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Characterization of the roles of PPP1R15A (GADD34) and PPP1R15B (CReP) in ER stress-induced apoptosis in vivo

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CHARACTERIZATION OF THE ROLES OF PPP1R15A (GADD34) AND PPP1R15B (CReP) IN ER STRESS-INDUCED APOPTOSIS IN VIVO

by

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DEDICATION

This thesis is dedicated to my family: my grandfather, grandmother, mother, and husband. My grandfather, who suffered through his last decade in this world with dementia, gifted me with his sense of humor, gentleness, love for music and God, and ultimately inspired me to pursue biology as I watched him decline from neurodegeneration. I could not be more grateful to contribute to science, even in this small way. My grandmother, who taught me that I could indeed do hard things and who I believe, given a different set of circumstances, would have gone far academically with her sharp mind and work ethic. My mom, who is always, always there for me and has given me more than I could ever list on this page. And my husband, who encourages me to grow always and loves endlessly; thank you for cheering me on in every step and in every pursuit.

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CHARACTERIZATION OF THE ROLES OF PPP1R15A (GADD34) AND PPP1R15B (CReP) IN ER STRESS-INDUCED APOPTOSIS IN VIVO

Abstract

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Failure to balance synthesis, folding and degradation of secreted proteins results in the accumulation of unfolded proteins in the endoplasmic reticulum, termed ER stress. Cells respond to ER stress by activating a signaling pathway known as the unfolded protein response (UPR). The UPR induces phosphorylation of eIF2α to attenuate global protein translation, allowing the ER to clear misfolded proteins. Opposing this function, eIF2α phosphatases contain a catalytic subunit, Protein Phosphatase 1, and either of two homologous scaffolding subunits, GADD34 and CReP. Inhibition of eIF2α phosphatases has been shown to prolong UPR signaling and promote survival in many cells types and has reduced the progression of neurodegenerative diseases in several mouse models. Despite the clinical significance of GADD34 and CReP, their precise roles in UPR signaling and ER stress-induced apoptosis are largely unknown. Zebrafish are an ideal model for studying eIF2α phosphatases in the UPR due to the efficiency of reverse genetics and the susceptibility of the caudal fin epidermal cells to ER stress-induced apoptosis. In acute ER stress, these cells undergo apoptosis mediated by p63-puma, but under chronic stress, apoptosis is mediated by CHOP, a downstream UPR target. We sought to determine the role of eIF2α phosphatases in both apoptotic programs by inducing eIF2α phosphatase loss- or gain-of-function and measuring the effect on apoptosis in response to

ER stress. Inhibition of both eIF2α phosphatases protected cells against both apoptotic responses, primarily through GADD34 inhibition. We speculate that survival is promoted through enhanced eIF2α phosphorylation, stalling the UPR in an early state and preventing accumulation of the late-stage target, CHOP. GADD34 overexpression protected cells against acute apoptosis, which we hypothesize is through a transient promotion of autophagy. Our results indicate that altering the influence of eIF2α phosphatases in the UPR may be a promising therapeutic for diseases exacerbated by ER stress.

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The Endoplasmic Reticulum

In all eukaryotic cells, the endoplasmic reticulum (ER) serves the important roles of protein translation, folding and processing of all secretory and membrane-bound proteins and calcium storage (Rao et al., 2004). The ER processes approximately one-third of the cell's proteins, so cells must regulate protein homeostasis in the ER, termed proteostasis, by controlling and balancing protein synthesis, assembly, trafficking, folding, and degradation (Wang et al., 2016). The ER must maintain a high level of quality and efficiency in protein folding for cells to survive and function normally, but because of the complex and intricate nature of protein folding, it is the most error-prone step in the expression of any particular gene (Wang et al., 2016). The ER facilitates protein folding by its oxidizing environment, which allows disulfide bond formation, and a high Ca^{2+} concentration, which acts as a folding buffer and is essential to chaperone protein function. Cells also must maintain a large amount of energy and nutrients to support the energy-demanding process of protein folding in order to maintain proteostasis. A cell's protein folding efficiency in the ER is incredibly sensitive to changes in intracellular environments or extracellular stimuli, and alterations that affect ER homeostasis can result in the accumulation of misfolded proteins.

