had significantly increased glutathionylation ( $p < 0.05$ , Figure 12). When comparing glutathionylation levels at different time points, the body had significantly more expression in the 96hpf fish ( $p < 0.05$ , Figure 13). The 48 and 72 hour fish all had equal levels of glutathionylation aside from the DMF wildtype fish at 48 hours being significantly higher ( $p < 0.05$ ).





**Figure 12.** Nrf2a activators alter glutathionylation levels. (A,C,E) BioGEE fluorescent intensity in the zebrafish body after exposure to Nrf2 activators (40 $\mu$ M SFN, 1 $\mu$ M tBHQ, 7 $\mu$ M DMF) at 48, 72, or 96hpf, respectively. (B,D,F) Representative images of the homozygous wild type (*nrf2a*<sup>+/+</sup>) x Tg(*insa:eGFP*) embryos at 48, 72, or 96hpf, respectively. Magenta depicts BioGEE and the FITC channel depicts the pancreatic beta cells. n=10 fish; Different letters indicate significant differences ( $p < 0.05$ ) from the wildtype DMSO control as determined by a ONEWAY ANOVA followed by a Fisher's LSD Post-Hoc Test.



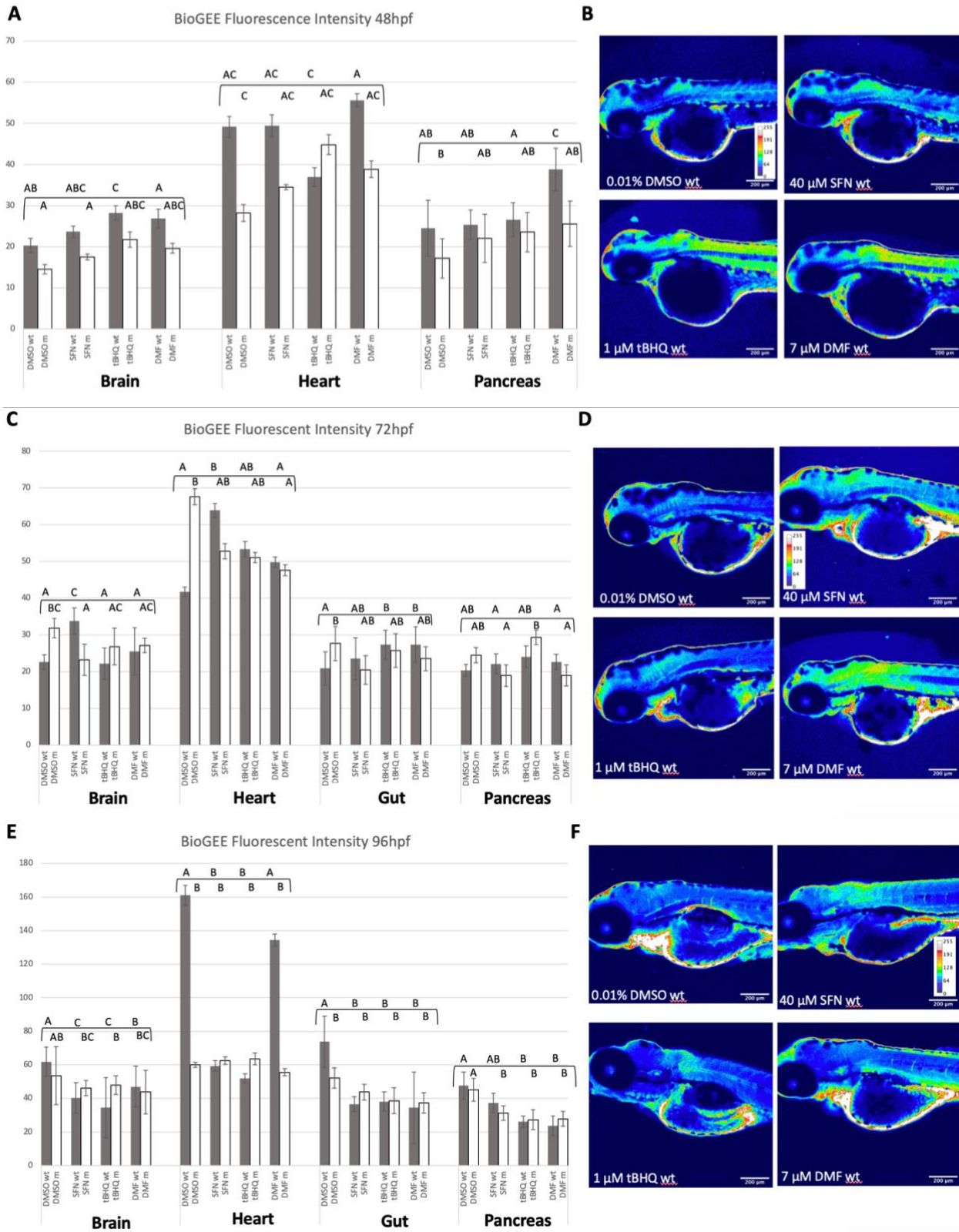
**Figure 13.** Glutathionylation increases at 96hpf (A) BioGEE fluorescent intensity in the zebrafish body after exposure to 40  $\mu$ M SFN at 48, 72, 96hpf. (B) BioGEE fluorescent intensity in the body after exposure to 1  $\mu$ M tBHQ at 48, 72, 96hpf. (C) BioGEE fluorescent intensity in the body after exposure to 7  $\mu$ M DMF at 48, 72, 96hpf. n=10 fish; Different letters indicate significant differences ( $p < 0.05$ ) from the wildtype DMSO control as determined by a ONE-WAY ANOVA followed by a Fisher's LSD Post-Hoc Test.



### ***3.2.2 Nrf2 activators alter glutathionylation levels in multiple organs***

To further understand how Nrf2 activators alter glutathionylation in the developing embryo, the same images used for the full body analysis were further analyzed to measure BioGEE levels in the following parts of the embryo: brain, heart, gut, and pancreas. As with the Nrf2 experiments, the 48hpf fish were measured for all regions apart from the gut. In the 48hpf SFN had no changes from the wildtype DMSO controls while tBHQ and DMF showed increases in glutathionylation at multiple organs (Figure 14a,b). tBHQ significantly increased BioGEE levels in the brain while DMF significantly increased BioGEE levels in the pancreas ( $p < 0.05$ ). With the 72hpf fish increases in glutathionylation were seen in all organs aside from the pancreas (Figure 14 c,d). Most notably, SFN significantly increased BioGEE in the brain and heart and both tBHQ and DMF significantly increased BioGEE in the gut ( $p < 0.05$ ). In the 96hpf fish all groups had significantly lower glutathionylation than the wildtype DMSO control for all organs aside from the yolk (Figure 14 e,f;  $p < 0.05$ ). Overall, the expression patterns seen at all three time-points matched the ones seen when looking at the full embryo.

When looking at differences between the wildtype and mutant fish, most time-points had decreased glutathionylation in the mutant fish or no change at all. However, there were a few significant increases in BioGEE seen in the mutant fish including the brain, heart, and gut of DMSO at 72hpf and the tBHQ mutant brain at 96hpf (Figure 14,  $p < 0.05$ ). Overall, the mutants seem to have more decreased glutathionylation when looking at individual organs instead of the whole embryo. The significant increase of the DMSO mutants was however seen in the whole embryos as well.

