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The Role of the Unfolded Protein Response in Fatty Liver Disease

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Dedication

This thesis is dedicated to my loving family and community of friends who have supported me endlessly. To my parents, thank you for being so unconditionally supportive, humorous, and kind. Baba, your educational journey and gentle nerdiness has always inspired me to follow what tickles my brain; Ma, I'm deeply grateful for your uncanny ability to intuit my thoughts and for all of your support – you both give me so much strength. Thank you both for always believing in me. To my older sister, Antara, thank you for shaping me into the person I am today. I look up to you so much. You are a reminder to me that it's always worth it to stand for what you believe in. I wouldn't be where I am without the warmth and inspiration from my family. To Vivian Zhao, thank you for being my person, I am eternally grateful you're my best friend. You've been through it all with me, and I can't imagine life without you and your little jokes. To Kara Wong, thank you for always supporting me, even from over a thousand miles aways – you are so brilliant and kind, I'm very proud of our elementary school selves for finding each other. I appreciate your patient attempts in trying to teach me statistics, and for always listening to me talk on and on about science and research! To Jenny Zheng, I am deeply grateful for our friendship from undergrad. You are so loving, attentive, and artistic – you always know how to ground me (and bake the best delicacies). To Courtney Yu, thank you for being a constant in my life, and for visiting me in Stockton during both undergrad and grad school. You've been there for so many milestones, so many laughs, I appreciate you so much. To Jennifer Morrow, all of our strolls on our lush campus and spontaneous encounters gave me so much joy and strength. Our consistent afternoon lunches are etched in my graduate experience; thank you for being community. To Bailey Whitlock, thank you for always being patient

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The Role of the Unfolded Protein Response in Fatty Liver Disease

Abstract

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The unfolded protein response (UPR) is composed of three highly conserved pathways (ATF6, IRE1, PERK). Cellular stressors induce protein misfolding and aggregation in the endoplasmic reticulum (ER). This signaling pathway maintains protein homeostasis when there is stress in the ER. When the UPR is activated, the eukaryotic initiation factor 2 alpha (eIF2α) becomes phosphorylated, which inhibits global mRNA translation. If ER stress remains chronically unmitigated, the UPR induces apoptosis. GADD34 and CReP shift in expression when the UPR is activated and work as phosphatases and dephosphorylate eIF2α in a feedback loop, allowing protein synthesis to resume. Several human diseases, including fatty liver disease (FLD) are affected by cell stress from improper protein folding and accumulation, making the UPR a therapeutic target. Previous studies have indicated the UPR to both cause or become activated by FLD, depending on the duration of cellular stress. At least 25% of humans worldwide have steatosis, and zebrafish are a powerful model organism for FLD studies. Their embryos are easily obtained, and the liver develops quickly in their transparent larvae, which allows us to visualize the development of fat in the liver. It is unknown how exactly the UPR is involved in inducing lipogenesis in hepatocytes. We sought to better understand the link between UPR activation and steatosis. Pharmacological treatments with various drugs, some of which induce ER stress, were administered over different durations in zebrafish embryos and

subsequently the expression of UPR network and lipogenesis genes were quantified through RT-qPCR. To visualize whether these drugs induced steatosis, zebrafish livers were stained with Oil Red O and imaged. Our results indicate that all chronic durations of pharmacological treatments resulted in fatty liver, and the expression of *atf6* decreased in response to treatment that prevents the dephosphorylation of eIF2α. This data provides insight pertaining to the activity of the UPR network during FLD in zebrafish models.

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CHAPTER 1: INTRODUCTION

The Endoplasmic Reticulum

Through the secretory pathway, integral membrane and secreted proteins travel through the endoplasmic reticulum (ER) and Golgi-apparatus and are exported to their programmed locations (Schwarz and Blower, 2016; Han and Kaufman, 2016). The ER organelle functions in eukaryotic cells to facilitate protein folding, synthesis, and modifications, which allows newly formed proteins to properly transport and function (Farhan and Rabouille, 2011). There are several families of molecular chaperone proteins, such as Hsp90, Hsp70, Hsp60, Hsp40 and small heat shock proteins (sHsps) that work to ensure proper protein folding by recognizing unfolded or misfolded proteins in the ER (Kosmaoglou et al., 2008). The oxidizing nature of the ER allows for a high Ca^{2+} concentration in this organelle; several ER chaperones properly and optimally function through low-affinity binding to calcium (Carreras-Sureda et al., 2018). Calreticulin is also one of the several ER proteins to modulate the calcium gradient in the ER, maintaining ER homeostasis.

Maintenance of ER homeostasis requires preventing the aggregation and accumulation of misfolded or unfolded proteins in the cell. Improperly folded proteins do not translocate from the ER to the Golgi, either remaining in the ER until conformationally corrected, or traveling to the cytosol via the endoplasmic reticulum-associated degradation (ERAD) process (Ozcan and Tabas, 2012). ER degradation enhancing alpha-mannosidase like protein 1 (EDEM1) and Derlin-1 proteins are two ERAD components working to handle the presence of misfolded proteins (Howarth et al., 2014). The presence of excessive misfolded proteins induces ER stress and

activates the unfolded protein response (UPR) signaling pathway to restore protein homeostasis (proteostasis) (Cao and Kaufman, 2012).

There are three branches of the UPR pathway that are present in all mammalian cells, but there exists cell-type-specific UPR signaling as different cell types have specific ER capacities and demands (van Ziel and Scheper, 2020). Subsequently, various human diseases are impacted uniquely by the UPR. Defects in protein-folding in the ER are implicated in autoimmune disorders, such as type-1 diabetes, inflammatory bowel disease, and rheumatoid arthritis, and cancers and neurodegenerative diseases (Cao and Kaufman, 2012). Hyperlipidemia can cause ER stress and induce metabolic diseases, such as diabetes and non-alcoholic fatty liver disease. The role of the UPR during metabolic diseases is the focus of this study.

The Unfolded Protein Response

The UPR is composed of three highly conserved signaling pathways (Activation Transcription Factor 6 (ATF6), Inositol-Requiring Enzyme 1a (IRE1a), Protein kinase R-like ER kinase (PERK)) among metazoans. Cellular stressors, ranging from oxidative stress, calcium dysregulation, glucose deprivation, viral infection, excess fat or cholesterol, alcohol abuse, mutations in specific proteins, and environmental toxins, induce protein misfolding and aggregation in the ER (Han and Kaufman, 2016). Heat shock protein 90 beta family member 1 (HSP90B1) is a chaperone in the ER involved in modulating the unfolded protein response (Liu and Li, 2008). Under normal cellular conditions, the ER chaperone binding immunoglobulin protein (BiP) is bound to three ER transmembrane UPR activator proteins: ATF6, IRE1a, and PERK, keeping them inactivated (Cao and Kaufman, 2012). BiP will bind to misfolded proteins in the ER during stress, allowing the activation of the UPR pathways. The duration of ER stress influences whether the UPR restores proteostasis or induces cellular apoptosis – the three UPR

branches attempt to alleviate ER stress under acute stress by decreasing the protein folding load in the ER and drive the activation of several downstream genes involved in apoptotic pathways under chronic stress (Walter and Ron, 2011).

ATF6 Branch

There are two isoforms of ATF6, ATF6α and ATF6β, the former being essential in activity against ER stress (Cao and Kaufman, 2012). When BiP is unbound from the ATF6 protein, ATF6 travels to the Golgi complex where it is cleaved and proteolytically processed by site-1 proteases (S1P) and site-2 proteases (S2P) (Wang and Kaufman, 2016). The cleaved ATF6 protein, now known as nAtf6 or p50Atf6, functions as a transcription factor in the nucleus and drives the genetic expression of numerous ERAD components and UPR targets, such as the XBP1 and BiP genes, to alleviate acute ER stress (Cao and Kaufman, 2012). ATF6α additionally functions to inhibit gluconeogenesis in hepatocytes by interrupting CREB-CRTC2 binding. The involvement of ATF6 and other UPR branches in the liver will be elaborated on in later sections of this thesis.

IRE1a Branch

This is the most conserved branch of the UPR, with IRE1a activity observed among eukaryotes from yeast to mammals (Zhang et al., 2016). The IRE1a pathway is activated upon the departure of BiP, which allows IRE1a proteins to trans-auto phosphorylate and form into a tetramer configuration. The X-box-binding protein 1 mRNA (*Xbp1*) is spliced in the cytosol by phosphorylated IRE1a proteins, resulting in an active transcription factor (*Xbp1s*) that also works to increase the expression of genes that encode ER chaperone proteins, ERAD proteins, and proteins involved in cellular inflammation (Cao and Kaufman, 2012). ATF6 and XBP1 have

been shown to heterodimerize during ER stress to upregulate the expression of the aforementioned genes.

PERK Branch

Akin to the ATF6 and IRE1a branches, the PERK pathway is activated when BiP releases PERK proteins upon detecting misfolded proteins. PERK then trans-auto phosphorylates. In the cytosol, activated PERK will phosphorylate the eukaryotic initiation factor 2 alpha (eIF2α) subunit at Ser51, which inhibits global mRNA translation, reducing the number of proteins entering the ER (Cao and Kaufman, 2012). The exception to this are mRNA transcripts with short open reading frames (ORFs) that are capable of translation when eIF2α is phosphorylated (Walter and Ron, 2011). These genes include *Atf4,* which encodes the activation transcription 4 (ATF4) protein. ATF4 drives the expression of *Ppp1r15a,* which encodes growth arrest and DNA damage-inducible protein (GADD34), an eIF2α phosphatase, as well as the expression of genes inducing autophagy, a process that restores cellular metabolism and homeostasis by clearing out aggregated proteins to restore proteostasis (Ryter et al., 2013). ATF4 also increases the expression of *Ddit3,* the gene encoding the CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) (Reid et al., 2016). If ER stress remains chronically unmitigated, the UPR induces apoptosis through upregulating CHOP expression.

CHOP activates various elements involved in cell death in both endogenous and exogenous pathways, activating Bim, a pro-apoptotic protein in the BCL-2 family, and death receptor 5 (DR5) (Hu et al., 2019; Kale et al., 2017). CHOP also induces the expression of *Ppp1r15a* (GADD34). Promoting GADD34 expression increases protein synthesis, which may induce oxidative stress when the ER is already undergoing stress from the presence of misfolded

proteins, which then may induce apoptosis by dysregulating ER calcium homeostasis (Caufman and Kao, 2012).

GADD34 and CReP: eIF2α phosphatases

The UPR is regulated by eIF2α phosphatases, which are composed of protein phosphatase 1 (PP1), the catalytic subunit that binds with either GADD34, or constitutive repressor of eIF2α phosphorylation (CReP), which are the scaffolding subunits (Connor et al., 2001; Rojas et al., 2015). GADD34/CReP increase in expression when the UPR is activated and work as phosphatases that dephosphorylate eIF2α in a feedback loop, allowing global protein synthesis to resume (Cao and Kaufman, 2012; Reid et al., 2016). GADD34 and CReP have structural homology at the C-terminal PP1 binding region (Reid et al., 2016).

GADD34 is known to be activated under conditions of ER stress, increasing in expression upon the activation of the PERK branch, specifically when ATF4 levels increase (Rojas et al., 2015). In a study by Reid et al. (2016), knocking out *GADD34* in stressed mouse embryonic fibroblasts (MEFs) prevented the typical progression and activity of the UPR due to the lack of protein synthesis in the cell. During chronic stress, the cell requires the dephosphorylation of eIF2α to allow for translational recovery, which GADD34 mitigates (Jousse et al., 2003). GADD34 plays a crucial role in regulating protein synthesis, but studies have shown that mice with homozygous GADD34 mutations survive without major phenotypic outcomes, whereas homozygous CReP mutations in mice and humans have demonstrated embryonic lethality, severe microcephaly, and other major defects due to translational dysfunction during development (Reid et al., 2016; Harding et al., 2009; Kernohan et al., 2015).

CReP, encoded by *Ppp1r15b*, is theorized to be constitutively active under normal cellular conditions (Jousse et al., 2003). There are studies that have shown otherwise, indicating

CReP expression to be regulated through UPR activation. IRE1α, which cleaves *Xbp1* as described earlier, has also been shown to cleave mRNA transcripts that have similar secondary structures as XBP1 – this process is referred to as regulated IRE1α-dependent decay (RIDD) (So et al., 2015). The study by So et al. (2015) demonstrated ER stress to lower CReP expression through RIDD. Unlike GADD34, CReP expression is seen to be independent of the levels of eIF2α, but it has been suggested that the translation of CReP may also increase in response to ER stress (Young et al., 2015).

The exact regulation of these two dynamically expressed feedback inhibitors of UPR signaling remain unclear. It is crucial to better understand the regulation of expression and activity of GADD34 and CReP and the expression of the UPR network as a whole, as the dysregulation of protein synthesis is the basis of several human diseases, some of which are exacerbated or alleviated by the cellular decision to either undergo apoptosis or resume protein translation during ER stress.

An Overview of the UPR and Human Diseases

Cancer

Cancer cells are characterized by their capacity for tumor growth and metastatic distribution throughout the body – chronic cellular proliferation, resistance to apoptotic signals, and activating invasion and metastasis are a few of the hallmarks of cancer (Hanahan and Weinberg, 2011). The activity of hyperactive oncogenes or loss-of-function mutations in tumor-suppressor genes may cause excess protein synthesis from increased metabolic requirements in cancer cells, causing ER stress (Wang and Kaufman, 2014). It is difficult to generalize the role of the UPR in cancers. This signaling pathway may either promote the survival of cancer cells through increasing their protein folding capacity, or induce apoptosis (Wang and Kaufman,

2016). A stressful tumor microenvironment requires additional immune and endothelial cells and UPR signaling may increase the production of cytokines, angiogenesis factors, and extracellular matrix (ECM) components (Baghban et al., 2020; Wang and Kaufman, 2014).

UPR signaling is detected in several cancers, including breast, colon, and gastric cancers, and hepatocellular carcinomas (HCC) – several studies have demonstrated adverse clinical outcomes with UPR activation during these diseases (McGrath et al., 2018; Patel et al., 2021; Dauer et al., 2019). The PERK branch has been demonstrated to induce cytoprotective autophagy in cells with high c-Myc oncogenic expression allowing for cell survival in melanomas (Bu and Diehl, 2016). PERK inhibitors are still being studied as potential therapeutic agents but their side effects are not yet fully understood clinically. Further research must be conducted to understand when PERK and other UPR branches serve to provide pro-apoptotic signaling rather than pro-survival activity in cancers.

Neurodegenerative Diseases

A hallmark of neurodegenerative diseases is the accumulation of misfolded and aggregated proteins in neurons and glial cells. Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and prion disease all share this characteristic, with evidence of UPR activation during disease (van Ziel and Scheper, 2020; Scheper and Hoozemans, 2015). Robust UPR activity causes apoptosis, and neuronal cell death is the basis of major symptoms in these diseases. Compared to non-neuronal cells, neurons are more sensitive to misfolded proteins and cannot regenerate from cell death; excess apoptosis is the driver of motor and cognitive impairments (Remondelli and Renna, 2017). Gene mutations causing the accumulation of mutant proteins in the ER may cause apoptosis in this way in neurons. It is known that mutated BiP causes neurodegeneration, but further research must be conducted to understand how other

specific mutations in the UPR signaling network induce neurodegenerative diseases (Wang and Kaufman, 2016). Targeting the UPR with pharmacological agents and gene therapy are approaches to try to ameliorate the devastating effects from these neurological disorders (Hughes and Mallucci, 2019).