Newly synthesized polypeptides enter the ER in an unfolded state through specialized ER membrane sites, translocons, which are complexes of ER membrane proteins that form a pore (Schnell et al., 2003). Inside the ER, protein folding is facilitated by resident chaperones, which are enzymes that catalyze protein-folding reactions or stabilize proteins in a state competent for folding (Gething and Sambrook, 1992 and Dill and Chan, 1997). Proteins that are correctly

folded and modified into their mature conformation are permitted to move through the secretory pathway; they are transported to the Golgi apparatus, where they are either sorted to be sent to various cellular compartments, recycled back to the ER, or secreted into the extracellular space (Alberts et al., 2002).

ER Stress

ER stress occurs when the load of proteins to be folded in the ER exceeds the chaperone capacity to accurately process the proteins, resulting in increased accumulation of misfolded proteins in the ER lumen (Ron and Walter, 2007). Protein misfolding negatively impacts cell function because necessary proteins are not able to leave the ER and perform their normal functions (Aridor and Balch, 1999). ER stress activates signal transduction pathways that cause a complex, multi-branched response in transcription and translation, known as the Unfolded Protein Response (UPR). Overall, the UPR leads to increased chaperone activity, decreased protein synthesis, and increased protein degradation by ER-associated protein degradation (ERAD). ER stress can be caused by a variety of intrinsic and extrinsic factors, including increased levels of protein synthesis, impaired ubiquitination and proteasomal degradation, deficient autophagy, energy deprivation, excessive or limited nutrients, dysregulated calcium levels or redox homeostasis, inflammation, and hypoxia (Wang et al., 2016).

The Unfolded Protein Response

Three UPR signaling pathways have been characterized that sense and respond to ER stress. The most conserved is the IRE1-XBP1 pathway (inositol-requiring protein 1α- X-box-binding protein 1) (Tirasophon et al., 1998). In this branch of the UPR, when the load of unfolded proteins in the ER lumen exceeds ER folding capacity, the protein-folding chaperone BiP (binding immunoglobulin protein) detaches from IRE1, which is a trans-ER membrane

bound protein. BiP detachment results in trans-autophosphorylation of the IRE1 homodimer, activating IRE1 endonuclease activity (Sidrauski and Walter, 1997). IRE1 can then unconventionally splice XBP1 mRNA, altering the reading frame for XBP1 translation which results in the synthesis of a transcription factor. The XBP1 transcription factor is highly active and activates target genes that encode protein chaperones and components of ERAD (Kopito, 1997; Travers et al., 2000). IRE1α also cleaves some mRNAs that contain XBP1-like secondary structures to contribute to mRNA degradation in a process known as regulated IRE1α-dependent decay (RIDD) (Hollien et al., 2009). In this process, mRNAs encoding transmembrane and secretory proteins are targeted to reduce ER stress by decreasing the incoming protein load.

The next UPR pathway is that of activating transcription factor ATF6. The ATF6 protein is also a trans-ER membrane protein, and upon BiP dissociation, it is translocated to the Golgi apparatus where it is cleaved to become an active transcription factor (Haze et al., 1999). ATF6α then goes on to the nucleus to activate the transcription of various genes involved in ER stress recovery, including the XBP1 gene, genes encoding protein chaperones such as BiP, ER protein 57 (Erp57), and glucose-regulated protein (GRP)94, and genes for ER-associated protein degradation functions (Yoshida et al., 2001; Wang et al., 2016).

The third pathway of the UPR is mediated by PKR-like ER kinase (PERK), which attenuates global protein translation to decrease the rate of proteins entering the ER. The global downregulation of protein synthesis conserves cellular resources and provides time to evaluate the severity of the protein misfolding and then reprograms gene expression either toward a pro-survival repair response or towards apoptosis when ER stress is continuous or severe (Andreev et al., 2015).