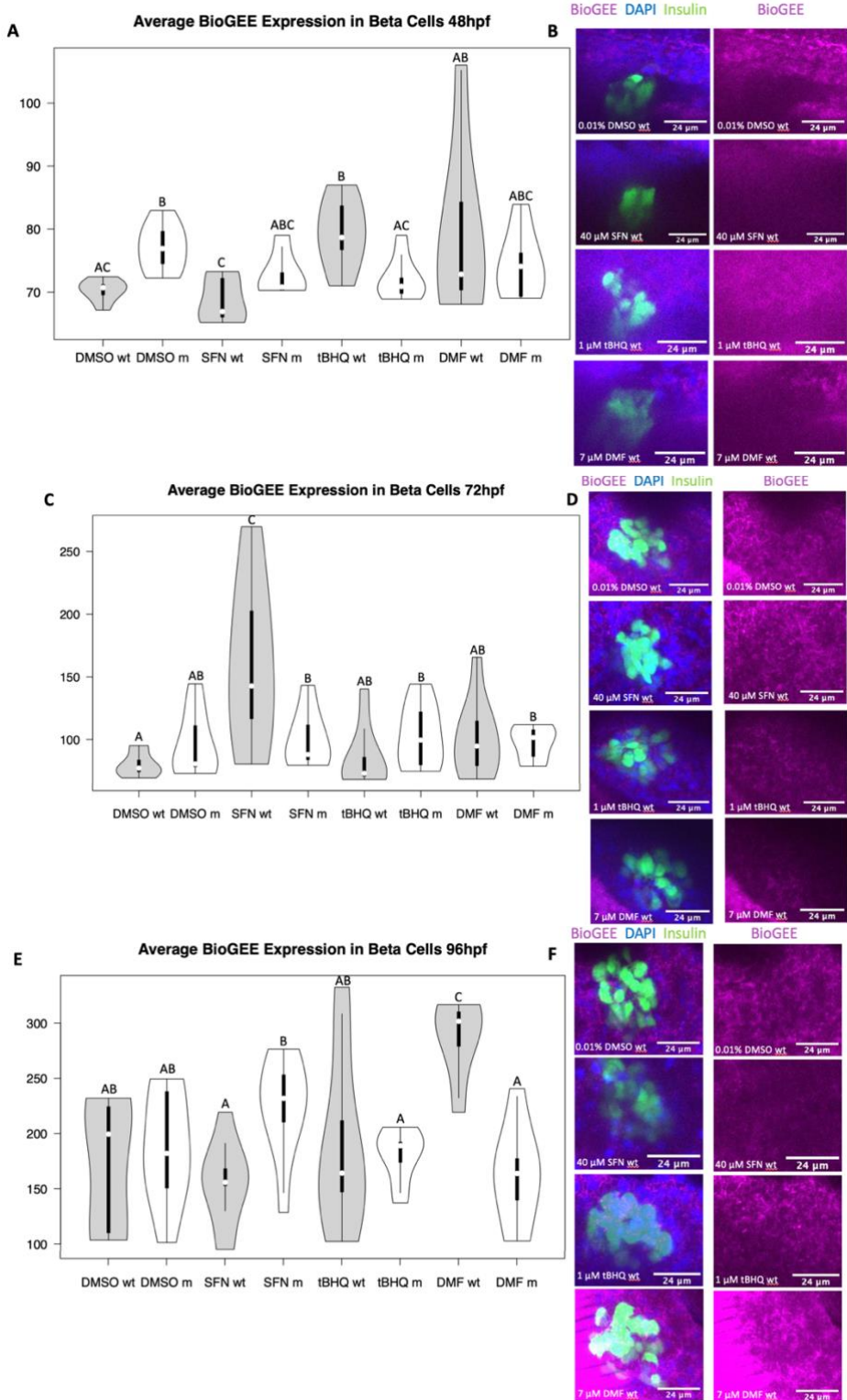


**Figure 14.** Nrf2 activators alter glutathionylation in multiple organs. (A,C,E) BioGEE fluorescent intensity in the brain, heart, gut and pancreas at 48, 72, and 96hpf, respectively. (B,D,F) Representative images of the homozygous wild type (*nrf2a<sup>+/+</sup>*) embryos at 48, 72, or 96hpf, respectively. All fish are displayed using a heatmap where blue is the lowest amount of BioGEE expression and white is the highest. n=10 fish; Lines represent the standard error mean. Different letters indicate significant differences ( $p < 0.05$ ) from the wildtype DMSO control as determined by a ONE-WAY ANOVA followed by a Fisher's LSD Post-Hoc Test.

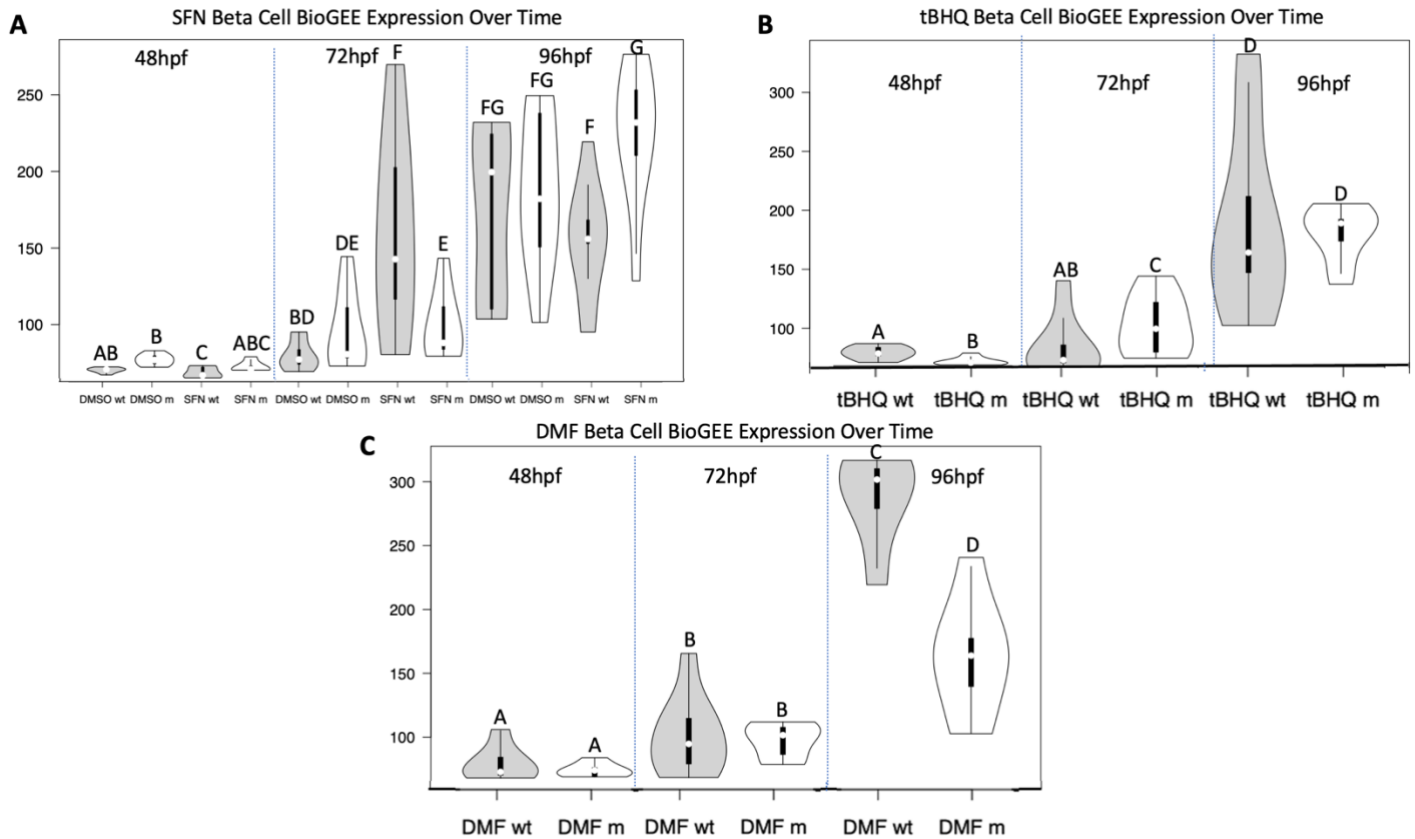
### ***3.2.3 Nrf2 activators alter glutathionylation levels in pancreatic beta cells***

To investigate how Nrf2 activators affect glutathionylation in the pancreatic beta cells zebrafish were exposed to either 40  $\mu$ M SFN, 1  $\mu$ M tBHQ, or 7  $\mu$ M DMF, and then z stacks were taken of their endocrine pancreas labelled with 100  $\mu$ M BioGEE. In the 48hpf fish the pro-oxidant tBHQ significantly increased glutathionylation, while the antioxidant enhancing molecule SFN significantly increased glutathionylation in the 72hpf fish ( $p < 0.05$ ; Figure 15a-d). There were no other notable changes from the control at these two time-points. For the 96hpf fish, tBHQ and SFN decreased glutathionylation, with the while DMF led to a significant increase ( $p < 0.05$ ; Figure 15e,f).