The UPR and Metabolic Diseases

The ER and Lipid Synthesis

In addition to regulating protein synthesis, the ER produces phospholipids and sterol fats (Basseri and Austin, 2012). The smooth ER is the primary site for cholesterol and lipid synthesis, which are both delivered from the ER to the rest of the cell (Jacquemyn et al., 2017). The ER membrane itself has a low concentration of cholesterols and sphingolipids, which is optimal for newly synthesized lipids and proteins to transport through and exit out of the ER (Meer et al., 2008). Several regulatory proteins influencing lipid metabolism reside in the ER (Han and Kaufman, 2016).

Sterol regulatory element binding proteins (SREBPs) are a group of transcription factors in the ER that regulate the expression of enzymes required for the synthesis of fatty acids (FA), triacylglycerol (TAG), and phospholipids (Eberlé et al., 2004). SREBPs have distinct roles during hepatic lipogenesis– there are three isoforms, with one gene encoding for both SREBP-1a and SREBP-1c, and another encoding for SREBP-2 (Amemiya-Kudo et al., 2002). A study by Shimano et al. (1996) overexpressing SREBP-1 in transgenic mice showed SREBP-1 to function by selectively activating fatty acid biosynthetic genes (Shimano et al., 1996). Transgenic mice expressing dominant-positive SREBP-2 in adipose and liver tissues demonstrated SREBP-2 to function as a regulatory activator of cholesterol synthesis (Horton et al., 1998).

Lipogenesis, Lipotoxicity, and the UPR

FA synthesis occurs in adipose and liver tissues (Pearce, 1983). Fatty acid synthase (Fasn) is an enzyme that moderates steps in the biochemical conversion of acetyl-CoA to palmitate during lipogenesis, the process of generating FAs from acetyl-CoA, which are then esterified to form TAGs (Howarth et al., 2014). Adipocytes store TAGs, releasing FAs by lipolysis when there is a low level of energy substrates available in the body (Li et al., 2009). The presence of excess FAs are mitigated by adipocytes to a certain level – when there is an overload of lipids in the body, FAs are esterified to TAGs, which accumulate in heart, liver, muscle, and pancreatic-β cells, causing lipotoxicity (Han and Kaufman, 2016).

Lipotoxicity causes ER stress (Gentile et al., 2011). The UPR can enhance lipogenesis and decrease fatty-acid oxidation through the IRE1a and PERK branches (Zheng et al., 2010). One cause of type-2 diabetes is when pancreatic-β cells lose their function from lipotoxicity and insulin resistance (Song et al., 2008). Apoptosis in pancreatic-β cells is a pathological feature of diabetes when there is an excessive need for insulin; a study by Song et al. (2008) showed CHOP deletions in mice to have improved β-cell mass and glycemic control (Tomita, 2016). Obese mice lacking IRE1a display increased insulin sensitivity compared to wild-type obese mice (Özcan et al., 2004). Loss-of-function mutations in *Xbp1* have shown to increase insulin sensitivity as well (Ozcan et al., 2004).

Non-alcoholic Fatty Liver Disease (NAFLD)

Non-alcoholic fatty liver disease (NAFLD), also known as hepatic steatosis, is characterized by excessive amounts of fat accumulation in the liver. When the mass of the liver is composed of at least five percent fat, one formally has NAFLD (El-Kader and El-Den Ashmawy, 2015). Up to 30% of the global population may be afflicted with NAFLD due to the

rise in obesity from social, political, and environmental factors (Schlegel 2012; Lee et al., 2019). This disease is associated with metabolic syndrome as well as mutations in components of the insulin signaling pathway promoting steatosis by increasing lipogenesis in the liver (El-Zayadi, 2008). What begins as NAFLD may progress to nonalcoholic steatohepatitis (NASH) due to additional fat and inflammation in the liver from increasingly severe metabolic syndrome, and from lipid peroxidation and collagen fiber deposition – these all may cause hepatocyte necrosis (Browning and Jorton, 2004). Up to 20% of NASH cases may progress to cirrhosis, which is the irreversible thickening of scar tissue in the liver (fibrosis) (Sheka et al., 2020). Cirrhosis may lead to hepatocellular carcinoma. NAFLD and NASH may be reversible through lifestyle modifications, but further progression of disease is not. There are no FDA approved therapeutics for NAFLD at this time. Animal models have so far not been able to demonstrate this disease progression between NAFLD and NASH.

It is known that the UPR is activated during FLD, but it remains unclear exactly how the three branches of the UPR can impact steatosis during acute and chronic cell stress. In a zebrafish genetic and biochemical study by Howarth et. al (2014), the overexpression of active, nuclear ATF6 (nATF6) induced FLD-like symptoms in zebrafish larvae and human hepatoma cells without causing ER stress. That study also demonstrated Atf6 driving the expression of Fasn epistatically, thereby stimulating lipogenesis, and that SREBP does not synergize with Atf6.

Research Overview

Zebrafish as Model Organisms

Danio rerio (zebrafish) larvae are the model system in this study. The zebrafish genome has been sequenced and it is established that 70% of human genes are orthologous with zebrafish

(Howe et al., 2013). These vertebrates can be used for genetic experiments to better study and understand human diseases – it is possible to conduct both loss and gain-of-function experiments using zebrafish embryos. Zebrafish are the preferred model organism for this research due to the ease in performing genetic expression and phenotypic research studies in them with minimal invasiveness in adult organisms.

Zebrafish embryos are easily obtained in great quantities, with hundreds of embryos being collected during spawning. The optical clarity provided during embryonic and larval development allows for experiments requiring phenotypic analysis and tissue imaging. They are a powerful model organism for FLD studies, as the liver develops quickly within three days post fertilization (dpf) in their transparent larvae. Studies have shown induction of all three UPR branches during conditions that evoke hepatocyte ER stress in zebrafish liver (Schlegel 2012). These animals undergo lipid packaging and transport with molecular mechanisms that are conserved among metazoans, furthering their capacity for pre-clinical NAFLD studies.

This research study will require raising zebrafish larvae to 4 dpf and 7 dpf in age before any drug treatments are administered. Preliminary studies have indicated steatosis to be worsened in zebrafish after treatment with the pharmacological agents salubrinal, tunicamycin, and thapsigargin. Salubrinal blocks the dephosphorylation of eIF2α, functioning as a pharmacological inhibitor of GADD34 and CReP (Boyce et al., 2005). Tunicamycin is a known activator of the UPR that functions by inhibiting N-glycosylation of proteins, resulting in the accumulation of misfolded proteins in the ER (Bassik and Kampmann, 2011). Another known ER stress activator, thapsigargin, functions by blocking the SERCA pump, depleting the ER of calcium (Lytton et al., 1991). This dysregulation of ER calcium levels induces UPR activity.

Genetic Expression Analysis

In this study, mRNA levels of genes involved in the unfolded protein response network (*ppp1r15a, ppp1r15b, ddit3, hspa5, atf6, ire1a, xbp1, perk, atf4)*, ER regulators and chaperones (*hsp90b1, calret, derl1, edem1*), and lipid synthesis components (*pck1, pklr, fasn2, srebp1, srebp2*) were analyzed with RT-qPCR to better understand the regulation of these genes during pharmacologically induced acute and chronic ER stress in 4 dpf wild-type zebrafish larvae. We hypothesize that the induction of ER stress will cause upregulation of the expression of genes encoding for ER components and most UPR and lipogenesis genes. Studying changes in downstream gene expression in response to inhibiting GADD34 and CReP will provide insights into their function.

Phenotypic Analysis

To visualize lipid accumulation in the liver in response to ER stress and UPR activation and manipulation, zebrafish larvae were raised to seven dpf prior to stress treatments. They were then treated with pharmacological activators of UPR and stained for lipids. The livers were then qualitatively scored for steatosis based upon intensity of the red pigment in the liver region. It is expected that all drugs but DMSO will cause steatosis in the liver, but it remains unclear how the inhibition of GADD34/CReP will affect the development and progression of steatosis in zebrafish embryos.

Together, data from these genetic expression and phenotypic studies will help determine and better shape our understanding of how components of the UPR work to induce lipogenesis during ER stress, and how modulating activity of the regulatory proteins of the UPR, GADD34 and CReP, may affect the rest of this network and development of steatosis. This knowledge may

assist in discovering therapeutics during fatty liver disease, a condition that is potentially reversible but currently incurable through medicinal treatments.

CHAPTER 2: MATERIALS AND METHODS

Zebrafish Maintenance

Zebrafish lines were fed twice a day, once in the early morning and again in the late afternoon, with tropical flakes and brine shrimp. The 9.5L fish tanks were filled with fish water (Table 1) from a circulating system kept at 28°C. This water was replaced weekly to prevent the buildup of waste products. Male and female fish were kept together in tanks and were exposed to a 14 hours light/10 hours dark cycle to encourage breeding.

Table 1.

Fish Water Recipe.

Reagent	Amount
$CaCl_2$	1.5g
Instant Ocean (Spectrum Brands)	4.5g
$NaHCO_3$	5g
DI Water	20 gal

Zebrafish Embryo Collection and Development

Zebrafish breeding tanks were set up with a barrier in the center and filled with fish water during the late afternoon prior to embryo collection. Fish were fed in their regular tanks then sorted by sex and placed into the breeding tanks with 2-5 females and males on each respective side of the divider. The next morning, fresh fish water was given to each breeding tank and then the divider was removed. Fish were left to mate for 20-45 minutes before being placed back into

their main tanks and fed. Embryos were collected from the breeding tanks and first cleaned with 1X E3 media (Table 2) in a mesh sieve and then placed into petri dishes filled with 1X E3 media. Embryos were sorted to ensure no dead embryos or debris was present and then were stored in an incubator at 28° C for either 4 or 7 days. Dead embryos and larvae were removed daily.

Table 2.

50x E3 Media Recipe.

Reagent	Amount
KCl	0.66g
$CaCl_2$	2.4g
$MgCl_2$	4.08g
NaCl	14.5g
Milli-Q Water	1L

Note. The 50x E3 media stock concentration was diluted to a working concentration of 1x E3 media.

Drug Treatments

Once 4 dpf, 5 larvae were pipetted into each well of a 24-well plate and excess 1x E3 media was removed. A total of 50 larvae were used for each drug treatment. 750µL of each 1X drug solution was pipetted to the appropriate well. Well plates were stored in the incubator at 28°C for the duration of the drug treatment. The duration of drug treatments varied from 2 hr-24 hr. DMSO (Sigma) was used as a vehicle control and ethanol was the positive control. Drugs were diluted (Table 3) with 1x E3 Media and used at the following concentrations: 1µM

thapsigargin (Sigma), 2.5µg/mL tunicamycin (Sigma), and 50 µM salubrinal (Sigma). At the end of the treatment, larvae were placed into microcentrifuge tubes and any excess liquid from the drug treatment was removed. Drug treated larvae were frozen at -80°C until they were used for RNA isolation.

Table 3.

Drug Dilutions.

Drug	Amount
Ethanol (1:62 dilution)	166µL in 10mL 1X E3 Media
DMSO (1:1000 dilution)	10µL in 10mL 1X E3 Media
Salubrinal (1:1000 dilution)	10µL in 10mL 1X E3 Media
Tunicamycin (1:10,000 dilution)	1µL in 10mL 1X E3 Media
Thapsigargin (1:20,000 dilution)	0.5µL in 10mL 1X E3 Media

Larvae used for phenotypic analysis using Oil Red O (Thermo Fischer Scientific) staining were exposed to the aforementioned drug treatments. When embryos reached 24 hpf, they were transferred to petri dishes with 2X 1-phenyl 2-thiourea (PTU) to remove their pigment to better allow for the visualization of the liver. The embryos continued developing in the incubator at 28°C. Once 7 dpf, they were subject to drug treatments and ready for Oil Red O staining.

Total RNA Isolation

The drug treated zebrafish larvae (4 dpf) were thawed and 500 µL of TRIzol (Invitrogen) was added to the microcentrifuge tube. The solution was homogenized with a p200 pipette, then an additional 200 µL of TRIzol was added and homogenized again. This step was duplicated

with another addition of 200µL of TRIzol if larvae did not completely homogenize. The solution was then incubated on ice for 15 minutes. To the tube, 200µL chloroform (Sigma) was added, and then the solution was capped and shaken vigorously by hand for 15 seconds. It was then left at room temperature for 2-3 minutes and spun in the centrifuge at maximum speed (16,000 x g) for 15 minutes. After spinning, only the aqueous layer containing RNA was pipetted to a sterilized, RNase free tube, and 500µL RNase-free isopropanol was pipetted in and mixed thoroughly. The solution was incubated on ice for ten minutes then centrifuged at maximum speed for 30 minutes. With a pipette, the supernatant liquid was discarded, and the remaining pellet was washed with 1000 µL of 75% RNase-free ethanol. The tube was centrifuged at maximum speed for five minutes and the supernatant was then discarded. The remaining pellet was air dried for 5 minutes and resuspended by pipetting and mixing 50 µL of RNase free water to the tube. The RNA was then incubated at 50-60°C for 10 minutes to fully resuspend the pellet in water. RNA concentration and purity was recorded with a NanoDrop UV Spectrophotometer. Only samples with a A260/280 value of 1.8 or higher were used for downstream analyses. The RNA was stored at -80°C until used for RT-qPCR.

RT-qPCR

Purified RNA was thawed and diluted to 100 ng/ul using RNase-free H_2O. A DNase treatment was performed to remove any genomic DNA (gDNA): 1µL DNase I buffer and 1µL DNase I Amp Grade Enzyme (Thermo Fischer Scientific) were pipetted to the diluted RNA and then incubated at room temperature for 15 minutes. To inactivate the enzyme, 1µL EDTA was added and the sample was heated at 65°C for 10 minutes.

Reverse transcription was conducted using 1 ug RNA input and the SuperScript IV kit (Invitrogen, USA) following the manufacturer's protocol. The reaction produced 22µL of cDNA.

To quantify the expression of specific genes, qPCR was performed with this cDNA. The following reagents were pipetted and mixed on a 96-well qPCR plate: 10µL iTaq SYBR, 2µL cDNA, 1µL forward primer (10µM), 1µL reverse primer (10µM), and 6µL RNase free H_2O. qPCR was performed on a CFX Real-Time PCR detection system (Bio-Rad). The thermocycler program began with a starting denaturation cycle for 30 seconds at 95°C. Next, the machine went for a denaturation step for 15 seconds at 95°C and an annealing and extension step for 30 seconds at 59°C, followed by a plate reading step – these three steps repeated for 40 cycles. The program then performed a melt curve analysis with 0.5°C increments every 2 to 5 seconds from 65°C-95°C.

dCT values were determined by normalizing the gene of interest (GOI) to eF1α (dCT = Ct (eF1α) - Ct (GOI), and ddCT values were calculated by normalizing to DMSO (ddCt = dCt treatment - dCt DMSO). Fold changes for biological and technical replicates were calculated with the following formula: 2^{ddCt} and then averaged.

Primer Design

Primer sequences (Table 3) for the following genes were designed by a previous graduate student: *eF1a* (reference gene), *ppp1r15a*, *ppp1r15b*, *ddit3*, and *hspa5* (Giresh 2022). All other sequences were obtained from a previously published paper (Howarth et al., 2014) and were modified to ensure the primers had a melting temperature (Tm) between 62°-74°C.

Table 4.

List of Primer Sequences.