The PERK Branch of the UPR

The branch of the UPR mediated by PKR-like ER-associated kinase (PERK) is the focus of this research. Overall, this branch works to inhibit global protein translation through the phosphorylation of eukaryotic translation initiation factor 2 (eIF2α). PERK is an ER-resident transmembrane kinase that is inactive during unstressed conditions through its association with BiP. Upon BiP dissociation, PERK oligomerizes, autophosphorylates, and then phosphorylates the α subunit of eIF2 at serine 51. Phosphorylation of eIF2α inhibits the activity of guanine nucleotide exchange factor, eIF2B, which is required for the formation of eukaryotic translational preinitiation complexes (Hinnebusch, 2000; Pavitt et al., 2012). This therefore inhibits the initiation step of protein synthesis across all transcriptomes and decreases the amount of mRNA entering the ER (Clemens, 1996). This transient reduction in the overall amount of proteins in the ER gives stressed cells an opportunity to clear misfolded proteins from the ER and protects cells against the potential toxicity of misfolded proteins (Brostrom and Brostrom, 1998).

During normal protein translation, for the 40S ribosome to recognize a start codon, eIF2 forms a ternary complex with GTP and Met-tRNAi which is loaded onto the 40S ribosome to form a 43S ribosome. This ribosome binds the 5' end of mRNA transcripts to initiate scanning downstream. After a start codon is recognized, eIF2-mediated GTP hydrolysis occurs and eIF2*GDP is released. For another initiation of translation to occur, eIF2B, a guanine exchange factor (GEF), then exchanges the GDP for GTP. When eIF2α is phosphorylated on serine 51, it forms a stable complex with eIF2B and increases the affinity of eIF2 for GDP, rapidly reducing the pool of active eIF2B, thus resulting in the general inhibition of protein synthesis

(Hinnebusch, 2014). Although phosphorylation of eIF2α acts to attenuate global protein synthesis, it selectively allows for more efficient translation of mRNAs that encode for stress-related proteins (Reid et al., 2016). Certain mRNA transcripts show favored translation in the state of reduced functional translation initiation complexes, typically those with short upstream open reading frames (uORFs) in their 5' untranslated region (Andreev et al., 2015). One such protein that becomes upregulated after eIF2α phosphorylation is activating transcription factor 4 (ATF4), which increases the capacity for protein transport in the ER and promotes the synthesis of multiple pro-survival genes (Harding et al., 2000). ATF4 enters the nucleus to activate UPR genes encoding proteins necessary for autophagy, the antioxidant response, amino acid biosynthesis and transport, increased protein chaperones, and the promotion of ERAD, which are hallmarks of the early UPR response (Harding et al., 2003; Han et al., 2013). ATF4 activates the transcription of the growth arrest and DNA damage-inducible protein 34 (GADD34; also known as PPP1R15A), which serves to promote eIF2α dephosphorylation and restore global protein translation. ATF4 also promotes the transcription of CHOP (CCAAT/enhancer-binding protein "C/EBP" homologous protein) by binding to and activating the CHOP promoter (Fawcett et al., 1999; Harding et al., 2000; Ma et al., 2002; Marciniak et al., 2004). CHOP expression is low at basal levels but is robustly induced in response to stress and is required for ER stress-induced apoptosis under conditions of chronic stress. In response to ER stress, Pyati et al. (2011) found CHOP to mediate a later-phase apoptotic pathway distinct from an acute cell death response mediated by p63 and puma. The PERK signaling pathway is therefore capable of promoting both an adaptive, pro-survival response, and an apoptotic response to ER stress. The severity and context of the ER stress, duration of UPR signaling, and cell type and environment are all

factors that contribute to the outcome of PERK signaling. The PERK pathway is outlined in Figure 1.