Similar to the full body findings, all exposure groups had differences between the Nrf2 wildtype and mutant zebrafish at various time-points. Both SFN and tBHQ had variability between the two genotypes at all three time-points, although the changes in tBHQ were not significant. For SFN there was an increase in the 48 hpf fish, a significant decrease in the 72hpf, and a significant increase in the 96hpf ( $p < 0.05$ ; Figure 15). DMSO and DMF only had significant variability at the 48- and 96-hour time-point respectively ( $p < 0.05$ ). When comparing glutathionylation levels at the different time-points, there was a steady increase between the three time-points. The 72hpf fish had significantly more glutathionylation than the 48hpf fish and the 96 hpf had significantly higher glutathionylation than the 72hpf fish ( $p < 0.05$ ; Figure 16).



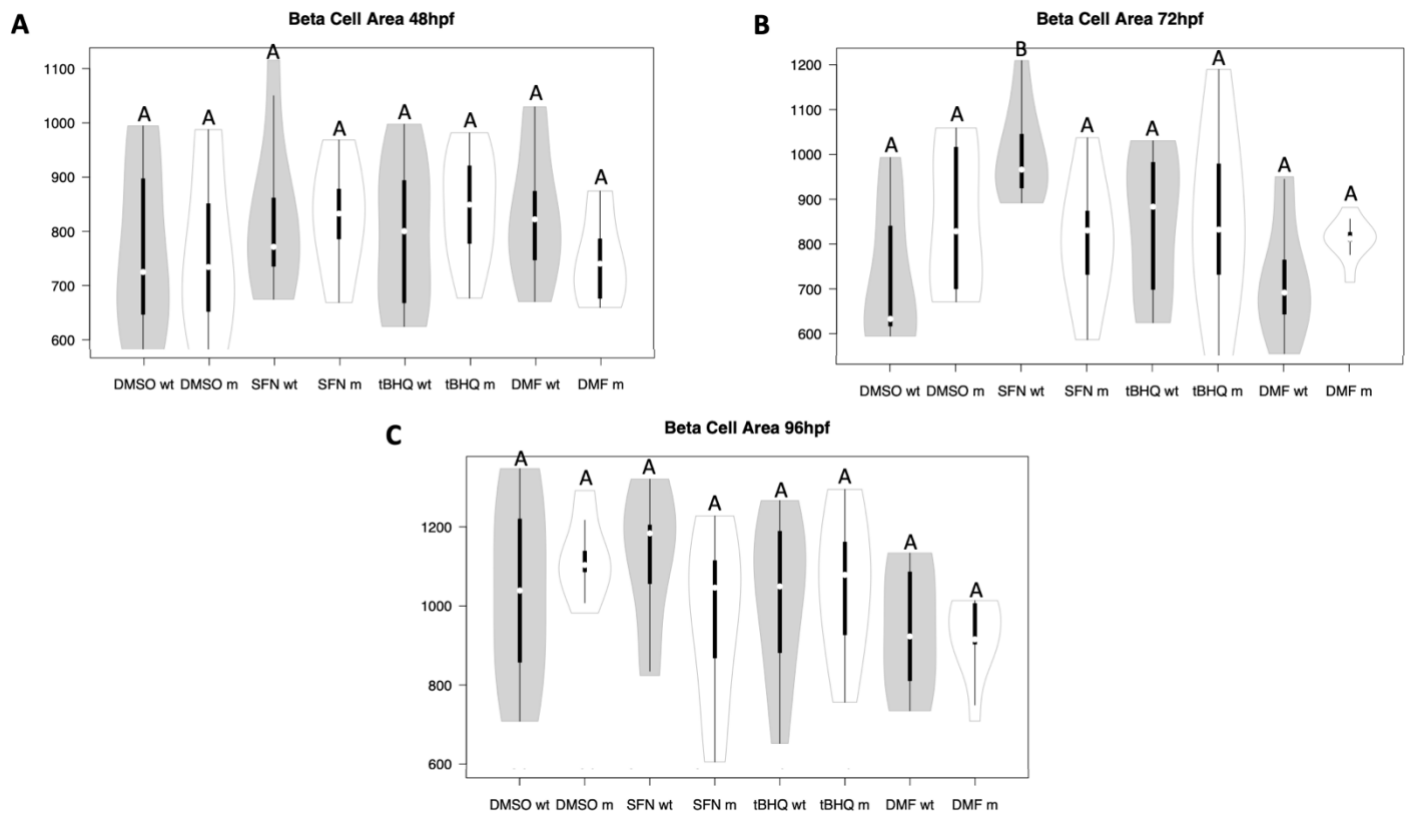
**Figure 15.** Nrf2a activators alter glutathionylation in beta cells. (A,C,E) BioGEE fluorescent intensity in the pancreatic beta cells of 48, 72, or 96hpf embryos after exposure to Nrf2 activators (40 $\mu$ M SFN, 1 $\mu$ M tBHQ, 7 $\mu$ M DMF), respectively. (B,D,F) Representative images of the homozygous wild type (*nrf2a*<sup>+/+</sup>) x Tg(*insa*:eGFP) zebrafish at 48, 72, or 96hpf, respectively. Images are the max intensity projection of the beta cell z stack where FITC represents the beta cells, magenta represents BioGEE, and DAPI represent the nuclei. n = 8-10 fish; Different letters indicate significant differences ( $p < 0.05$ ) from the wildtype DMSO control as determined by a ONE-WAY ANOVA followed by a Fisher's LSD Post-Hoc Test



**Figure 16.** Glutathionylation increases in a time dependent manner. (A) BioGEE fluorescent intensity in the pancreatic beta cells after exposure to 40  $\mu$ M SFN at 48, 72, and 96hpf. (B) BioGEE fluorescent intensity in the beta cells after exposure to 1  $\mu$ M tBHQ at 48, 72, 96hpf. (C) BioGEE fluorescent intensity in the beta cells after exposure to 7  $\mu$ M DMF at 48, 72, 96hpf. n=10 fish; Different letters indicate significant differences ( $p < 0.05$ ) from the wildtype DMSO control as determined by a ONE-WAY ANOVA followed by a Fisher's LSD Post-Hoc Test.

### 3.2.4 SFN increased beta cell area at 72hpf following an exposure at 48hpf

To further investigate the impact of exposure to Nrf2 activators later on in development, the area of the beta cells exposed to BioGEE was measured. With both the 48- and 96-hour beta cells, there were no significant changes in beta cell area (Figure 17 a,b). At the 72-hour time-point the wildtype SFN fish significantly increased beta cell area (Figure 17 c). This increase was rescued in the SFN mutant zebrafish.



**Figure 17.** SFN increases beta cell area at 72hpf. (A,B,C) Beta cell area at 48, 72, and 96hpf, respectively. n=10 fish; Different letters indicate significant differences ( $p < 0.05$ ) from the wildtype DMSO control as determined by a ONE-WAY ANOVA followed by a Fisher's LSD Post-Hoc Test.

#### 4. DISCUSSION

During development redox homeostasis plays an important role in cell fate decision and proper organogenesis. Disrupting redox homeostasis, can lead to phenotypic changes during development such as teratogenesis and beta cell dysregulation. It can also lead to diseases in humans later in life including cancer, neurodegenerative diseases, and rheumatoid arthritis. In this study we investigate one of the main regulators of redox homeostasis, Nrf2, during zebrafish development. Our findings support that Nrf2 activators increase Nrf2 translocation and alter glutathionylation at all three critical windows of development (24, 48, 72hpf). Expression patterns of both Nrf2 and glutathionylation also varied between time-points suggesting different phenotypic outcomes depending on when exposure occurs during development.