Gene	Protein name	Primer Sequence (5'-3')	Tm
ppp1r15a	Growth arrest and DNA damage-inducible protein (GADD34)	F: TCGTCTGTCAGCTCCAGAAC R: GCGGATCTGCTCGCATAACT	62°C
ppp1r15b	Constitutive repressor of eif2(phosphorylation (CReP)	F: TCGTCTGTCAGCTCCAGAAC R: GCGGATCTGCTCGCATAACT	62°C
ddit3	C/EBP homologous protein (Chop)	F: CACCTCATCCGGAGATCTCC R: GACGCTGAGGAGCAGGATGA	64°C
hspa5	Binding immunoglobin protein (BiP)	F: GAGAATCGACAGCCGCAATG R: CCGCACTGCCGTACAGTTTG	64°C
ef1a	Eukaryotic translation initiation factor 1A	F: CTGGAGGCCAGCTCAAACAT R: TCAAGAAGAGTAGTACCGCTAGCATT	62°C 74°C
atf6	Activating transcription factor 6	F: CTGCCTGTGGTGAAACCTCCACCTG R: AGTGTCATGGTGACCACAGGAGATGTTG	67°C 65°C
ire1a	Inositol-requiring enzyme 1a	F: TGACGTGGTGGAAGTTGGTA R: TGCAGAAGTAACGGATCACATTGGGATGTTCATCTG	67° 66°C
xbp1	X-box-binding protein 1	F: CAGAGACTGGGGTTGGATACCTTGGAAAC R: TGCAGGGCCAGGGCTGTGAGTATCC	65°C 70°C

(Table 3 Continued)

perk	PKR-like ER kinase	F: GTCTTGGTGGGCTCTGAAGAGTTCGAT R: CTCACCTGTGAGCCTTCTCCGTCTTT	65°C
atf4	Activating transcription factor 4	F: CCAGCGCTCTGCTGCCATCGACAG R: CCAGCGCTCTGCTGCCATCGACAG	70°C 65°C
hsp90b1	Heat shock protein 90 beta family member 1	F: GAGGAAGCTTCTGACTACCTTGAGCTGG R: CAGCTCCCAATCCCACACAGTCTTCTC	65°C 66°C
calret	Calreticulin	F: GCTGCAGTGTGCTTTATTTCTGCACTGGCC R: GCATAAAACCGAGCATCTTGACTTGTTTGCAGAC	68°C 65°C
derl1	Derlin-1	F: GATCTGGGCGGTCGCTCCTTCCTC R: GGAACAGAGCCCTCGTCACTTTTCCAG	69°C 66°C
edem1	ER degradation enhancing alpha-mannosidase like protein 1	F: GACAGCAGAAACCCTCAAGCGAAGACC R: GCCTCAAAGACTTGAACAGTGGAGTCCTTGTC	66°C
pck1	Phosphoenolpyruvate carboxykinase 1	F: GGAAACTCACTGCTGGGGAAGAAGTGC R: GTCTCCCACACACTCCACCTTCC	66°C 65°C
pklr	Pyruvate kinase, liver And RBC	F: TCCTGGAGCATCTGTGTCTGCTGGA R: CTGATGAGTGCCATGAGAGAAGTTGAGTCTGGC	67°C
fasn2	Fatty acid synthase	F: GCAGAGAAAGCTTGCCAAACAGGGAGT R: GATCAGCTCCGTCCAGTCCACCTG	66°C
srebp1	Sterol regulatory element-binding protein 1	F: GCCCACTCTTCTGGTGTGGCTGCT R: CCCAAAGCCTTCAGACACGTCCTC	69°C 65°C

(Table 3 Continued)

srebp2	Sterol regulatory element-binding protein 2	F: CAGACACTCACACAAGCACACACGCAG R: GACCTGGTTCTGGATGAATCGTGAGGG	67°C 65°C

Oil Red O Staining

Larvae Fixation

Up to 10-15 drug treated 7 dpf zebrafish larvae were placed in microcentrifuge tubes with 4% paraformaldehyde (PFA) (Sigma) for 1-4 hours at room temperature to fixate the specimen. With 1000µL 1X PBS the samples were washed for 2 minutes 3 times. The fixed larvae were stored in 1X PBS at 4°C for up to 1 month prior to staining.

Lipid Staining

Larvae were incubated in 85% propylene glycol followed by 100% propylene glycol prior to staining with Oil Red O (Sigma) overnight. After staining, embryos were washed in 100% propylene glycol, 85% propylene glycol, and PBS, after which they were stored in 80% glycerol until imaging.

Steatosis Scoring and Imaging

Following the oil red O staining protocol, larvae were imaged with a LEICA M80 dissection stereo microscope and software from the Leica Application Suite X (LAS X) version 3.7.4.23463. Individual larvae were qualitatively scored as having no steatosis or steatosis based upon the presence and intensity of pink to red colored droplets in the liver.

Statistical Analysis

All RT-qPCR data was statistically analyzed using JMP v12 (SAS, USA). The effect of drug treatment type, duration, and their interaction on gene expression (dCt values) was evaluated using multivariate general linear models (GLM). Shapiro-Wilk's test was used when determining if model residuals were normally distributed. Pairs of treatment groups and durations were then compared through post-hoc Student's t-tests. For genes analyzed exclusively at one time point, ANOVA with post-hoc Student's t-test was used to compare pairs of treatments. Levene's test was used to analyze if variances were equivalent between treatment groups; for results that did not meet model assumptions, nonparametric tests (Kruskall-Wallis test followed by post-hoc Wilcoxon test) were used. With the data from the Oil Red O protocol, Fisher's exact tests with 2x2 contingency tables were performed with GraphPad software. For both techniques, results with p-values that were 0.05 or less were deemed as statistically significant and denoted with an asterisk (*) on the figures.

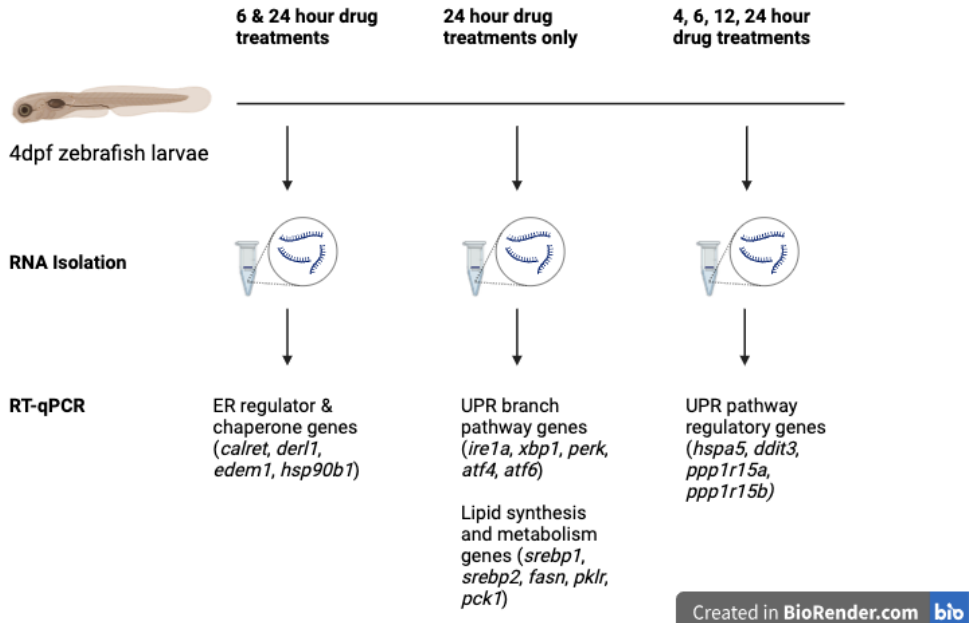
CHAPTER 3: RESULTS

Gene Expression in 4 dpf Zebrafish

The mRNA expression of ER regulators and chaperones, the UPR network, and lipogenic regulator genes were measured in this study. Gene expression from ERAD components, ER regulatory enzymes, and all three branches of the UPR as well as key, downstream targets of the PERK pathway that regulate the pathway or activate pro-apoptotic pathways were analyzed. A variety of core, metabolic genes that are linked to FLD were studied as well, some of which are activated by UPR components (Ozcan and Tabas, 2012). By looking at the expression of these genes together, a better baseline understanding of how the UPR influences lipogenesis during FLD may be achieved.

Figure 1

Schematic Representation of Drug Treatment Durations for Various Gene Groupings



Prior to quantifying gene expression, 4dpf zebrafish larvae were treated with drugs for acute and chronic durations that either induce ER stress or inhibit eIF2α phosphatases and then RT-qPCR was performed.

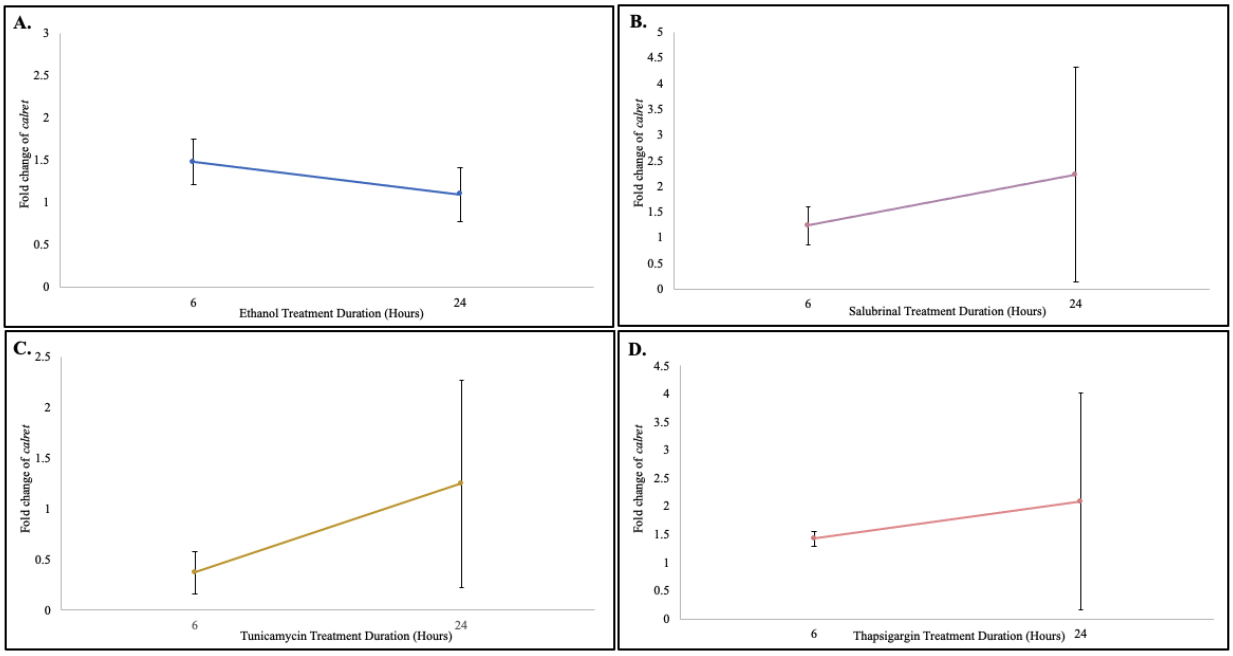
Gene Expression of ER Regulators and Chaperones

Calret encodes the calreticulin protein, which is associated with maintaining ER homeostasis by regulating calcium levels in the organelle (Carreras-Sureda et al., 2018). Expression of *calret* was not affected by the interaction between treatment type and duration ($p=0.99$); it also did not significantly differ between treatment types compared to DMSO ($p=0.57$) (Figure 2). The duration of drug treatment (6 hrs and 24 hrs) affected *calret* expression ($F_{1, 20}= 8.68$, $p=0.01$).

Both *derl1*, which encodes for Derlin-1 proteins, and *edem1*, which encodes for ER degradation enhancing alpha-mannosidase like protein 1, are components of the ERAD process— they serve to clear out the harmful presence of excessive misfolded proteins (Howarth et al., 2014; Kadowaki et al., 2018). We hypothesized that ER stressors may upregulate their expression. Expression of *derl1* was affected by treatment ($F_{4, 112.6}= 10.5$, $p= 0.007$), duration ($F_{1, 63.6}= 23.7$, $p= 0.0004$), and by interaction between treatment and duration ($F_{4, 36.9}= 3.4$, $p= 0.043$). Tunicamycin treatment downregulated *derl1* expression; there was a 0.005 fold change compared to DMSO at 6 hrs ($p= 0.0034$) and a 0.14 fold change compared to DMSO at 24 hrs ($p= 0.048$) (Figure 3C). Either acute or chronic treatment with ethanol, thapsigargin, and salubrinal did not affect *derl1* expression ($p > 0.05$) (Figure 3A-B, D).

Figure 2

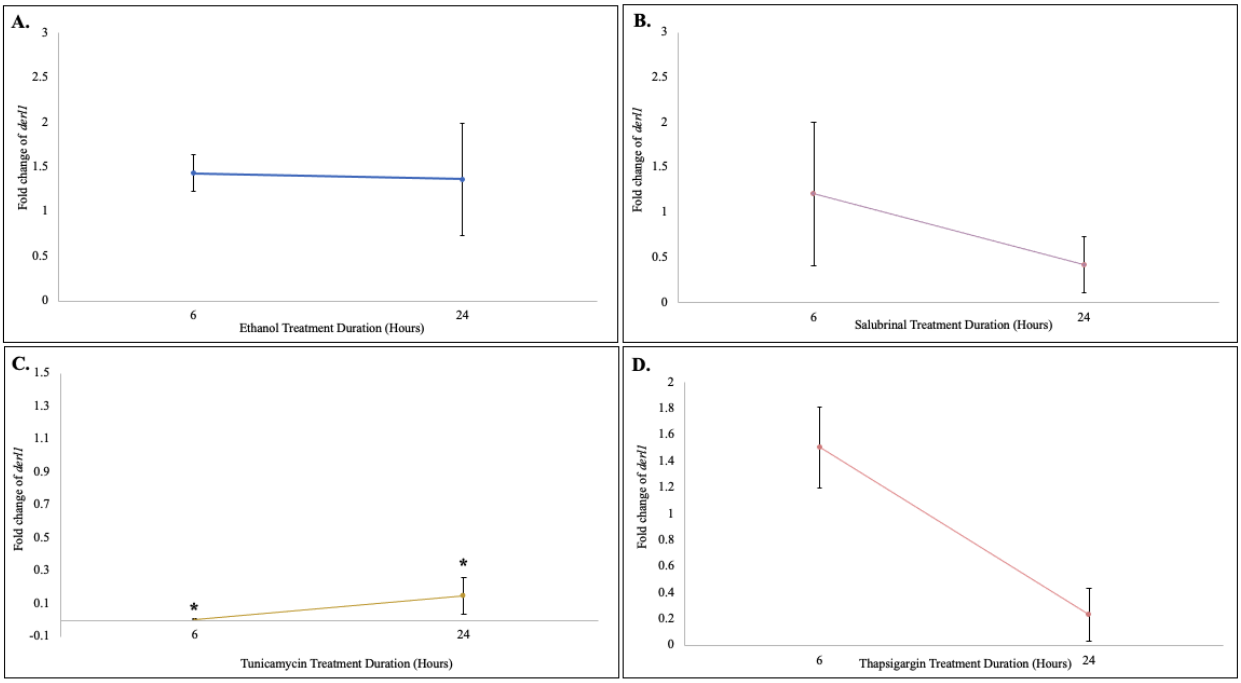
Expression of calret Upon 6hr and 24hr Drug Treatments



Note. Fold change in expression of *calret* in response to 6 hr and 24 hr drug treatments. Zebrafish larvae (4 dpf) were treated with DMSO (control), ethanol, salubrinal, tunicamycin, and thapsigargin for 6 and 24 hours. Error bars represent standard error values of fold change values (n = 3 experiments at 6hrs, n = 2 experiments at 24 hrs). Acute and chronic drug treatments did not affect *calret* expression (GLM and post-hoc Student's t-tests, $p > 0.5$). **A.** *calret* expression post ethanol treatment (350mM). **B.** *calret* expression post salubrinal treatment (50µM). **C.** *calret* expression post tunicamycin treatment (2.5µg/mL). **D.** *calret* expression post thapsigargin treatment (1µM).