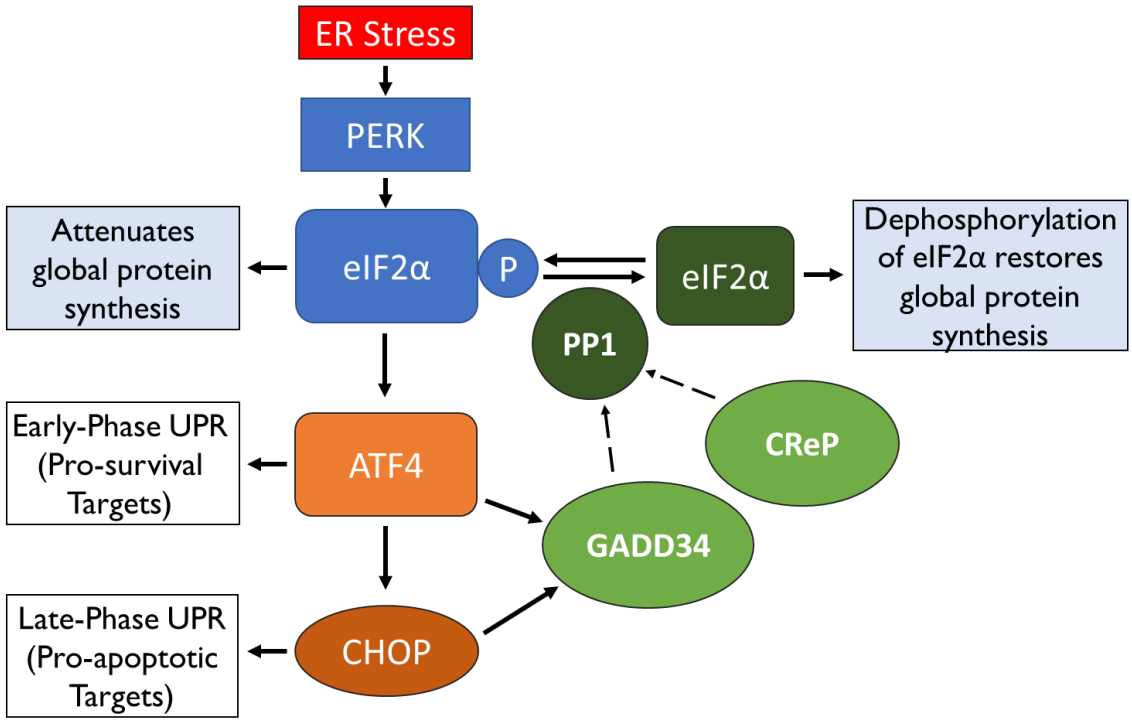

Figure 1: Model of the PERK pathway of UPR signaling. In response to ER stress, the phosphorylation of eIF2α by PERK results in global protein translation attenuation, and the upregulation of a pro-survival gene cascade through ATF4 activation. ATF4 also promotes CHOP activation, which can lead to a pro-apoptotic pathway in conditions of prolonged ER stress. GADD34 and CReP are two targeting subunits that interact with PP1 to dephosphorylate eIF2α, with GADD34 being a potent feedback inhibitor of PERK signaling.

Although CHOP accumulation correlates with apoptosis, both ATF4 and CHOP mRNAs and proteins have short half-lives, so strong and chronic PERK activation is necessary to promote high levels of CHOP (Wang and Kaufman., 2014). The human CHOP protein contains transcriptional activation/repression domains at its N-terminus and a C-terminal basic-leucine zipper (bZIP) domain containing a DNA-binding region followed by a leucine zipper motif for dimerization (Oyadomari and Mari, 2004; Ron and Habener, 1992; Ubeda, 1996). CHOP's bZIP

domain allows it to form heterodimers with other C/EBPs and another bZIP group, the CREB/ATF family, and has been reported to be required for processes in CHOP-induced apoptosis (Matsumoto et al., 1996; Maytin et al., 2001). In addition to acting as a transcription factor, CHOP also initiates cellular effects through protein-protein interactions (Oyadomari and Mari, 2004). The precise mechanism(s) by which CHOP initiates its apoptotic response is not fully known, but many studies have found various ways in which CHOP sensitizes ER-stressed cells to apoptosis. CHOP plays a role in inducing GADD34 expression, potentially through direct binding to the GADD34 promoter (Marciniak et al., 2004). Through GADD34 activation, CHOP promotes the reversal of the translational repression initiated by the PERK pathway. Increased protein synthesis can contribute to the ER stress of the cell and predispose cells to CHOP-mediated apoptosis in conditions of continuous or severe ER stress (Han et al., 2013). CHOP is involved in significantly disrupting redox homeostasis, which prepares stressed cells for apoptosis (McCullough et al., 2001; Marciniak et al., 2004). CHOP has been found to deplete cells of glutathione, an important intracellular scavenger of ROS (McCullough et al., 2001), and a direct target gene of CHOP, ERO1α, promotes oxidizing conditions in the ER and an accumulation of reactive oxygen species, contributing to protein misfolding and death in stressed cells (Marciniak et al., 2004). CHOP has also been found to sensitize cells to ER stress-induced apoptosis through inhibiting transcription of the anti-apoptotic protein Bcl2, upregulating the pro-apoptotic protein Bim, promoting translocation of the pro-apoptotic effector protein Bax from the cytosol to the mitochondria, and targeting CDK2 and CDK4 for degradation to cause cell growth arrest (McCullough et al., 2001, Wang et al., 2001). However, studies by Han et al. did not detect either CHOP or ATF4 to occupy the promoter regions of any pro-apoptotic genes upon ER stress induction in mouse embryonic fibroblast cells (MEFs).