When looking at how Nrf2 activators impact Nrf2 expression immediately after exposure, there were no significant changes in Nrf2 in the body. However, there was a variance in Nrf2 expression when looking at different organs individually with tBHQ having significantly more Nrf2 in the brain at 48hpf and DMSO having significantly more Nrf2 in the gut and heart at 72hpf. This supports findings that the redox potential and antioxidant defenses vary from tissue to tissue making certain organs more sensitive to redox imbalance than others (Pi, 2010). Furthermore, the organs that were measured develop at different time-points and thus may have different redox environments at the time of Nrf2 activation; The brain and heart are already functional at 24hpf while at 72hpf the liver has just become functional and the exocrine pancreas is still extending (Ruzicka, 2019).

Differences in Nrf2 expression were also found when looking at the pancreatic beta cells. All three Nrf2 activators increased Nrf2 expression at 24hpf while all had significantly less expression at 72hpf. Worth noting, decreased Nrf2 expression at 72hpf seemed to be localized to

the beta cells with there being a clear decrease in fluorescence in the beta cells in comparison to the exocrine pancreas (Figure 9e). This is also a different expression pattern than what was seen in the full body and supports the fact that beta cells, in particular, are known to be more susceptible to oxidative stress and redox modulation than other cell types due to inherently lower antioxidant defenses (Pi, 2010). Also interesting was the fact that total Nrf2 fluorescence levels in the body was significantly lower at 72hpf while the beta cells had significantly less Nrf2 at 48hpf. The beta cells at 48hpf having less Nrf2 overall parallels previous research that found the beta cells to be most susceptible to redox modulation at the 48hpf time-point resulting in aberrant pancreas morphology (Rastogi, 2021).

For aim 1 I hypothesized that exposure to Nrf2 activators increases Nrf2 expression based on the fact that SFN, tBHQ, and DMF have all been previously shown to interact with Keap1 leading to an increase in Nrf2 stabilization (Robledinos-Anton, 2019; Kubo, 2017). However, most of our findings showed no significant change or even a decrease in Nrf2 stabilization with the 72hpf beta cells. One possible explanation for this is Nrf2 is stabilized at some point after exposure, but not at the time of fixation, or that Nrf2 is initially stabilized followed by *de novo* Nrf2 expression. This is supported by western blots looking at the cytosolic and nuclear fractions of Nrf2 after exposure to SFN or tBHQ for 48hpf. After the exposure, the amount of nuclear fraction of Nrf2 increased, but the total amount of Nrf2 between the cytosol and nucleus seemed to decrease or stay the same (Chen 2018, Wei, 2019). These findings are also supported by preliminary time-lapse translocation experiments done in the Timme-Laragy lab where we found that Nrf2-GFP human kidney cells exposed to tBHQ translocated into the nucleus over-time, but the total expression of Nrf2-GFP decreased throughout the 15 hour time-lapse. Previous studies have found that 6 hours is a sufficient amount of time for tBHQ to



activate Nrf2 and upregulate antioxidant defense genes (Li, 2005), but not much is currently known about how much or for how long Nrf2 is stabilized in the cytosol during this time period. It is also not fully known to what extent *de novo* Nrf2 is involved in the initial Nrf2 activation.

Another explanation is that dose times or amounts may not be enough to alter redox homeostasis; There may be some level of Nrf2 stabilization and/or activation, but not enough to have significant effects. To test whether Nrf2 is translocating into the nucleus when exposed to SFN, DMF, and tBHQ, colocalization of Nrf2 and DAPI in the endocrine pancreas was measured. At 24 and 72hpf all three Nrf2 activators increased Nrf2 translocation to the nucleus, tBHQ significantly, and at 48hpf SFN and DMF significantly increased translocation. The one exception to the observed increase in Nrf2 nuclear translocation in the beta cells was with the tBHQ exposed embryos at 48hpf where there was a significant decrease in colocalization. 48hpf was also the timepoint where SFN and DMF had the most significant increase in Nrf2 translocation and the amount of colocalization overall was higher at 48hpf than the other timepoints. The overall increase in Nrf2 translocation seen at this time-point may be due to the increased susceptibility of beta cells to oxidative stress. Previous research has found 48hpf to be a critical window for beta cell development; redox modulation had the most significant effect on beta cell area and morphology at this time-point. tBHQ, a pro-oxidant, was found to significantly decrease beta cell area which would suggest decreased proliferation and a more oxidized environment. On the other hand, SFN, an antioxidant promoting compound, led to a significant increase in beta cell area which would suggest increased proliferation in a more reduced state (Rastogi, 2021; Hansen, 2015). These findings line up with our colocalization results where tBHQ seems to have decreased Nrf2 translocation at 48hpf while SFN and DMF had an increase.

While with the Nrf2 experiments the embryos were examined immediately after exposure, for the BioGEE experiments the fish were examined 24-hours later to link changes in Nrf2 expression to eventual altered glutathionylation. When looking at BioGEE levels in the body, Nrf2 activators had increased BioGEE levels at the 48 and 72-hour time-point followed by a significant decrease in BioGEE levels at 96hpf. This supports the aim 2 hypothesis that the time-point in which glutathionylation is measured will greatly impact the effect of Nrf2 activators. This same expression pattern was also seen when looking at different organs individually as well as the pancreatic beta cells. This differed from the Nrf2 expression patterns which tended to be more organ specific. One notable exception to these findings was DMF exposed embryos at 96hpf where there was a significant increase in BioGEE levels in the beta cells as well as an increase in the heart in comparison to the body. These findings line up with the hypothesis that DMF will have more varied glutathionylation levels because it can interact with GSH independently from Nrf2. As stated previously, DMF rapidly interacts with GSH and initially lowers the cellular GSH levels. However, within 24 hours GSH levels increase 2-fold and GSH recycling is upregulated (Brennan, 2015; Hoffman, 2017). Since the fish used for BioGEE were fixed 24 hours after initial exposure, DMF is expected to increase cellular GSH levels by this time-point. This provides free GSH which could potentially explain the increase in glutathionylation whether it be to modify proteins or store GSH.

When looking at differences in BioGEE between the three time-points, there was a time dependent increase in glutathionylation in both the body and beta cells. The 96hpf embryos, in particular, had a significant increase with it having 2-3 folds higher expression than the 48hpf embryos. Interestingly, SFN and tBHQ seem to significantly reduce this affect while DMF had BioGEE levels similar to those of the control again. The pattern of BioGEE expression over time

is supported by previous HPLC measurement performed by the Timme-Laragy lab measuring glutathionylated proteins in zebrafish embryos over development. These finding does not line up with the notion that glutathionylation typically increases when oxidative stress is present as the 96hpf larvae have a more reduced environment (Timme-Laragy, 2013). Instead, an alternative explanation is an increase in GSH storage at 96hpf since there is also an increase in the total amount of cellular GSH at this time-point.

Interestingly, SFN was the only compound to significantly increase glutathionylation in the beta cells at 72hpf (exposed at 48hpf) and that correlated with a significant increase in beta cell area. This increase lines up with previous findings where SFN was also found to significantly increase beta cell area at 96hpf following an exposure at 48hpf. The increase in area also seems to be Nrf2 dependent as the same change was not seen in the mutant zebrafish. This is again supported by previous findings where changes in pancreas area and morphology were not seen in the Nrf2 mutant fish.

When comparing the Nrf2 and BioGEE results, BioGEE levels seemed to mostly change independently from changes in Nrf2 expression. This does not support the first part of the aim 2 hypothesis where redox state was inferred to be the main regulator of glutathionylation. If that were the case, then increases in glutathionylation would correlate to decreases in Nrf2 expression. These findings instead suggests that oxidative stress alone does not regulate glutathionylation. Instead, it seems that changing GSH levels and enzyme production during development play a greater role in glutathionylation than previously thought. As stated previously, one alternative explanation for the BioGEE expression patterns is for storage of GSH, possibly facilitated by the enzyme Gstp. To see if our findings of increased glutathionylation could be due to increased *gstp* expression, a preliminary RT-qPCR experiment

was performed. From our preliminary qPCR data, we found that all groups increased *gstp* expression aside from the 48hpf DMF group. This supports that changes in BioGEE expression levels may be impacted by *gstp* expression.