Figure 3

Expression of derl1 Upon 6hr and 24hr Drug Treatments

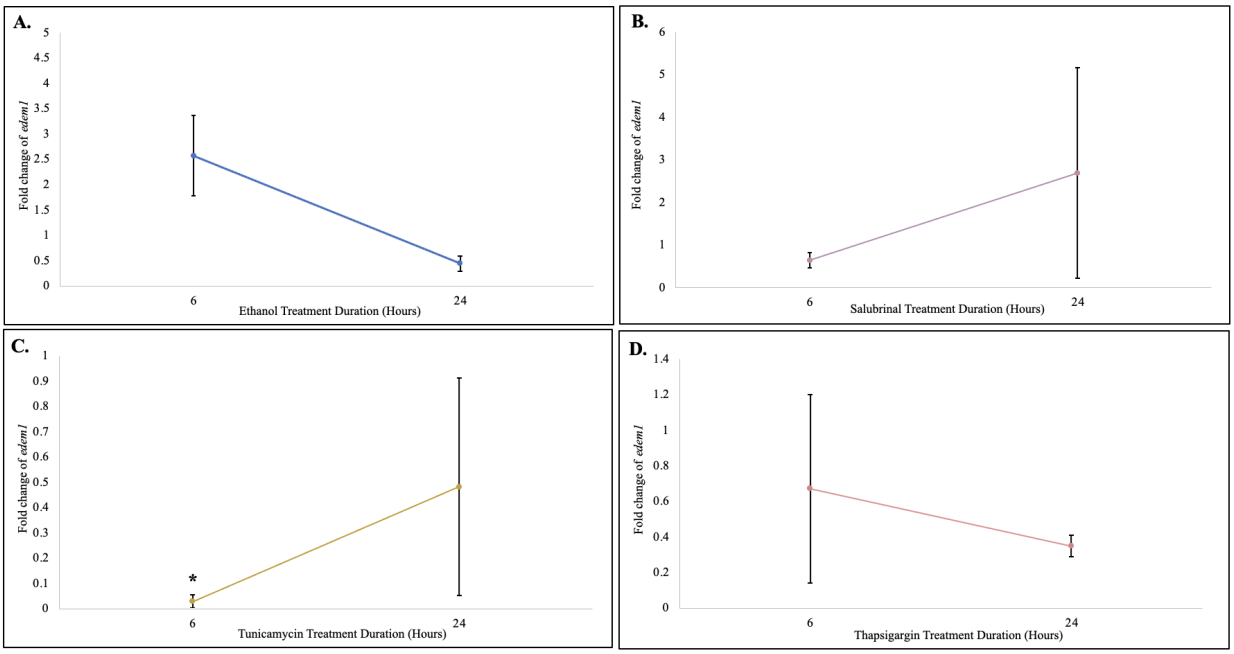


Note. Fold change in expression of *derl1* in response to 6 hr and 24 hr drug treatments. Zebrafish larvae (4 dpf) were treated with DMSO (control), ethanol, salubrinal, tunicamycin, and thapsigargin for 6 and 24 hours. Error bars represent standard error values of fold change values (n = 3 experiments at 6hrs, n = 2 experiments at 24 hrs). Asterisks denote significant differences between *derl1* expression compared to DMSO treatment (GLM and post-hoc Student's t-tests, $p < 0.05$). **A.** *derl1* expression post ethanol treatment (350mM). **B.** *derl1* expression post salubrinal treatment (50µM). **C.** *derl1* expression post tunicamycin treatment (2.5µg/mL). **D.** *derl1* expression post thapsigargin treatment (1µM).

Expression of *edem1* was affected by treatment type ($F_{4, 90}$= 4.24, p= 0.023), but was not influenced by duration (p= 0.29), or treatment and duration crossed (p= 0.35) (Figure 4). *Edem1* expression decreased 0.03 fold compared to DMSO after 6hrs of tunicamycin treatment (p = 0.0022), while all other treatments at 6hrs or 24hrs did not influence expression (p > 0.05).

Figure 4

Expression of edem1 Upon 6hr and 24hr Drug Treatments



Note. Fold change in expression of *edem1* in response to 6 hr and 24 hr drug treatments. Zebrafish larvae (4 dpf) were treated with DMSO (control), ethanol, salubrinal, tunicamycin, and thapsigargin for 6 and 24 hours. Error bars represent standard error values of fold change values (n = 3 experiments at 6hrs, n = 2 experiments at 24 hrs). Asterisks denote significant differences between *edem1* expression compared to DMSO treatment (GLM and post-hoc Student's t-tests, p < 0.05). **A.** *edem1* expression post ethanol treatment (350mM). **B.** *edem1* expression post

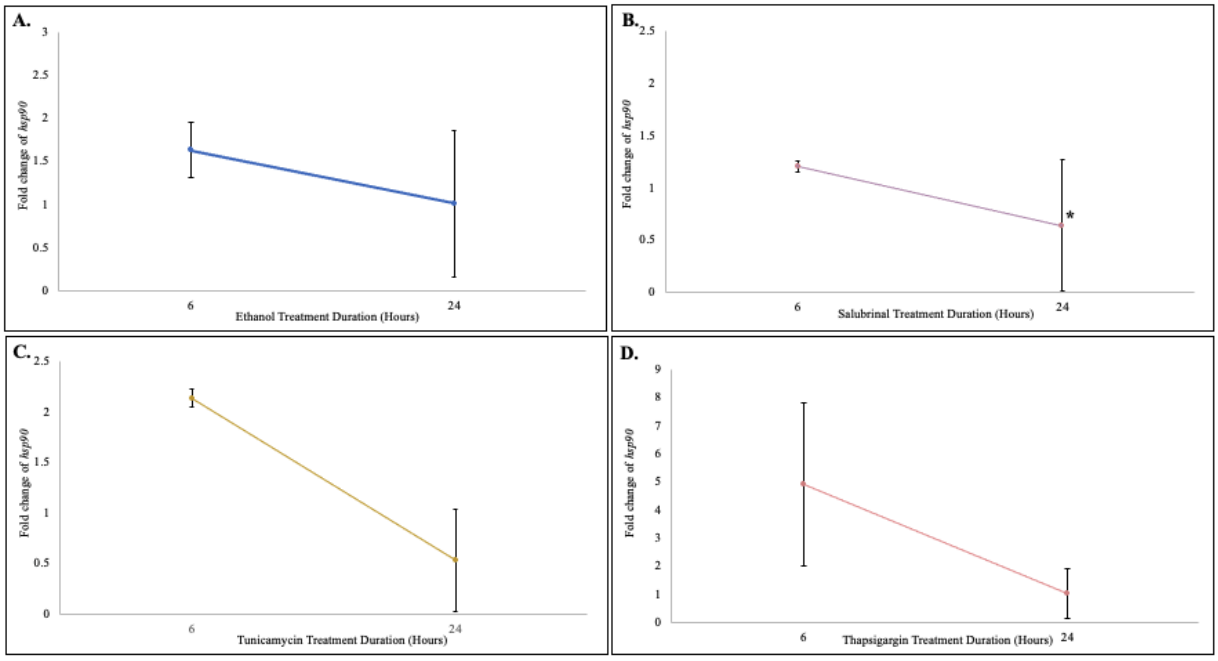
(Figure 4 Continued)

salubrinal treatment (50µM). **C.** *edem1* expression post tunicamycin treatment (2.5µg/mL). **D.** *edem1* expression post thapsigargin treatment (1µM).

Hsp90b1 expression was not influenced by treatment type (p= 0.33) and treatment and duration crossed (p= 0.11), but was significantly affected by duration ($F_{1, 8.40} = 7.34$, p= 0.019) (Figure 5). With 24hrs of salubrinal treatment, *hsp90b1* decreased 0.64 fold compared to DMSO (p=0.0315). All other treatments at 6hrs and 24hrs did not influence its expression (p > 0.05).

Figure 5

Expression of hsp90b1 Upon 6hr and 24hr Drug Treatments



Note. Fold change in expression of *hsp90b1* in response to 6 hr and 24 hr drug treatments. Zebrafish larvae (4 dpf) were treated with DMSO (control), ethanol, salubrinal, tunicamycin, and thapsigargin for 6 and 24 hours. Error bars represent standard error values of fold change values

(Figure 5 Continued)

(n = 2 experiments at 6hrs, n = 2 experiments at 24 hrs). Asterisks denote significant differences between *edem1* expression compared to DMSO treatment (GLM and post-hoc Student's t-tests, $p < 0.05$). **A.** *hsp90b1* expression post ethanol treatment (350mM). **B.** *hsp90b1* expression post salubrinal treatment (50µM). **C.** *hsp90b1* expression post tunicamycin treatment (2.5µg/mL). **D.** *hsp90b1* expression post thapsigargin treatment (1µM).

Gene Expression of the UPR

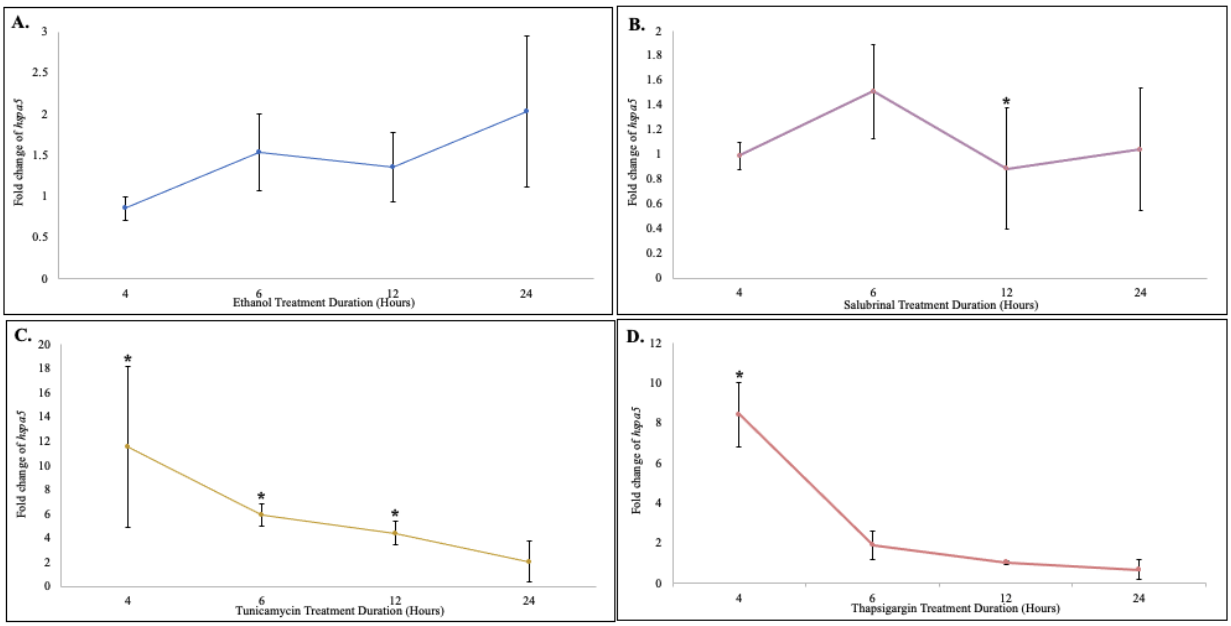
Upon detecting ER stress, BiP releases IRE1a, PERK, and ATF6 proteins, allowing the UPR to activate. BiP proteins are encoded by the *hspa5* (heat shock protein family A (Hsp70) member 5) gene, which is activated both by the presence of misfolded proteins during ER stress, and by activation of genes downstream in the UPR, such as *atf6* (Cao and Kaufman, 2012). Here, 4 dpf zebrafish larvae were exposed to pharmacological stressors and expression of hspa5 was measured over 24 hours to measure the time-course of BiP activation during ER stress and UPR activation. Expression of *hspa5* was affected by duration of stressor treatments ($F_{3, 40.82}= 12.85$, $p=0.014$), treatment type ($F_{4, 25.2}= 5.95$, $p=0.001$), and by duration and treatment crossed ($F_{12,33.0}= 2.60$, $p= 0.001$) (Figure 6).

Tunicamycin treatment significantly altered *hspa5* expression compared to DMSO ($p= 0.0034$) with acute durations causing higher fold changes (Figure 6C). After 4 hours of tunicamycin treatment, *hspa5* expression increased by 11.5 fold compared to DMSO ($p= 0.001$); at 6 hours, expression was increased by 5.9 fold ($p= 0.001$), and by 4.4 fold at 12 hours compared to DMSO ($p= 0.02$). There was no significant expression change in *hspa5* after 24 hours of tunicamycin treatment compared to DMSO treatment ($p= 0.26$). Thapsigargin treatment

increased *hspa5* expression by 8.4 fold at 4hrs compared to DMSO (p= 0.006); treatments of longer durations (6hrs, 12hrs, and 24hrs) caused expression to then return toward baseline expression as they did not induce significant expression compared to DMSO (p= 0.40, p= 0.97, p= 0.091, respectively) (Figure 6D). After 12hrs of salubrinal treatment, *hspa5* expression was downregulated 0.89 fold relative to DMSO (p= 0.042) (Figure 6B). All other salubrinal and ethanol treatments did not influence *hspa5* expression (p > 0.5) (Figure 6A, B).

Figure 6

Expression of hspa5 Upon 4hr, 6hr, 12hr, and 24hr Drug Treatments



Note. Fold change in expression of *hspa5* in response to 4 hr, 6 hr, 12 hr, and 24 hr drug treatments. 4 dpf zebrafish larvae were treated with DMSO (control), ethanol, salubrinal, tunicamycin, and thapsigargin for 4, 6, 12, and 24 hours. Error bars represent standard error values of fold change values (n= 3 experiments). Asterisks denote significant differences between *hspa5* expression compared to DMSO treatment (GLM and post-hoc Student's t-test, $p < 0.05$) **A.** *hspa5* expression post ethanol treatment (350mM). **B.** *hspa5* expression post

(Figure 6 Continued)

salubrinal treatment (50μM). **C.** *hspa5* expression post tunicamycin treatment (2.5μg/mL). **D.** *hspa5* expression post thapsigargin treatment (1μM).

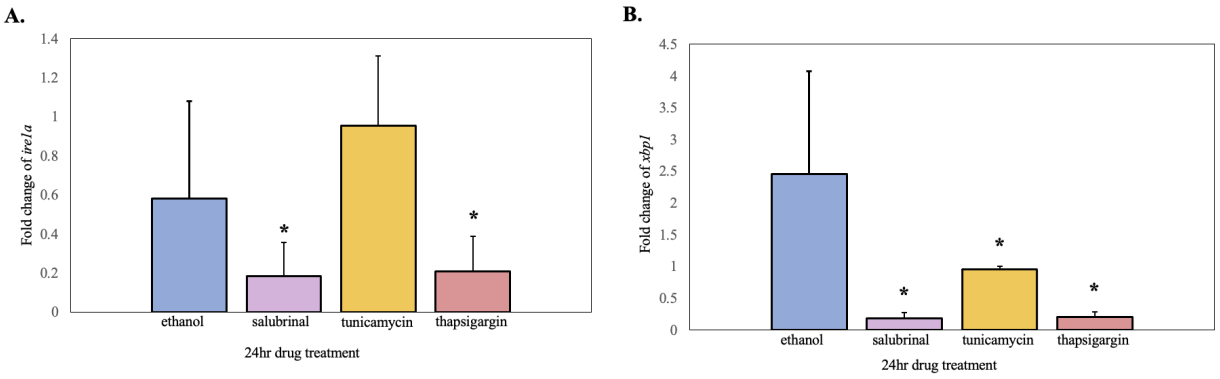
Expression of genes involved in the ire1a, perk, and atf6 pathways

With the exception of downstream genes in the PERK pathway, expression of the majority of UPR genes was analyzed only after inducing chronic stress (24 hr durations of treatments).