Rather, ATF4 and CHOP were seen to form heterodimers that upregulate genes involved in the UPR for functions like autophagy, mRNA translation, protein synthesis, ATP depletion, and oxidative stress. These data suggest that cell death is indirectly promoted by CHOP through its primary role of supporting the restoration of protein synthesis.

Translational resistance to ER stress. The increased translation of certain mRNAs during ER stress has not been found to be based on the number, organization, depth, or specific sequence of the uORFs in the 5' leaders, and instead appears to utilize diverse mechanisms in each gene transcript (Andreev et al., 2015). Two main mechanisms have been proposed: first, alleviation of scanning ribosome obstruction, in which a weak kozak context of an uORF allows for scanning ribosomes in conditions of high initiation rates to obstruct the start site of the CDS (proposed for CHOP), and second, delayed reinitiation, where the slower loading of the ternary complex on the 40S ribosome during ER stress promotes some uORFs to be scanned past, making reinitiation more likely to occur at later uORFs (attributed to ATF4) (Palam et al., 2011; Hinnebusch, 1997).

eIF2α Phosphatases

The activity of PERK is opposed by eIF2α phosphatases, which are multi-subunit proteins that complex with Protein Phosphatase 1 (PP1), the catalytic subunit, and either of two homologous scaffolding subunits, GADD34 or CReP (constitutive repressor of eIF2α phosphorylation; also known as PPP1R15B) (Connor et al., 2001; Jousse et al., 2003). These protein phosphatases act to re-initiate protein synthesis upon ER stress recovery, but the dynamics of their specific roles and interactions in this process are still poorly understood. The primary goal of this research is to further the understanding of the unique and similar roles of GADD34 and CReP in response to ER stress *in vivo*.

Growth arrest and DNA damage-inducible protein 34, GADD34, acts as a negative feedback inhibitor of UPR signaling through interaction with PP1 to dephosphorylate eIF2α (Novoa et al., 2001). Induction of GADD34 expression by ER stress is dependent on the activity of PERK, similar to ATF4 (Novoa et al., 2001). Human GADD34 is a 674 amino acid PP1 regulatory subunit that directs PP1 substrate recognition and localization to the ER following ER stress (Brush et al., 2003). To promote eIF2α dephosphorylation, GADD34 targets PP1 to the ER to form an eIF2α phosphatase complex, which relies upon the ability of GADD34 to bind to PP1 and localize to the ER. PP1 binding requires the conserved C-terminal KVRF PP1-binding motif and a RARA motif C-terminal to the KVRF motif, whereas ER localization is determined by the N-terminal 180 amino acids of GADD34 (Brush et al., 2003). GADD34 is a member of the GADD gene family, which are genes whose expression is induced by DNA damage, growth factor deprivation, and other forms of cell stress (Zhan et al., 1994).

GADD34 is responsible for progressing the UPR from the early stage into the late stage in MEFs (Reid et al., 2016). The early stage is marked by translation suppression and degradation of misfolded proteins and occurs over the initial 30 minutes to 1 hour of ER stress. The later stage beginning around 4 hours post-ER stress focuses on enhancing ER protein folding capacity through increasing the synthesis of chaperones (Reid et al., 2014). Around 4 hours after stress induction, GADD34 is also essential for the return of the mRNAs encoding ER-targeted proteins that are released from the ER 30 minutes after stress (Reid et al., 2016). While GADD34 functions to progress the UPR around 4 hours after stress, cells that lack GADD34 have been shown to be able to compensate, but the transition then occurs much later (Reid et al., 2016). Cells lacking GADD34 show elevated eIF2α phosphorylation still at 8 hours after ER stress induction and slowly decline at later time points, around 18-24 hours post-stress induction,