When looking at differences between the wildtype Nrf2 fish and the Nrf2 mutant fish with a single point mutation rendering Nrf2 unable to properly interact with the ARE, Nrf2 expression was mostly equal to the wildtype fish apart from a few significant decreases. This mostly supports the hypothesis that mutating the binding domain should not impact Nrf2 protein levels, but also suggests some low levels of Nrf2 self-regulation. When looking at BioGEE expression, there were both decreases and increases in BioGEE levels in the mutant fish in the body and beta cells at all time-points. There was a lot more variability between the mutant and wildtype fish when looking at BioGEE levels in comparison to Nrf2 levels as predicted in the hypothesis. This suggests that although it does not seem that oxidative stress is the sole regulator of glutathionylation, Nrf2 does still play an important role in its regulation. A fully functioning Nrf2 is needed to properly upregulate enzymes involved in increasing cellular GSH levels and those involved in the storage of GSH such as *Gstp*. More studies need to be performed to further elucidate the relationship between Nrf2 and glutathionylation during development possibly using a full Nrf2 knock-out model to see if we get the predicted increase in glutathionylation.

Previous research in our lab found that exposure to Nrf2 activators SFN and tBHQ at 48hpf did not change Nrf2 expression or glutathionylation at 96hpf, but exposure to these same compounds led to changes in endocrine pancreas area and morphology at the same timepoint. Our findings help better understand what is going on between Nrf2 and GSH during this window of development to cause those phenotypic changes. At 48hpf there was significant decrease in Nrf2 expression in the beta cells along with a significant increase in Nrf2 translocation into the

nucleus. Furthermore, SFN significantly increased glutathionylation at 48hpf and, as with the previous studies, increased beta cell area. Our findings also suggests that alterations to the redox homeostasis at certain critical windows can lead to phenotypic changes even when physiological steady state is reached again shortly after. Whether the shift in redox modulation is leads to a permanent disease phenotypic or is still able to be rescued later in development is not known and is potentially tissue specific (Kotas, 2015).

In conclusion, exposure to SFN, tBHQ, and DMF did not tend to significantly alter Nrf2 expression in the body, but did lead to organ specific changes depending on the timepoint suggesting tissue-specific sensitivity to Nrf2 activation. Nrf2 activators also increased Nrf2 translocation into the nucleus of beta cells and led to an increase in glutathionylation at the 48 and 72hpf time-point followed by a significant decrease at 96hpf. Interestingly BioGEE expression patterns did not tend to line up with Nrf2 expression. The increase in glutathionylation can instead be partially explained by the increase in *gstp* expression seen in all three Nrf2 activators suggesting an increase in GSH storage. This is further supported by the fact that the total amount of glutathionylation was greatest at 96hpf when the amount of cellular GSH is also high. BioGEE expression did, however, significantly change in the Nrf2 mutant fish suggesting that Nrf2 does play a role in regulating glutathionylation. Although the relationship between Nrf2 expression and glutathionylation during development needs to be explored further, our data suggests that the effects of Nrf2 activation on Nrf2 expression and glutathionylation depend on the timing of exposure to the perturbing chemicals and the tissue type. Once, this relationship is fully understood in the zebrafish vertebrate model, the critical windows and tissue sensitivity in human development can be better studied. With the current research continuing to find links between oxidative stress and adverse human health outcomes such as diabetes, cancer,

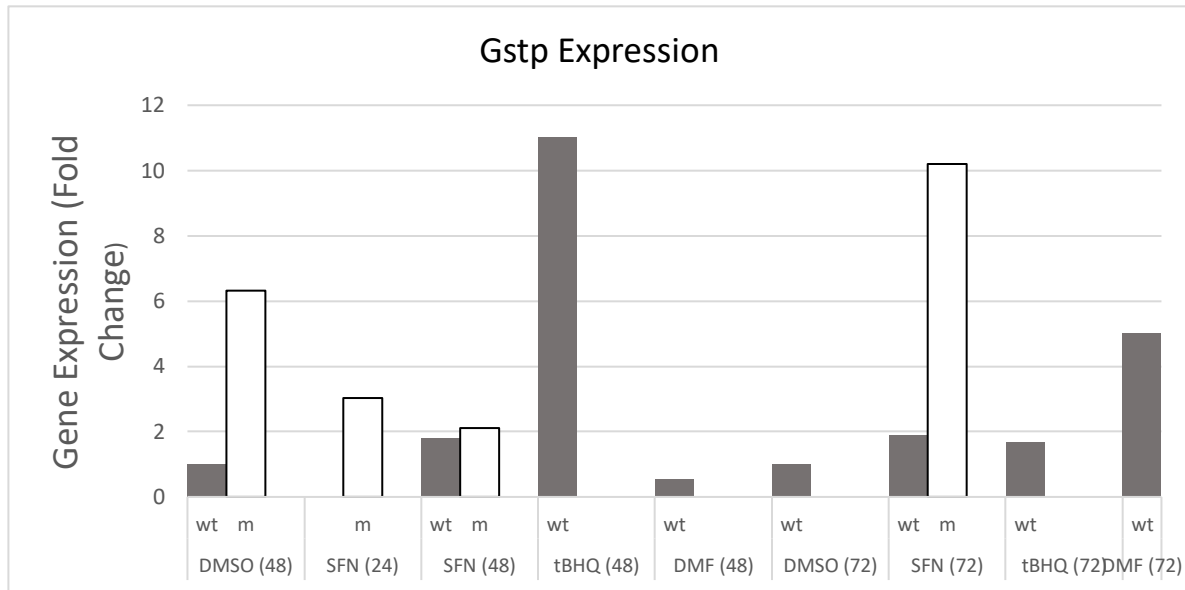
and neurodegenerative disorders as well as the prevalence of environmental toxicants linked to oxidative stress in humans, it is important to better understand the effect of Nrf2 activation during development and how that impacts GSH in the cell. Finding these windows of development where redox homeostasis is most sensitive in humans can allow for possible preventative and/or protective measures to oxidative stress during development.