One of the three UPR sensors, IRE1a, encoded by *ire1a*, splices *xbp1* mRNA in the cytosol—this activates *xbp1*, which then functions as the transcription factor *xbp1s* and drives the expression of genes alleviating ER stress (Cao and Kaufman, 2012). Expression of *ire1a* was not affected by 24hrs of ethanol treatment ($p= 0.58$) or by tunicamycin treatment ($p= 0.23$) compared to DMSO (Figure 7A). After 24hrs of salubrinal treatment, *ire1a* expression decreased 0.19 fold, and thapsigargin caused a 0.21 fold decrease ($p = 0.0095$) relative to DMSO. Similarly, *xbp1* was not affected by 24hrs of ethanol treatment compared to DMSO ($p= 0.46$). All other treatments lowered *xbp1* expression compared to DMSO; salubrinal decreased it by 0.15 fold ($p < 0.001$), tunicamycin by 0.084 fold ($p < 0.001$), and thapsigargin by 0.36 fold ($p < 0.001$) (Figure 7B). Neither gene was differentially expressed in response to ethanol treatment, which is consistent with other studies (Howarth et al., 2014).

Figure 7.

Expression of ire1a and xbp1 Upon 24hr Drug Treatments



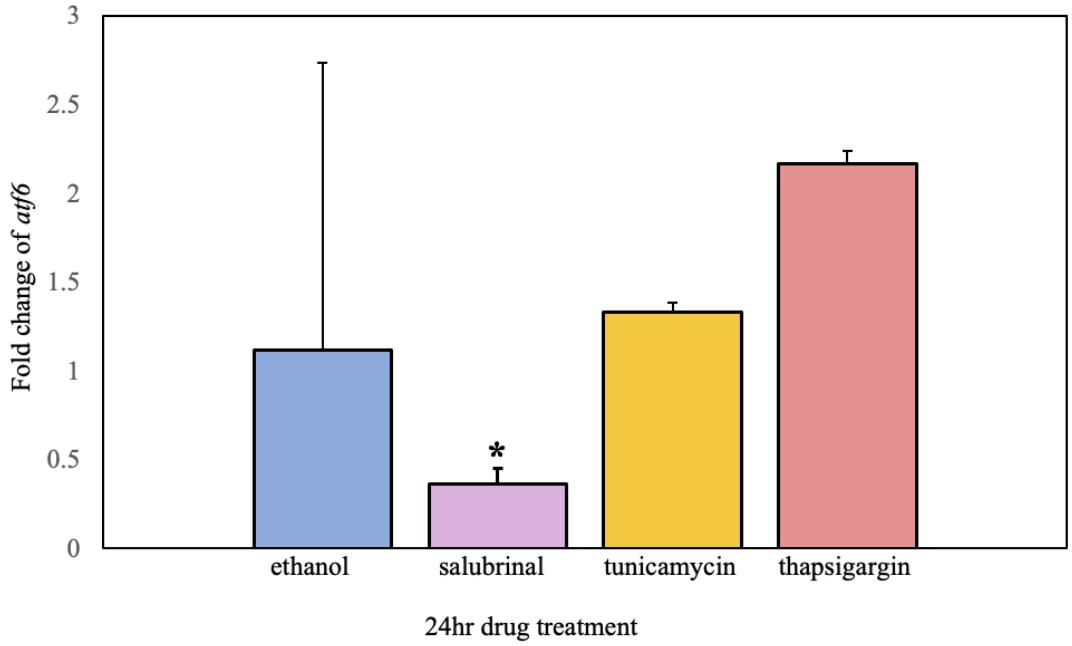
Note. Fold change in expression of *ire1a* and *xbp1* in response to 24 hr drug treatments. Zebrafish larvae (4 dpf) were treated with DMSO (control), ethanol, salubrinal, tunicamycin, and thapsigargin for 24 hours. Error bars represent standard error values of fold change values (n = 3 experiments). Post-hoc student's t-tests were performed to determine statistical significance ($p < 0.05$, denoted with *). **A.** *ire1a* expression post ethanol treatment (350mM), salubrinal treatment (50µM), tunicamycin treatment (2.5µg/mL), and (thapsigargin treatment (1µM). **B.** *xbp1* expression post ethanol treatment (350mM), salubrinal treatment (50µM), tunicamycin treatment (2.5µg/mL), and (thapsigargin treatment (1µM).

The ATF6 branch of the UPR was studied next. The activity of ATF6, which is encoded by *atf6* (Activating transcription factor 6), is implicated in the development of alcoholic fatty liver disease and overexpression of *atf6* was shown in a study by Howarth et. al (2014) to cause FLD under acute stress while reducing FLD from chronic stress. In this study, 24hrs of exposure to tunicamycin did not have significant changes in *atf6* expression compared to DMSO ($p= 0.87$), and neither did thapsigargin treatment ($p= 0.51$) (Figure 8). Treatment with ethanol for 24hrs did not significantly affect its expression compared to DMSO, with only a 1.1 fold

increase (p= 0.042). After 24hrs of salubrinal treatment, there was 0.37 fold decrease in *atf6* expression compared to DMSO treatment (p= 0.048).

Figure 8.

Expression of atf6 Upon 24hr Drug Treatments

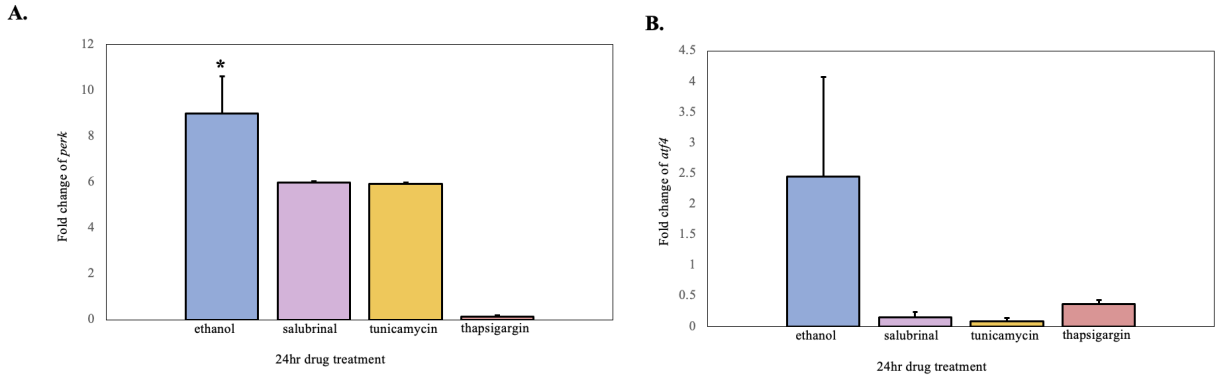


Note. atf6 expression after 24 hr drug treatments. 4 dpf zebrafish larvae were treated with DMSO (control), ethanol, salubrinal, tunicamycin, and thapsigargin for 24 hours. Error bars represent standard error values of fold change values (n= 2 experiments). Post-hoc student's t-tests were performed to determine statistical significance ($p < 0.05$, denoted with *). *atf6* expression post ethanol treatment (350mM), salubrinal treatment (50µM), tunicamycin treatment (2.5µg/mL), and thapsigargin treatment (1µM).

Perk (Protein kinase R (PKR)-like endoplasmic reticulum kinase) and *atf4* (Activating transcription factor 4) expression were next studied. In the PERK branch of the UPR, PERK proteins phosphorylate eIF2α during ER stress, which causes the inhibition of global protein translation; *atf4* mRNA is an exception to this, and is translated when eIF2α is phosphorylated. After 24hrs of ethanol treatment, expression of *perk* increased 9.0 fold compared to DMSO ($p= 0.047$); thapsigargin treatment downregulated its expression by 0.14 fold compared to DMSO ($p= 0.042$). Salubrinal and thapsigargin treatment did not significantly affect *perk* expression ($p= 0.073$ for both treatments). None of the drug treatments had an impact on *atf4* expression compared to DMSO ($p > 0.05$) (Figure 9B).

Figure 9.

Expression of perk and atf4 Upon 24hr Drug Treatments



Note. Fold change in expression of *perk* and *atf4* in response to 24 hr drug treatments. Zebrafish larvae (4 dpf) were treated with DMSO (control), ethanol, salubrinal, tunicamycin, and thapsigargin for 24 hours. Error bars represent standard error values of fold change values ($n = 2$ experiments). Post-hoc student's t-tests were performed to determine statistical significance ($p < 0.05$, denoted with *). **A.** *perk* expression post ethanol treatment (350mM), salubrinal treatment (50μM), tunicamycin treatment (2.5μg/mL), and (thapsigargin treatment (1μM). **B.**

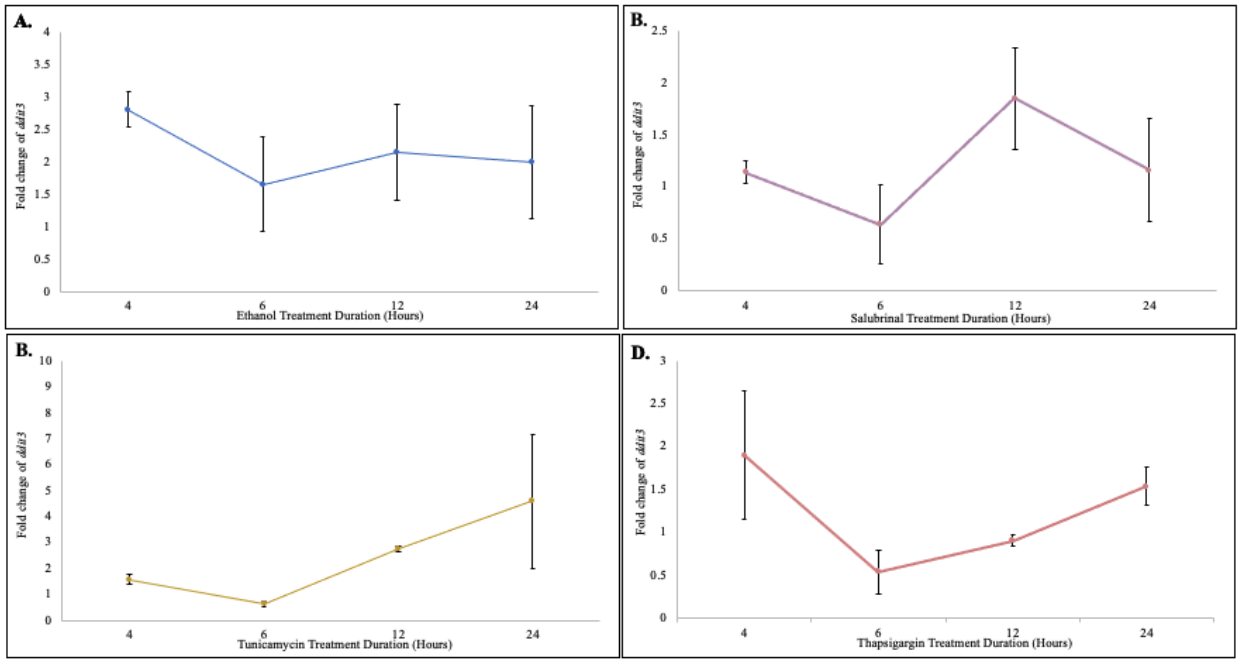
(Figure 9 Continued)

atf4 expression post ethanol treatment (350mM), salubrinal treatment (50µM), tunicamycin treatment (2.5µg/mL), and thapsigargin treatment (1µM).

Next in the PERK branch, *ddit3* (DNA damage-inducible transcript 3 protein) encodes CHOP proteins, which are the primary pro-apoptotic targets of the PERK pathway and subsequently it was studied at several acute and chronic time points, given the devastating nature of excess apoptosis in metabolic diseases. *Ddit3* is driven in expression from *atf4* activation, and was measured in expression after drug treatments at several time points (4, 6, 12, and 24 hrs).

Figure 10.

Expression of ddit3 Upon 4hr, 6hr, 12hr, and 24hr Drug Treatments



Note. Fold change in expression of *ddit3* in response to 4 hr, 6 hr, 12 hr, and 24 hr drug treatments. 4 dpf zebrafish larvae were treated with DMSO (control), ethanol, salubrinal,

(Figure 10 Continued)

tunicamycin, and thapsigargin for 4, 6, 12, and 24 hours. Error bars represent standard error values of fold change values (n= 3 experiments). Acute and chronic drug treatments did not affect *ddit3* expression (GLM and post-hoc Student's t-tests, $p < 0.05$) **A.** *ddit3* expression post ethanol treatment (350mM). **B.** *ddit3* expression post salubrinal treatment (50µM). **C.** *ddit3* expression post tunicamycin treatment (2.5µg/mL). **D.** *ddit3* expression post thapsigargin treatment (1µM).

Expression of *ddit3* was not significantly affected by treatment duration (p= 0.21), treatment type (p=0.75), or by the interaction between duration and treatment (p= 0.64) (Figure 10).

Gene expression of UPR Regulators

The UPR stalls protein translation in the cell through PERK phosphorylating eIF2α; to allow for global protein translation to resume, either GADD34 or CReP bind to the PP1 protein and dephosphorylate eIF2α. The mechanisms of the activation of GADD34 (Growth arrest and DNA damage-inducible protein) and CReP (Constitutive repressor of eIF2α phosphorylation) during the UPR are still being researched. Due to their regulatory mechanisms in the UPR, it is crucial to understand the timing of their activation, thus drug treatments with time points ranging from 4-24 hrs were used to analyze the dynamics of their expression.