## 5. FUTURE DIRECTIONS

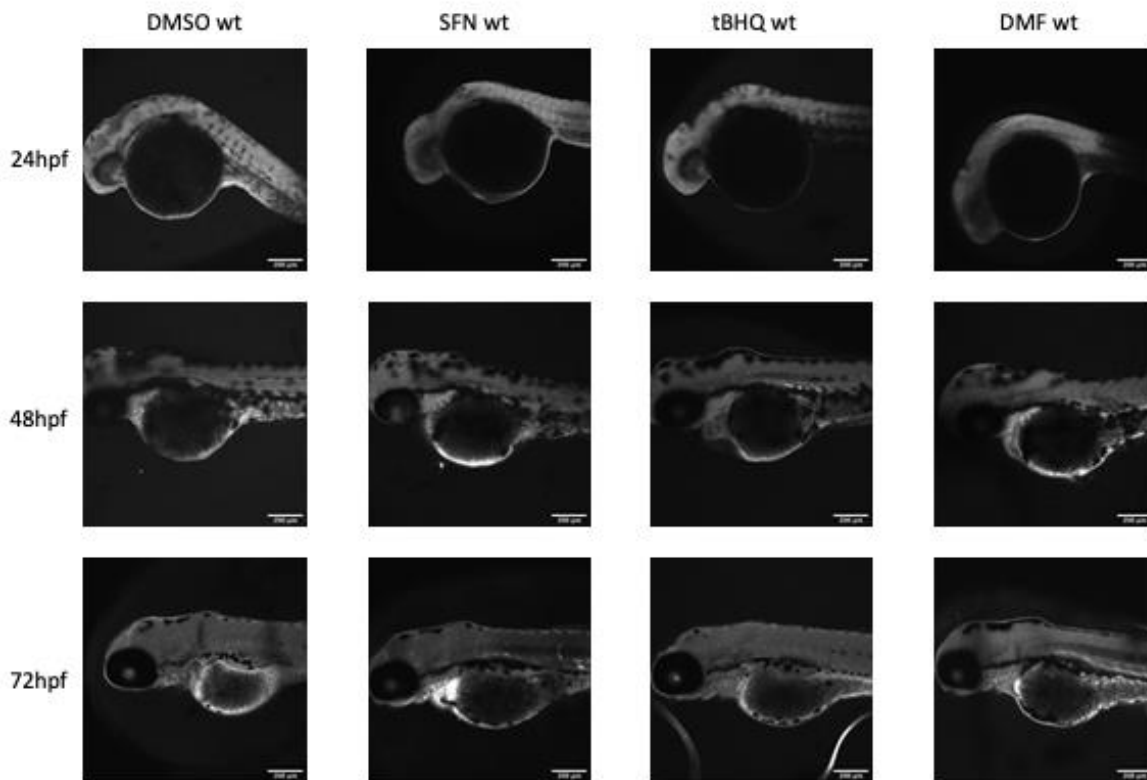
Preliminary results from our lab looking at anti-Nrf2 and BioGEE staining in the liver of 96hpf zebrafish found that the liver had different expression patterns than the previously characterized endocrine pancreas. Due to this fact, there are plans to take liver z-stacks of the fish used in this project to see if a similar pattern is seen. To further study the effect of the Nrf2 activators, different concentrations can also be used. For all three exposure groups, the doses chosen were on the higher end of sub-lethal doses. If multiple lower doses are used, then potential dose-dependent changes can be investigated. Since DMF has been previously found to interact with the Nrf2 pathway differently depending on the concentration, this data would be especially interesting in the DMF exposures. Finally, preliminary experiments have been performed measuring the effect of Nrf2 activators on *gstp* expression using RT-qPCR. Preliminary findings show an increase *gstp* expression for all measured exposure groups (Fig S1). However, it is also important to note that *gstp* expression was greatly increased in the Nrf2 mutant fish. The Nrf2 mutant fish have Nrf2 that is unable to properly act as a transcription factor and although some compensation from other members of the Nrf family is expected, the amount of expression should not be greater. Due to this fact and the missing exposure groups, more trials of this experiment need to be done before any definitive conclusions are made.

Furthermore, the protocol used for RNA extraction needs to be optimized in order to find the correct number of fish needed per sample for the younger time-points. Further studies can also be done to better understand the relationship between Nrf2 protein levels, glutathione redox potential, and gene expression. Some notable genes that would be good to explore include Nrf2a and Nrf2b to further study self/cross-regulation, other glutathione related genes like glutathione reductase, and antioxidant genes known to be upregulated when Nrf2 is activated.

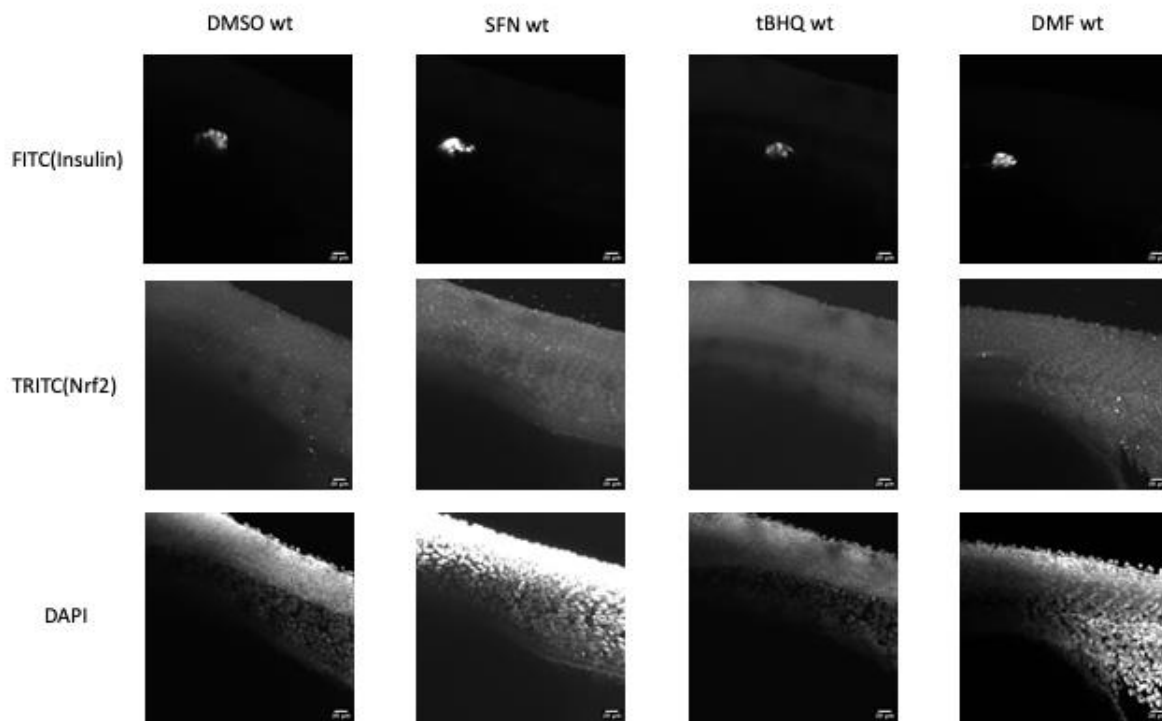
### APPENDIX - SUPPLEMENTARY DATA



**Figure S1.** *Gstp* expression following exposure to Nrf2 activators (40 $\mu$ M SFN, 1 $\mu$ M tBHQ, 7 $\mu$ M DMF). All graphs are represented as a fold change in comparison to the DMSO control of their respective timepoint. n= 1 biological and 2 technical replicates.

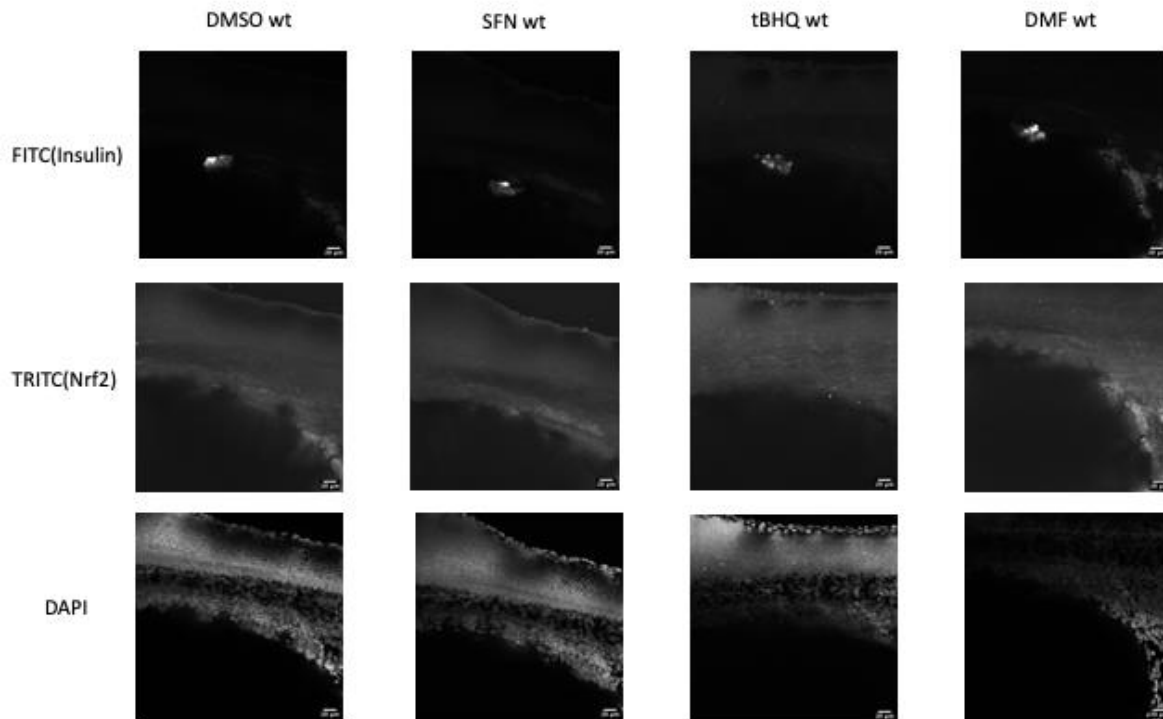


**Figure S2.** Grayscale Images of zebrafish embryos taken at 10x on the TRITC channel (Nrf2) from images in figure 2. Scale bars represent 200  $\mu$ m.

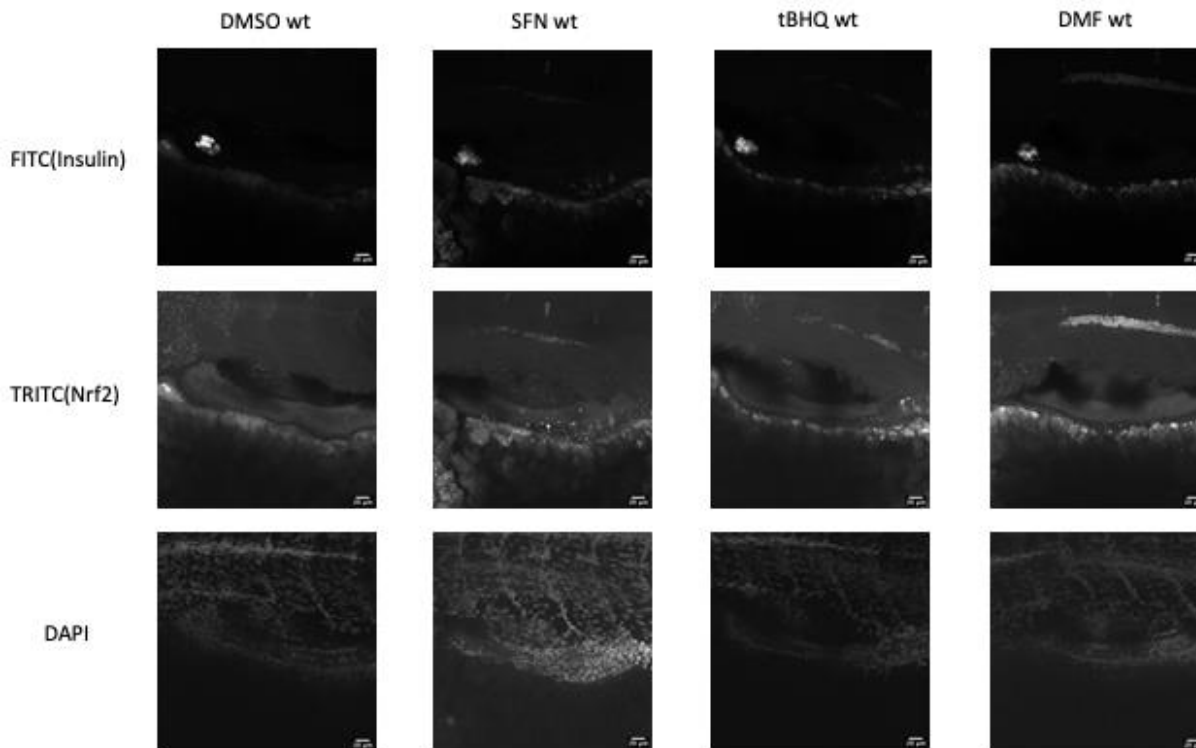


**Figure S3.** Grayscale split channel images of 40x beta cells at 24hpf (figure 5b). All scale bars represent 20  $\mu$ m.