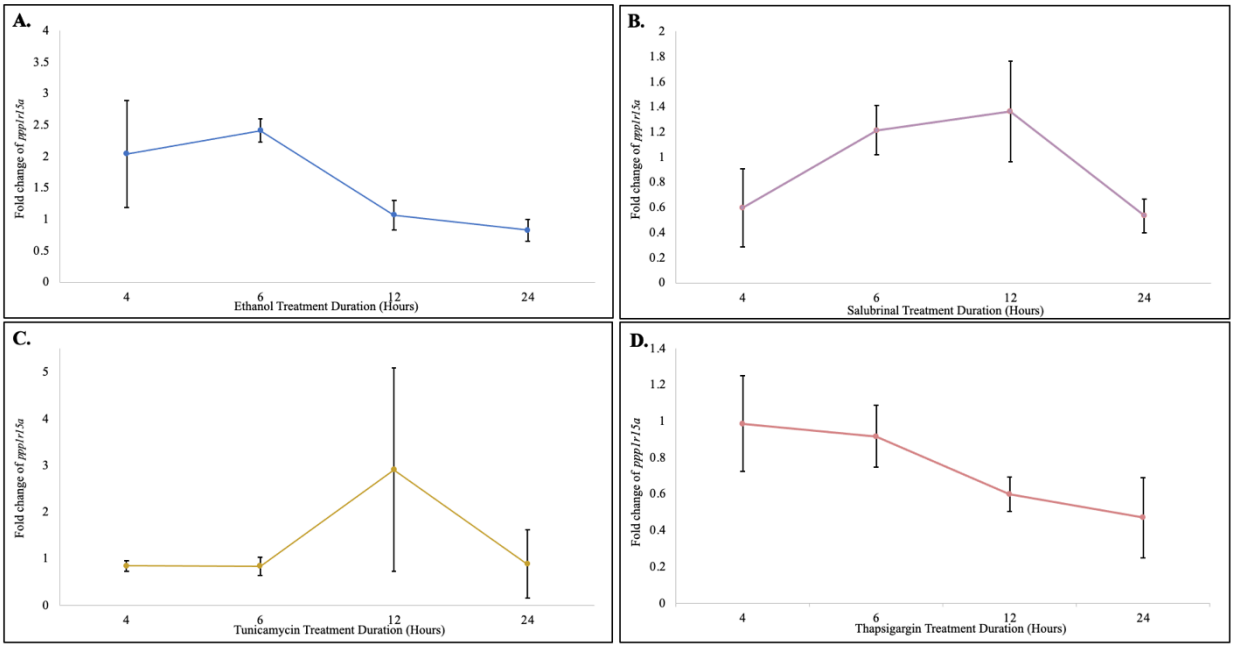
Ppp1r15a (Protein phosphatase 1, regulatory subunit 15A), which encodes GADD34, a protein downstream in the PERK branch, serves as a feedback inhibitor of the UPR. *Ppp1r15a* expression did not change in response to pharmacological treatment relative to DMSO in this study ($F_{4, 3.56}= 0.69$, p=0.60), even with salubrinal, the pharmacological inhibitor of GADD34

and CReP (Figure 11). The duration of treatment did not influence expression (p= 0.34), and neither did the interaction between treatment and duration (p= 0.83). While no treatment was statistically significant compared to DMSO, ethanol treatment for 4hrs increased *ppp1r15a* expression by 2.0 fold, while 6hrs of treatment increased gene expression by 2.4 fold. After 12hrs of ethanol treatment, gene expression did not change, and 24hrs of treatment decreased gene expression by 0.83 fold ($p > 0.05$). After 4hrs of salubrinal treatment, gene expression decreased by 0.60 fold; 6hr and 12hr treatments did not change gene expression, and 24hr treatments decreased expression by 0.53 fold ($p > 0.05$). Tunicamycin treatment at 4hrs and 6hrs decreased expression by 0.84 fold, while 12hrs of treatment increased expression by 2.91 fold; expression decreased again by 0.89 fold after 24hrs of treatment ($p > 0.05$). After 4hrs and 6hrs of thapsigargin treatment, gene expression did not change; 12hrs of treatment decreased expression by 0.60 fold, while 24hrs decreased expression by 0.47 fold ($p > 0.05$).

Ppp1r15b (Protein Phosphatase 1 regulatory subunit 15B) encodes CReP. It remains unclear whether *ppp1r15b* is constitutively active, or if ER stress and other branches of the UPR may influence its activity. There were no significant differences in expression of this gene between acute and chronic duration of treatment ($F_{3, 4.49}= 1.16$, p=0.34), treatment type ($F_{4, 1.83}=$ 0.31, p=0.87), and treatment and duration crossed ($F_{12, 6.22}= 0.35$, p=0.97) (Figure 12). Treatments did not induce significant expression change compared to DMSO; 4hrs of ethanol treatment increased *ppp1r15b* expression by 2.2 fold, and 6-24hr treatments did not influence expression ($p > 0.05$). Salubrinal treatment at 4hrs did not influence expression; 6hrs of treatment decreased expression by 0.67 fold, 12hrs decreased expression by 0.82 fold, and 24hrs decreased expression by 0.45 fold ($p > 0.05$).

Figure 11.

Expression of ppp1r15a Upon 4hr, 6hr, 12hr, and 24hr Drug Treatments



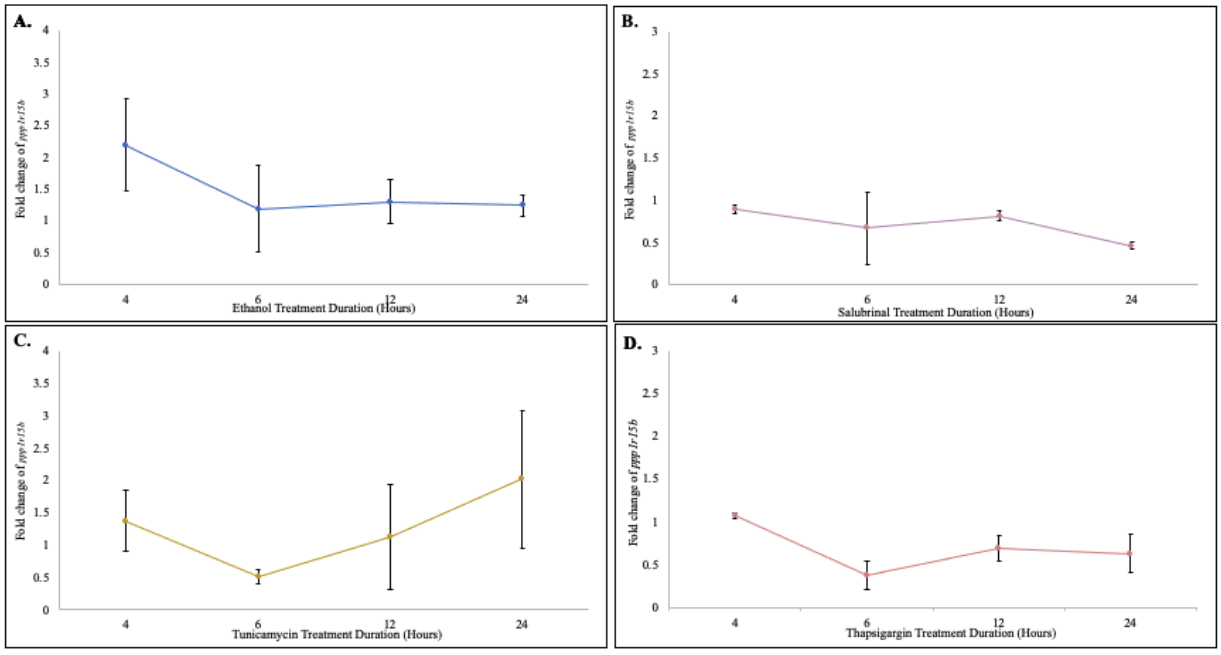
Note. Fold change in expression of *ppp1r15a* in response to 4 hr, 6 hr, 12 hr, and 24 hr drug treatments. 4 dpf zebrafish larvae were treated with DMSO (control), ethanol, salubrinal, tunicamycin, and thapsigargin for 4, 6, 12, and 24 hours. Error bars represent standard error values of fold change values (n= 3 experiments). Acute and chronic drug treatments did not affect *ppp1r15a* expression (GLM and post-hoc Student's t-tests, $p < 0.05$) **A.** *ppp1r15a* expression post ethanol treatment (350mM). **B.** *ppp1r15a* expression post salubrinal treatment (50µM). **C.** *ppp1r15a* expression post tunicamycin treatment (2.5µg/mL). **D.** *ppp1r15a* expression post thapsigargin treatment (1µM).

Tunicamycin treatment did not influence gene expression after 4hrs and 12hrs of treatment; 6hrs of treatment decreased expression by 0.51 fold, and 24hrs increased expression by 2.0 fold (p >

0.05). After 4hrs of thapsigargin treatment, expression did not change; 6hrs decreased expression by 0.37 fold, and both 12hrs and 24hrs decreased expression by 0.69 fold ($p > 0.05$).

Figure 12.

Expression of ppp1r15b Upon 4hr, 6hr, 12hr, and 24hr Drug Treatments



Note. Fold change in expression of *ppp1r15b* in response to 4 hr, 6 hr, 12 hr, and 24 hr drug treatments. 4 dpf zebrafish larvae were treated with DMSO (control), ethanol, salubrinal, tunicamycin, and thapsigargin for 4, 6, 12, and 24 hours. Error bars represent standard error values of fold change values (n= 3 experiments). Acute and chronic drug treatments did not affect *ppp1r15b* expression (GLM and post-hoc Student's t-tests, $p < 0.05$) **A.** *ppp1r15b* expression post ethanol treatment (350mM). **B.** *ppp1r15b* expression post salubrinal treatment (50µM). **C.** *ppp1r15b* expression post tunicamycin treatment (2.5µg/mL). **D.** *ppp1r15b* expression post thapsigargin treatment (1µM).

Expression of lipid synthesis and metabolism genes

Given the importance of the ER during lipid metabolism and synthesis, it is crucial to understand how expression of genes encoding key enzymes that control the synthesis of fatty acids and cholesterols in this organelle respond to chronic durations (24 hrs) of both ER stress and GADD34/CReP inhibition. SREBP enzymes play an influential role in maintaining lipid homeostasis— activated, nuclear SREBP functions to drive the expression of lipid metabolism genes such as *fasn*, the gene encoding FAS (Moslehi and Hamidi-zad, 2018; Bengoechea-Alonso and Ericsson, 2007). In adipocytes and hepatocytes, FAS is an enzyme that catalyzes the de novo synthesis of fatty acids that may be used to produce TAGs as energy storage (Jensen-Urstad and Semenkovich, 2011).

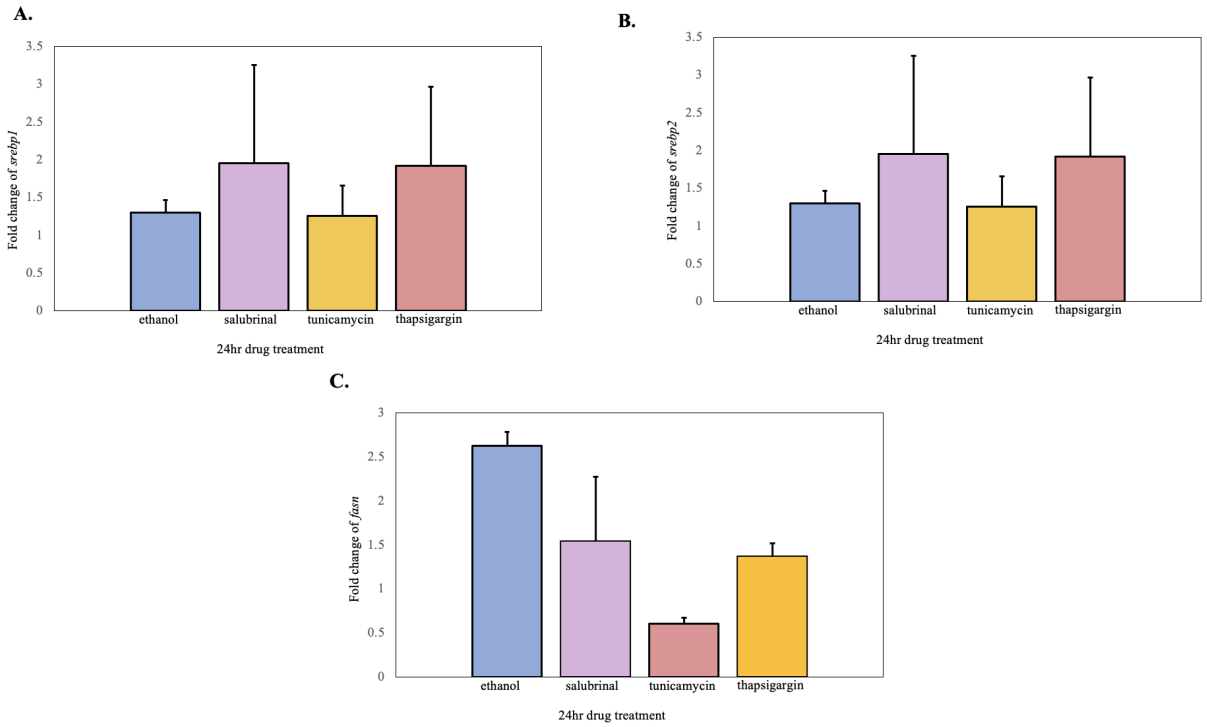
Expression of *srebp1* (Sterol regulatory element-binding protein 1) was not influenced by 24 hrs of ethanol ($p= 0.70$), salubrinal ($p= 0.49$), tunicamycin ($p= 0.85$), or thapsigargin treatment compared to DMSO ($p= 0.62$) (Figure 13A). Similarly, *srebp2* expression did not differ from 24 hr treatment of ethanol ($p= 0.31$), salubrinal ($p= 0.27$), tunicamycin ($p= 0.26$), or thapsigargin ($p= 0.60$) relative to DMSO (Figure 13B); *fasn* expression was not influenced by treatments either ($p > 0.05$) (Figure 13C).

The Howarth et al. (2014) study demonstrated that genes encoding glycolysis enzymes, such as *pklr* (Pyruvate kinase, liver and red blood cell (RBC)), decrease in expression in response to ethanol in zebrafish, and that expression of genes encoding glyceroneogenesis enzymes, such as *pck1* (Phosphoenolpyruvate carboxykinase 1), are upregulated. This study demonstrated the same trend for *pklr*: 24 hrs of ethanol treatment decreased gene expression by 0.39 fold compared to DMSO ($p= 0.019$), while salubrinal ($p= 0.68$), tunicamycin ($p= 0.26$), and

thapsigargin (p= 0.95) did not influence expression relative to DMSO (Figure 14A). *Pck1* expression was not significantly influenced by treatments (p > 0.05) compared to DMSO (Figure 14B).

Figure 13.

Expression of srebp1, *srebp2*, and *fasn Upon 24hr Drug Treatments*



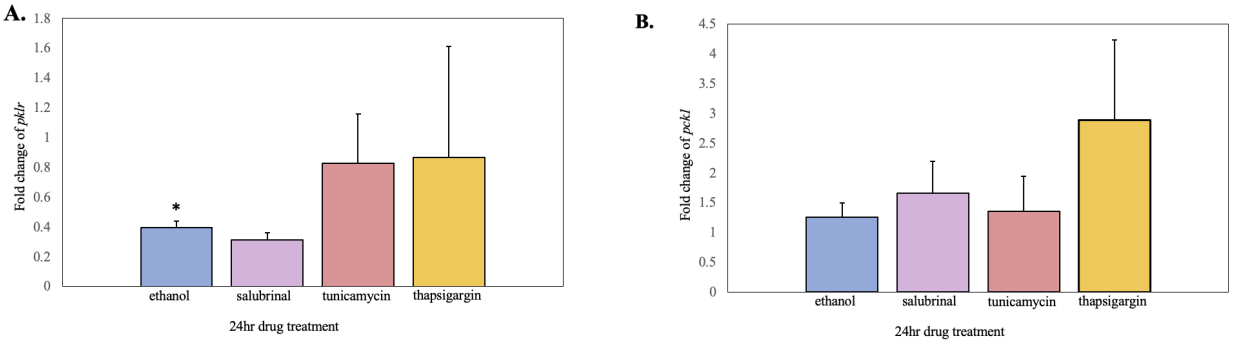
Note. Fold change in expression of *srebp1*, *srebp2*, and *fasn* in response to 24 hr drug treatments. Zebrafish larvae (4 dpf) were treated with DMSO (control), ethanol, salubrinal, tunicamycin, and thapsigargin for 24 hours. Error bars represent standard error values of fold change values (n = 2 experiments). Post-hoc student's t-tests were performed to determine statistical significance ($p<0.05$, denoted with *). **A.** *srebp1* expression post ethanol treatment (350mM), salubrinal treatment (50µM), tunicamycin treatment (2.5µg/mL), and (thapsigargin treatment (1µM). **B.** *srebp2* expression post ethanol treatment (350mM), salubrinal treatment (50µM), tunicamycin

(Figure 13 Continued)

treatment (2.5µg/mL), and thapsigargin treatment (1µM). **B.** *fasn* expression post ethanol treatment (350mM), salubrinal treatment (50µM), tunicamycin treatment (2.5µg/mL), and thapsigargin treatment (1µM).

Figure 14.