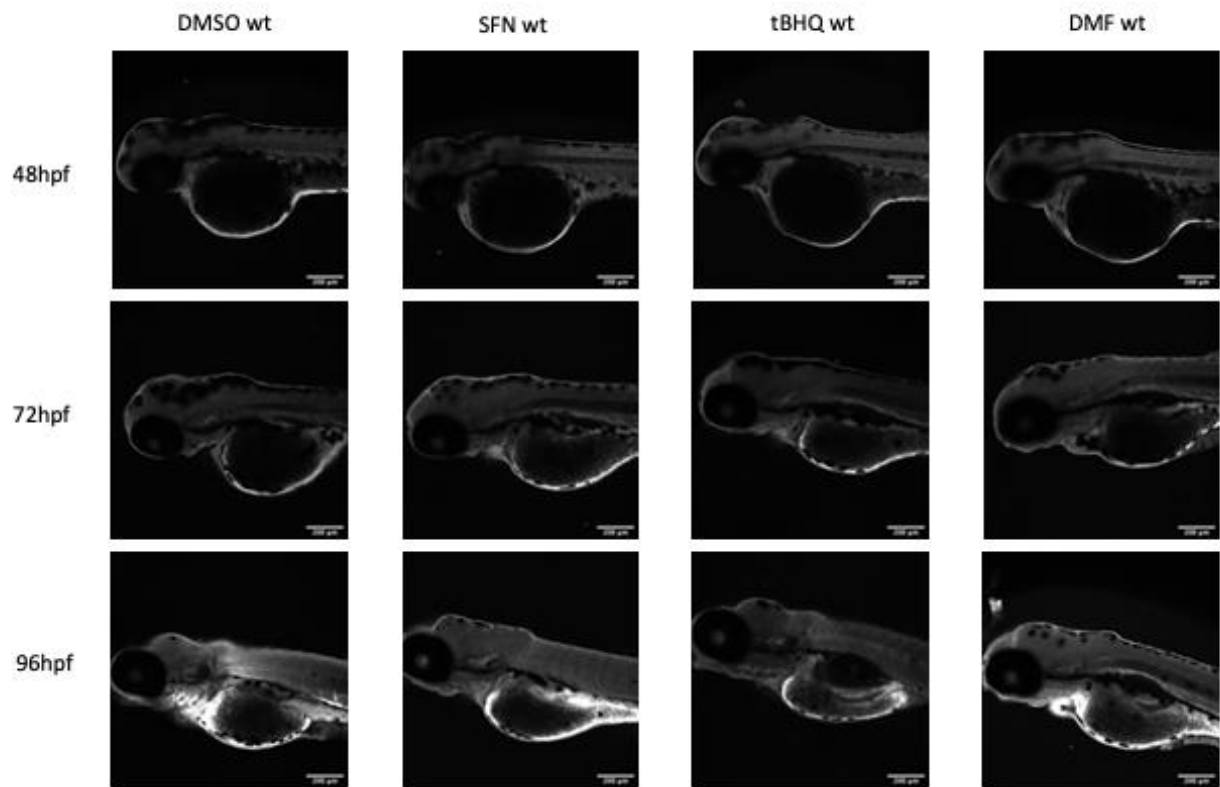




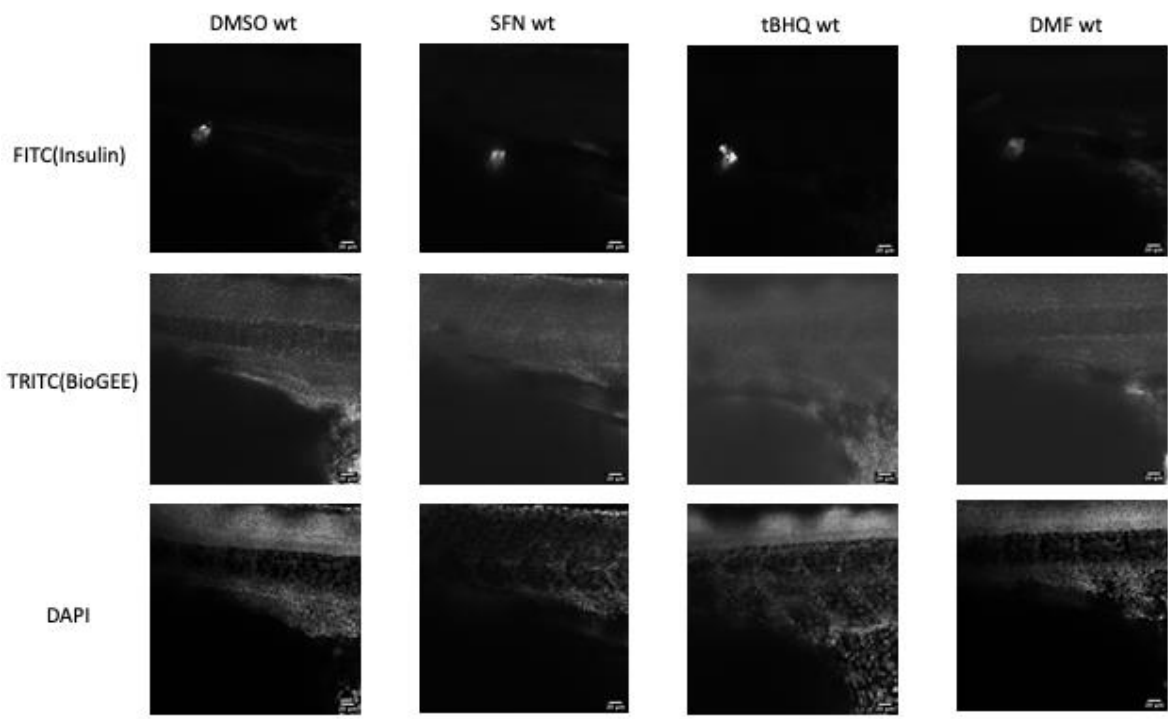
**Figure S4.** Grayscale split channel images of 40x beta cells at 48hpf (figure 5d). All scale bars represent 20  $\mu$ m.



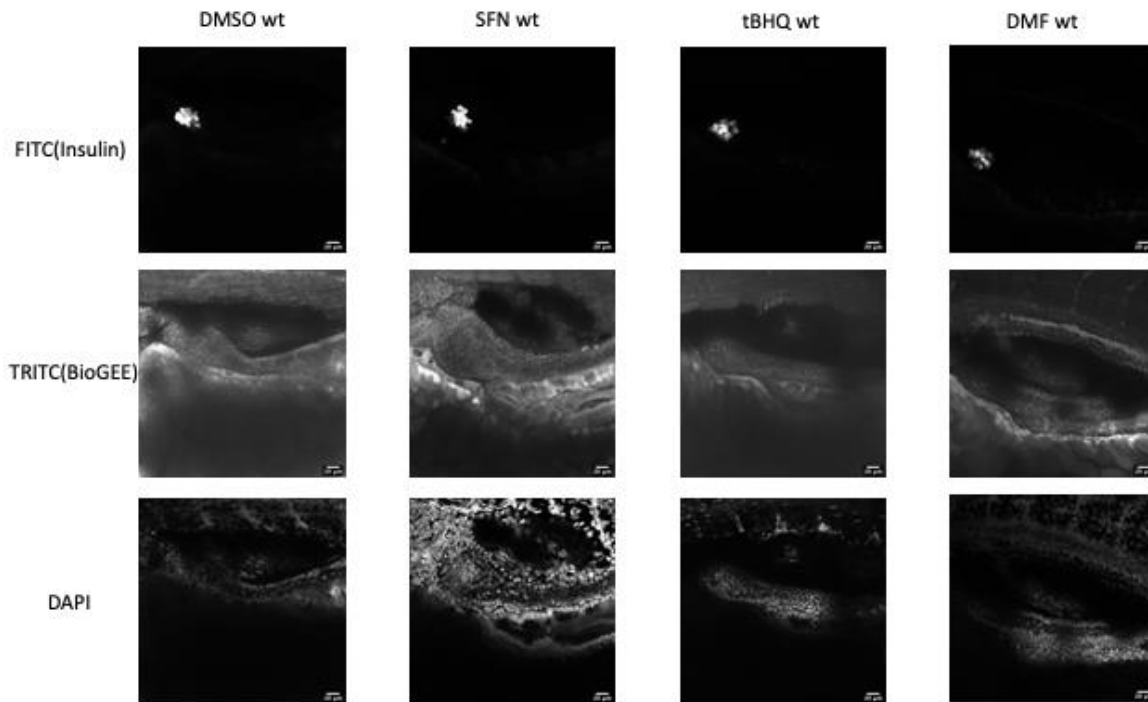
**Figure S5.** Grayscale split channel images of 40x beta cells at 72hpf (figure 5f). All scale bars represent 20  $\mu$ m.



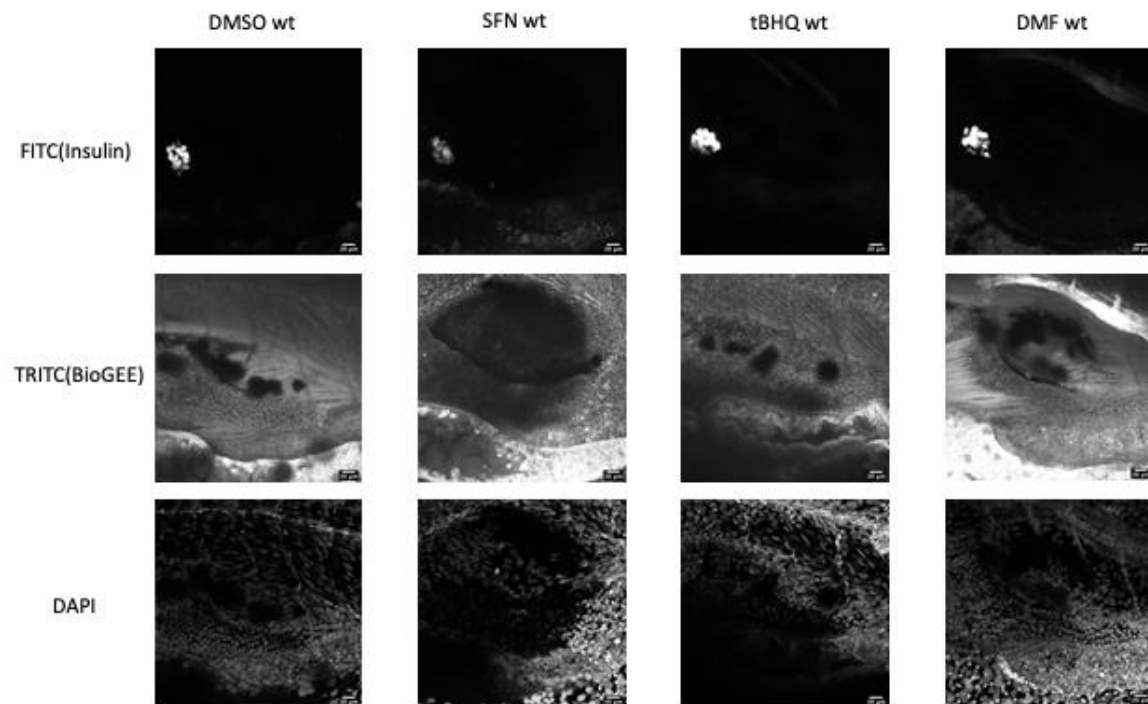
**Figure S6.** Grayscale Images of zebrafish embryos taken at 10x on the TRITC channel (BioGEE) from images in figure 8. Scale bars represent 200  $\mu$ m.



**Figure S7.** Grayscale split channel images of 40x beta cells at 48hpf (figure 11b). All scale bars represent 20  $\mu$ m.



**Figure S8.** Grayscale split channel images of 40x beta cells at 72hpf (figure 11d). All scale bars represent 20  $\mu$ m.



**Figure S9.** Grayscale split channel images of 40x beta cells at 96hpf (figure 11f). All scale bars represent 20  $\mu$ m.

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