Expression of pklr and pck1 Upon 24hr Drug Treatments



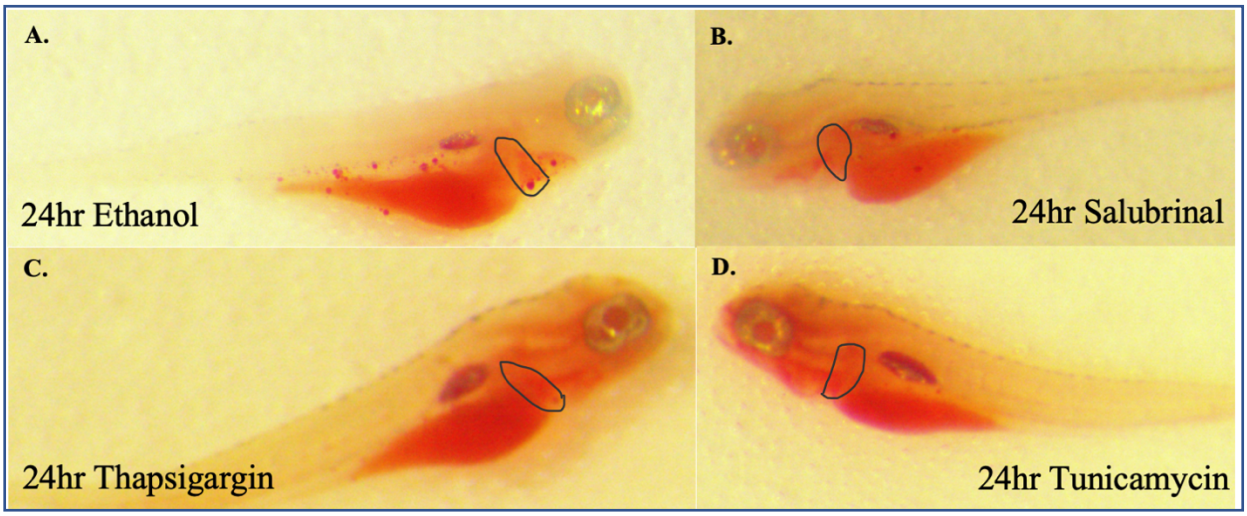
Note. Fold change in expression of *pklr* and *pck1* in response to 24 hr drug treatments. Zebrafish larvae (4 dpf) were treated with DMSO (control), ethanol, salubrinal, tunicamycin, and thapsigargin for 24 hours. Error bars represent standard error values of fold change values (n = 3 experiments). Post-hoc student's t-tests were performed to determine statistical significance ($p<0.05$, denoted with *). **A.** *pklr* expression post ethanol treatment (350mM), salubrinal treatment (50µM), tunicamycin treatment (2.5µg/mL), and (thapsigargin treatment (1µM). **B.** *pck1* expression post ethanol treatment (350mM), salubrinal treatment (50µM), tunicamycin treatment (2.5µg/mL), and thapsigargin treatment (1µM).

Phenotypic Analysis of Zebrafish Livers for Steatosis

Fatty liver disease is characterized by the accumulation of excess lipids in the liver. Zebrafish larvae are optically clear, allowing for liver tissue to be stained and then imaged. Additionally, zebrafish larvae develop livers by 3 dpf. Oil Red O staining allows for lipids to be visualized with ease. It is of interest to see how various ER stressors and GADD34/CReP inhibitors may affect development of FLD. Initially, experiments were conducted on 4 dpf larvae, but the yolk sac (located to the left of the liver) obscured the liver, which made it difficult to detect steatosis (Figure 15).

Figure 15.

4dpf Zebrafish Larvae Stained with Oil Red O After 24 hr Drug Treatments



Note. 4 dpf zebrafish larvae were treated with ethanol, salubrinal, tunicamycin, and thapsigargin for 24 hours then stained with Oil Red O. The liver is outlined in black and lipid droplets are stained red. **A.** 4 dpf WT larvae treated with ethanol for 24hrs. **B.** 4 dpf WT larvae treated with salubrinal for 24 hrs.

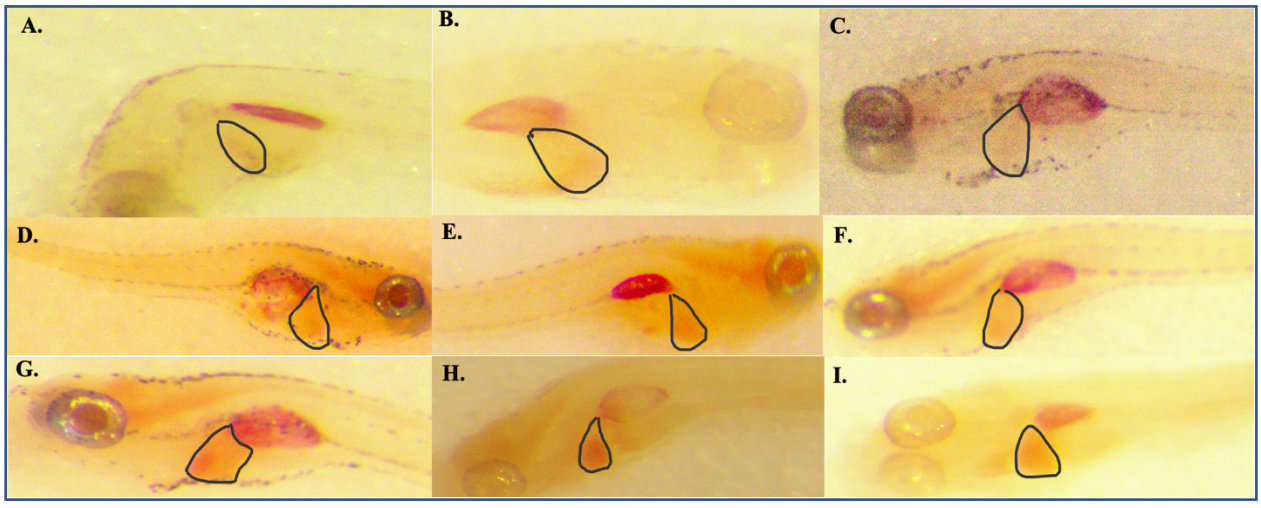
(Figure 15 Continued)

C. 4 dpf WT larvae treated with thapsigargin for 24 hrs. **D.** 4 dpf WT larvae treated with tunicamycin for 24 hrs.

Experiments were instead performed on larvae that were 7 dpf, as the yolk dissipates by then, allowing for more easily scorable larvae (Jardine and Litvak, 2003). The larvae were scored as having "steatosis" or "no steatosis", but they were additionally categorized with a third "mild steatosis" category to allow for more nuance in the categorical analysis of the effects of different drugs (Figures 16-17).

Figure 16.

Scoring Rubric of 7dpf Zebrafish Larvae Stained with Oil Red O after 4hr and 24hr Drug Treatments.



Note. Scoring rubric of 7dpf zebrafish larvae stained with Oil Red O after 4hr and 24hr drug treatments. 7dpf zebrafish larvae were treated with DMSO, ethanol, salubrinal, tunicamycin, or thapsigargin for 4 hours or 24 hours then stained with Oil Red O. The liver is outlined in black

(Figure 16 Continued)

and lipid droplets are stained red. **A-C.** 7dpf WT larvae scored as having no steatosis. **D-F.** 7dpf WT larvae scored as having mild steatosis. **G-I.** 7dpf WT larvae scored as having severe steatosis.

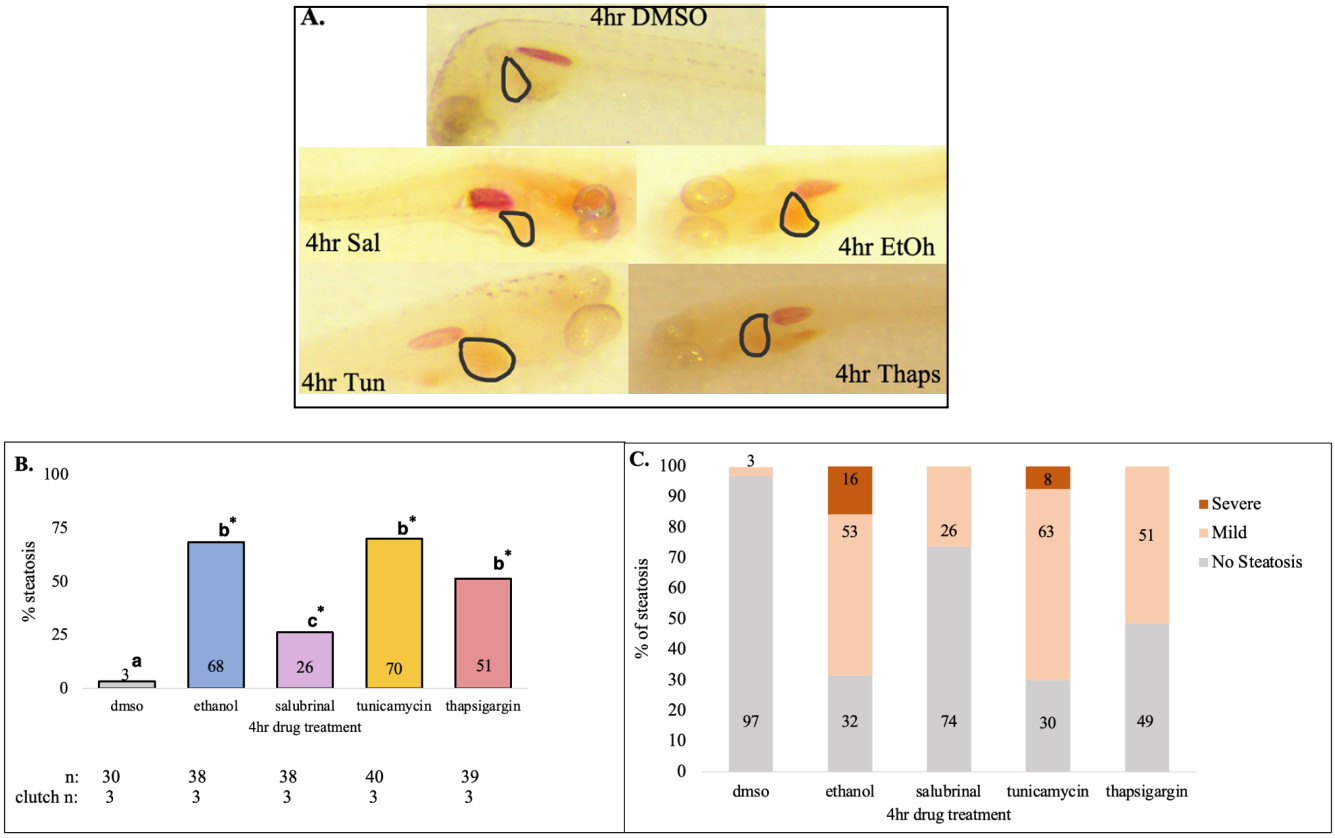
Treatment with 4hrs of DMSO did not induce steatosis in any 7dpf larvae other than one outlier (Figure 17B-C). Ethanol treatment for 4hrs caused 68.4% of all larvae to develop steatosis, a significantly higher fraction of embryos to develop steatosis compared to DMSO ($p= 0.0087$) (Figure 17B). Among the ethanol treated larvae, 52.6% of them demonstrated mild steatosis, and 15.8% of them developed severe steatosis (Figure 17C). After 4hrs of salubrinal treatment, 26.3% of larvae developed fat droplets in the liver, which is significant compared to DMSO treatment ($p= 0.017$). All salubrinal treated larvae demonstrated mild steatosis, but none showed severe steatosis (Figure 17C). Tunicamycin treatment for 4hrs caused the most robust phenotype, with 70% of all treated larvae exhibiting steatosis, an extremely significant increase in comparison to DMSO treated larvae ($p < 0.0001$) (Figure 17B). Of the tunicamycin treated larvae with steatosis, 62.5% had mild steatosis and 7.5% had severe steatosis (Figure 17C). After 4hrs of thapsigargin treatment, 51.3% of all larvae developed steatosis, a significant difference in phenotype compared to DMSO treatment ($p < 0.0001$); all had mild steatosis (Figure 17B-C).

After 24hrs of DMSO treatment, only 5.6% of embryos developed mild steatosis (Figure 18B-C). Ethanol treatment for 24hrs caused steatosis in 83% of all treated larvae, which is a significant increase compared to DMSO treated larvae ($p < 0.0001$); 51% of the larvae demonstrated mild steatosis and 31% showed severe steatosis (Figures 18B-C). Salubrinal treatment resulted in 81% of livers having steatosis, which is significant when compared to

larvae treated with DMSO ($p < 0.0001$), with 47% of them showing mild steatosis and 34% developing severe steatosis (Figures 18B-C). Tunicamycin resulted in the most steatosis, with 97% of larvae showing steatosis, with 41% having mild steatosis and 57% with severe steatosis; this was a significant increase compared to DMSO treated larvae ($p < 0.0001$) (Figures 18B-C). Thapsigargin resulted in 76% of all samples having steatosis, 47% of which were mild and 29% being severe; this treatment also resulted in significantly higher amounts of steatosis in comparison to DMSO treated larvae ($p < 0.0001$) (Figures 18B-C).

Figure 17.

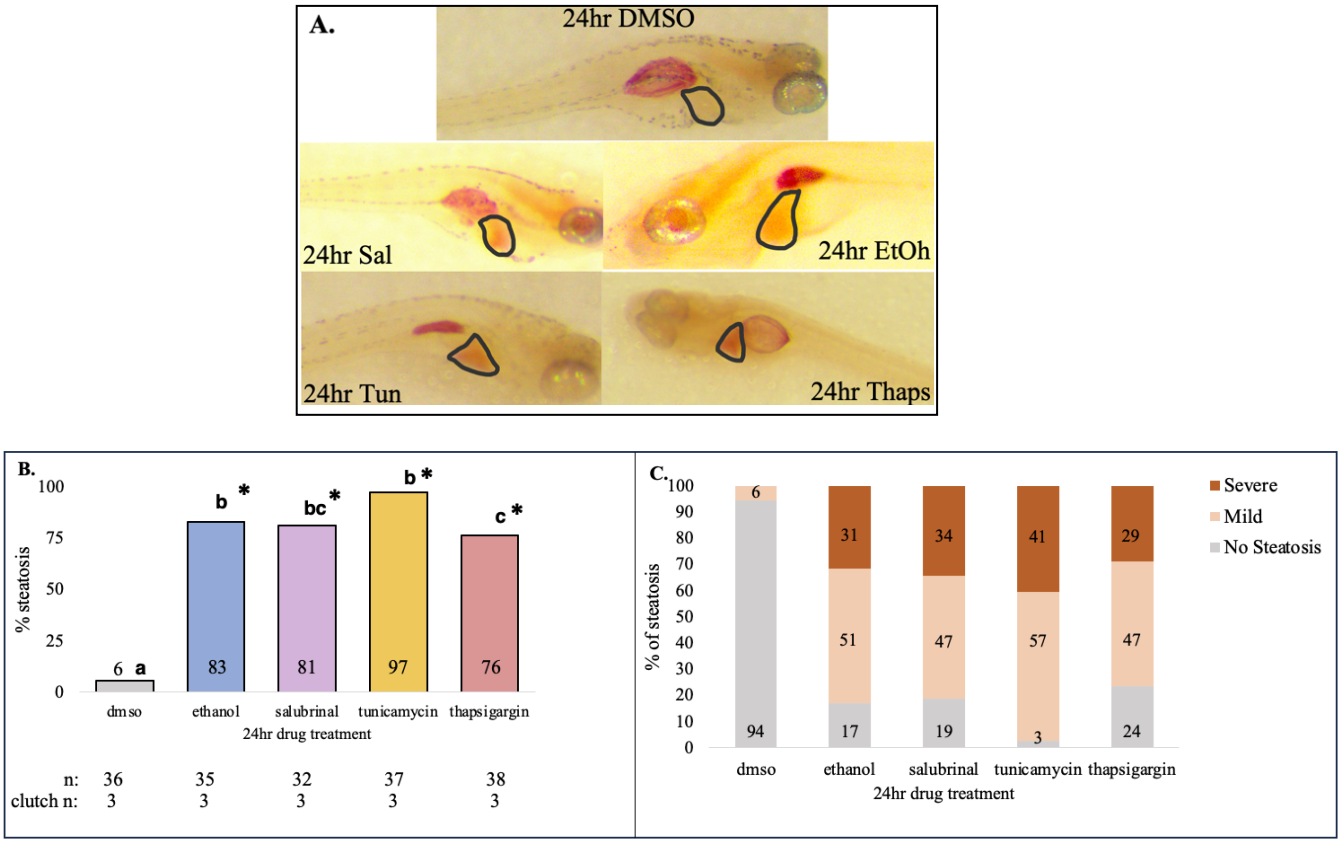
7dpf Zebrafish Larvae with Steatosis After 4hr Drug Treatments



Note. 7dpf zebrafish larvae were treated with DMSO (control), ethanol, salubrinal, tunicamycin, or thapsigargin for 4 hours, then stained with Oil Red O. **A.** 7dpf larvae stained with Oil Red O after being treated with either DMSO (control), ethanol, salubrinal, tunicamycin, or thapsigargin for 4hrs. The liver is outlined in black. **B.** The larvae livers were qualitatively analyzed and scored as having steatosis or no steatosis. Letters indicate significant differences between treatment groups. Fisher's Exact Test was performed to determine statistical significance compared to DMSO treatment ($p < 0.05$, denoted with *). "Clutch n" and "n" are in reference to the number of clutches and the total number of larvae scored. **C.** The same 7dpf 4hr drug treated larvae from figure 17B were scored into three categories: no steatosis, mild steatosis, or severe steatosis, based on the intensity of the color of stained livers.

Figure 18.

7dpf Zebrafish Larvae with Steatosis After 24hr Drug Treatments



Note. 7dpf zebrafish larvae with steatosis after 24hr drug treatments. 7dpf zebrafish larvae were treated with DMSO (control), ethanol, salubrinal, tunicamycin, or thapsigargin for 24 hours then stained with Oil Red O. **A.** 7dpf larvae stained with Oil Red O after being treated with either DMSO (control), ethanol, salubrinal, tunicamycin, or thapsigargin for 24hrs. The liver is outlined in black. **B.** The larvae livers were qualitatively analyzed and scored as having steatosis or no steatosis. Letters indicate significant differences between treatment groups. Fisher's Exact Test was performed to determine statistical significance compared to DMSO treatment ($p < 0.05$, denoted with *). "Clutch n" and "n" are in reference to the number of clutches and the total number of larvae scored. **C.** The same 7dpf 24hr drug treated larvae from figure 17B were scored

(Figure 18 Continued)

into three categories: no steatosis, mild steatosis, or severe steatosis, based on the intensity of the color of stained livers.

CHAPTER 4: DISCUSSION

The UPR plays a significant role in promoting cell survival or cell death under stressful conditions, which may be protective or detrimental during disease conditions (Wang and Kaufman, 2016). There is an apparent link between UPR activation and steatosis, but it is not clearly defined at this time. In order to better understand the role of UPR activation in the development of fatty liver disease, zebrafish larvae were treated with known pharmacological inducers of ER stress and a pharmacological inhibitor of the regulatory proteins GADD34 and CReP for various durations, and the expression of genes encoding ER chaperones, components of the UPR network, and lipogenesis enzymes were quantified. In addition, the effects of these manipulations on development of steatosis were determined using lipid staining and phenotypic analysis of treated embryos. The results from this study demonstrated that chronic (24 hr) exposure to ethanol, salubrinal, tunicamycin, and thapsigargin induce steatosis, with both distinct and overlapping patterns of gene expression changes in response to these treatments.

Alcohol is a known inducer of fatty liver as it is primarily metabolized in hepatocytes, and excess consumption of it is correlated with the activation of the UPR, thus ethanol was used in this research as a positive control in both gene expression and phenotypic analysis experiments (Ji 2012; Howarth et. al, 2014). Tunicamycin and thapsigargin are drugs commonly used as ER stressors to induce the UPR. Their mechanisms of action vary – tunicamycin inhibits the N-glycosylation of proteins, which results in the accumulation of several misfolded proteins in the ER (Breitling and Aebi, 2013). Thapsigargin depletes the ER of calcium through blocking the SERCA pump, which severely affects molecular chaperone proteins that are dependent on

calcium to function (Lytton et al., 1991). Salubrinal is a drug that inhibits GADD34 and CReP from complexing with PP1, which prevents the dephosphorylation of eIF2α (Boyce et al., 2005).

It was hypothesized that ER regulator and chaperone genes would increase in expression from exposure to ER stressors, tunicamycin and thapsigargin. This study demonstrated the following genes to primarily not have statistically significant changes in expression following acute (6 hrs) or chronic (24 hrs) treatment with ethanol, tunicamycin, or thapsigargin: *derl1*, *calret*, *edem1*, and *hsp90b1*. Given the mechanism of thapsigargin blocking the SERCA pump, depleting the ER of calcium, it was expected that *calret* would be upregulated in response to thapsigargin. A limitation of this study was that there were only two experiments completed to measure the expression of these genes during chronic stress – more replicates should be completed, and drug concentrations could potentially be modified to better understand whether these treatments affect their expression. Salubrinal only significantly influenced *hsp90b1*, causing it to decrease in expression after chronic treatment; while the outcome is understood, the exact mechanism of this effect of salubrinal is unknown, but it does not inherently cause ER stress. A different study demonstrated increased expression of *hsp90b1* to be associated with poor survival in patients with hepatitis-B associated HCC (Yang et al., 2015).

Dichotomous Roles of the UPR in FLD

A study by Vacaru et al. (2014) demonstrated the activation of the UPR from stress conditions in zebrafish larvae to positively correlate with the occurrence of steatosis. This same study revealed a difference between an “adaptive” and “stressed” UPR: when larvae are initially exposed to thapsigargin treatments and then to tunicamycin for acute durations, results have shown the UPR to be adaptive and to lower the incidence of steatosis (Vacaru et al., 2014). Meanwhile, treatments only consisting of tunicamycin in the Vacaru et al. study demonstrated

robust activation of the UPR which resulted in severe steatosis. An adaptative UPR allows for protein translation to resume, whereas the stressed UPR model induces apoptotic pathways. A study by Howarth et al. (2014) demonstrated the upregulation in expression of UPR effector genes from the livers of 5dpf and 14dpf transgenic zebrafish with overexpressed nAtf6 – they replicated this same experiment in WT zebrafish larvae exposed to ethanol and found similar results in gene expression, with both zebrafish systems developing steatosis.

ATF6 and SREBPs have been demonstrated to be cleaved and activated upon ER stress by the same proteases; it is of interest to determine whether any of the UPR branches may drive the activation of SREBPs during FLD (Ye et al., 2000; Howarth et al., 2014). The study by Howarth et al. (2014) demonstrated *atf6* to have an epistatic interaction not with *srebp1* or *srebp2*, but with *fasn* in zebrafish livers. Atf6 in zebrafish has demonstrated dichotomous roles in FLD as well – during chronic stress, a lack of Atf6 prevents steatosis, but during acute ER stress, its activity can prevent steatosis (Cinaroglu et al., 2011). It is of interest to see how the expression of *atf6* is influenced by not just ER stressors, but salubrinal as well. The results from this thesis demonstrated *atf6* expression to not significantly change in response to chronic durations of treatment ER stressors, but it was found to decrease in expression from salubrinal treatment compared to DMSO. Seeing this downregulation of *atf6* expression may partially explain the worsening of steatosis from salubrinal treatments. Salubrinal is an eIF2α phosphatase inhibitor, and these results with this treatment increasing *atf6* expression indicate crosstalk between the UPR branches, which has previously been documented, but is not fully defined (Walter et al., 2018). The Howarth et al. (2014) study demonstrated ATF6 to serve as a key regulator of steatosis, and it is interesting and novel to see salubrinal decrease its expression.

The following genes are implicated in lipid metabolism but did not change in expression in response to drug treatments for 24 hrs: *srebp1*, *srebp2*, *fasn*, *pklr,* and *pck1*. It was expected that *fasn* would increase in expression as a response to ethanol, but the 2.5 fold increase compared to DMSO was not significantly different. Additionally, given that *fasn* has been implicated to be driven in expression from *atf6*, it is interesting to see that salubrinal did not impact its expression.

This study's results did not indicate significant changes in expression in *pppr1r15a* or in *ppp1r15b* from salubrinal or ER stress inducing treatments. While salubrinal works to inhibit GADD34 and CReP from dephosphorylating eIF2α, the mechanism of action of salubrinal is currently unknown; cellular insults are at times ameliorated from salubrinal treatments while exacerbated among different cell types (Matsuoka and Komoike, 2015). Results of Oil Red O staining in 7 dpf zebrafish larvae indicated that there is significant presence of steatosis in the liver upon treatment with salubrinal; 81% of all larvae were detected to have steatosis after 24hr treatments of salubrinal. In metabolic diseases, inhibiting the activity of eIF2α phosphatases may worsen clinical outcomes. A study in GADD34-deficient mice by Nishio and Isobe (2015) demonstrated these mice to develop obesity, NAFLD, and insulin resistance over the course of their lives; their study also revealed young GADD34-deficient mice that were fed high fat diets to have high levels of steatosis in comparison to wild type (WT) mice.

A previous study conducted in the Weiser lab revealed that during acute ER stress, GADD34 activity is protective against apoptosis in caudal fin cells in zebrafish embryos, while knocking GADD34 out during chronic ER stress led to a protective response in these cells (Hicks 2019). A study by Wang et al. (2019) demonstrated that salubrinal ameliorates neural defects in mice, whereas blocking eIF2α phosphatases worsens FLD. It is clear that the impacts

of GADD34 and the activity of eIF2α phosphatases vary depending on tissue type and the duration of cellular stress. Much remains unclear about the activation of the other major eIF2 phosphatase, CReP. A separate study from the Weiser lab revealed in 1dpf zebrafish larvae that the expression of GADD34 and CReP genes both increased as a response to tunicamycin and thapsigargin treatment; this study later revealed CReP expression to decrease by chronic stress, whereas GADD34 remain elevated (Giresh 2022). The lack of significant changes in gene expression in UPR effector genes in response to ER stressors in this study may be potentially due to the UPR oscillating between activation and deactivation (Gomez and Rukowski, 2016). It must be noted that these results with salubrinal are preliminary in nature and should be repeated using a combinatorial drug treatment that induces ER stress.

Increased cell death in hepatocytes and pancreatic-β cells is a critical mechanism of inducing NAFLD (Alkhouri et al., 2011). Other studies in mice have indicated the deletion of ATF4 and CHOP to reduce disease outcomes in the liver from excess ethanol (Ji et al., 2005; Magne et al., 2011). The PERK pathway is suggested to be activated by ethanol, with PERK proteins detected and *ATF4* upregulated during alcoholic steatohepatitis in mice (Hao et al., 2021). The findings from this study did not indicate significant changes in expression of *perk* or *atf4* from salubrinal, thapsigargin, or tunicamycin in 4dpf zebrafish larvae, but *perk* did increase in expression significantly from chronic ethanol treatments. Only two experiments were conducted analyzing *perk* expression; salubrinal treatment was indicating an increase in *perk* expression, but these results were only approaching significance ($p= 0.073$), and more repeat experiments should be conducted. The effects of drug treatments on the PERK pathway have not been properly evaluated through gene expression studies, as much of this pathway is influenced by post-translational phosphorylation. *Ddit3* (CHOP) did not change in expression compared to

ethanol, salubrinal, and tunicamycin treatments. CHOP is the key downstream apoptotic target of the PERK pathway, and its activation may be influential on the liver phenotype, worsening steatosis; these results are atypical, and should be replicated after longer durations of treatments to determine whether *ddit3* has a role on steatosis in zebrafish.

From the IRE1a pathway, *ire1a* was surprisingly shown to decrease in expression in response to chronic thapsigargin treatment, which was not anticipated given the ER stress inducing nature of the drug; salubrinal did decrease the expression as well, compared to DMSO. Downstream in this branch, *xbp1* expression decreased from salubrinal, tunicamycin, and thapsigargin. A study done in IRE1a deficient mice indicated worsening steatosis; the expression of this branch of the UPR works to maintain lipid homeostasis as IRE1a degrades miRNAs that induce lipid metabolism (Wang et al., 2018).

In this study, both acute and chronic durations of tunicamycin and thapsigargin treatment caused steatosis in the majority of zebrafish larvae, while salubrinal was shown to induce steatosis primarily from chronic durations of treatment. The results from salubrinal treatment indicate that blocking eIF2α phosphatase activity can worsen FLD in zebrafish livers.

Future Studies

The detection of steatosis from all of the treatments administered is indicative that genes responding to ER stress in the UPR play a role in affecting lipid metabolism. This study provided information about the expression of UPR genes in zebrafish larvae; more studies must be done to definitively understand the role of regulatory proteins in this pathway exacerbating steatosis. There are several directions that can be taken for future studies.

Oil Red O analysis was completed on wild-type zebrafish with pharmacological agents; more data from mutant GADD34 and CReP zebrafish that are exposed to ER stressors and are

phenotypically and molecularly analyzed may be insightful to understand how this pathway functions-- this data can then be compared to the wild-type results from this study to see if there are significant differences between them. If there are, these studies may provide definitive proof that GADD34 exacerbates steatosis in zebrafish livers; differences between GADD34 and CReP may also be detected during the context of disease. Heterozygous and homozygous mutant zebrafish lines could be used for this, as well as the usage of morpholinos designed against these regulatory proteins; these results can be used to compare against results from salubrinal usage. Phenotypic analysis should also be conducted after prolonged stress treatments of a minimum of 48hrs.

More RT-qPCR can be performed to better understand the expression of UPR and lipogenic genes—genes from both of these categories could be studied at more chronic time-points, such as 48 hrs, to continue understanding the effects of prolonged stress on the liver. It may also be interesting to dissect the livers from 4dpf-7dpf zebrafish for data regarding gene expression to see the differences in expression from just the liver versus the entire larvae. Additionally, gene expression should be measured after performing combinatorial drug treatments with salubrinal and ER stressors to see if GADD34 and CReP expression are reduced by salubrinal when the UPR is robustly activated – a study by Vacaru et al. (2014) demonstrated activated UPR to either induce or protect against steatosis, depending on the activity of different target genes. Combination drug treatments are protective in caudal fin cells, but may worsen the phenotype in the liver. Other drugs of interest that should be used in future studies include ISRIB, a different eIF2α phosphatase inhibitor, and Sephin, a drug that solely targets and inhibits GADD34 activity (Tsytler et al., 2011; Das et al., 2015). Several results in this study using thapsigargin did not shift gene expression when it was hypothesized that expression should

increase; repeat experiments should be conducted with fresh thapsigargin on the possibility of the stock being used was faulty, and incapable of inducing ER stress adequately.

If GADD34/CReP are knocked out, which could be done with morpholinos injected in zebrafish embryos, it is of interest to determine if the lack of these regulatory proteins worsen the effects of stress or ethanol, and how these knockouts may influence gene expression patterns.

Fatty liver disease has no cure outside of changes in lifestyle during the beginning stages of this disease, which may be unsustainable for many populations of people for a wide variety of reasons with emphasis on sociopolitical factors and less so from individual behavior (Saklayen, 2018). This disease and other metabolic diseases are steeply rising in incidence globally. Understanding the exact mechanisms behind the UPR affecting lipogenesis may lead to the development and regulation of therapeutics that target this pathway.

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