as other mechanisms are activated that reduce eIF2α phosphorylation (Reid et al., 2016). In accordance with prolonged elevation of eIF2α-P without GADD34, protein synthesis is suppressed for much longer, only showing a moderate increase at 24 hours, in contrast to WT cells which show nearly full recovery of protein synthesis by 8 hours (Reid et al., 2016). Prolonged elevation of phosphorylated AKT, an inhibitor of PERK, and therefore a decline in phosphorylated PERK, seems to contribute to this partial recovery of protein synthesis when GADD34 is not present (Reid et al., 2016).

The decline in eIF2α phosphorylation seen in response to ER stress prior to GADD34 protein detection indicated that another mechanism was at work to initially contribute to this decline. CReP, named "Constitutive Repressor of eIF2α Phosphorylation" was found to have homology in the C-terminal end to GADD34 and could also bind PP1c, and was given its name due to its presence in unstressed cells and its primary function of regulating basal levels of eIF2α phosphorylation (Jousse et al., 2003). The half-life of CReP is short, at approximately 45 minutes. Studies show that in the absence of CReP, levels of phosphorylated eIF2α are higher and have a delayed decline, showing that CReP plays a role in the ER stress response (Jousse et al., 2003). Also supporting this role of CReP during ER stress, CReP has been shown to prevent the accumulation of CHOP. RNAi targeting CReP resulted in increased CHOP, but not when cells were overexpressing GADD34. This suggests that there is functional redundancy between GADD34 and CReP (Jousse et al., 2003). CReP mRNA levels have also been shown to compensate for a lack of GADD34; Reid et al. (2016) showed a significant 10-fold increase in CReP mRNA compared to WT over 16 hours post-stress induction, suggesting that a lack of GADD34 results in enhanced transcription of the CReP gene or stabilization of CReP mRNA. CReP mRNA is also one of the main substrates of IRE1α-dependent decay during UPR

activation, which contributes to elevating the levels of phosphorylated eIF2α independent of PERK kinase activity (So et al., 2015). While CReP is important for basal regulation of eIF2α phosphorylation, its involvement in UPR signaling in response to ER stress has been well supported, albeit not well characterized at this time. These findings suggest that the eIF2α phosphatases may be coordinately regulated to manage phosphorylated eIF2α levels during ER stress.

Genetic knockout of GADD34 and CReP in mice has revealed the primary function of these eIF2α phosphatases and the resulting phenotypes in mammalian physiology (Harding et al., 2009; Kojima et al., 2003; Nishio and Isobe, 2015). In studies by Harding et al., (2009) mutant mice homozygous for a knockout allele of the GADD34 gene survived to adulthood and were fertile, with the only defects detectable upon treatment with an ER or physiological stressor. Mutant mice homozygous for a knockout allele of *Ppp1r15b* (CReP) were about half the size of WT at birth, pale in color, and failed to nurse, with none surviving past the first day of postnatal life. The pallor was explained by low hematocrit and red blood cell count with abnormalities in red cell size and shape. The livers showed compensatory proliferation in blood precursors, implicating an important role for CReP in fetal erythropoiesis. Mutating serine 51 on eIF2α was sufficient to rescue the CReP mutant mice in size and red blood cell counts. Also shown in liver cytoplasmic extracts was elevated GADD34, indicating compensation for CReP deficiency. Embryos that lacked all PPP1R15 function failed to form a blastocyst cavity, grow, or hatch from the zona pellucida and did not develop past the preimplantation period. This is likely due to a failure to deal with the significant increase in protein synthesis that occurs at the 2-8 cell stage. In this stage, genes for the eIF2 subunits, GADD34 and CReP, are activated and genes that inhibit translation are repressed, such as PERK. Again, double homozygous mutants lacking

GADD34 and CReP were able to be rescued by the serine-51 mutation on eIF2α. These studies revealed that eIF2α dephosphorylation is the essential function of the PPP1R15 family but also highlighted distinct roles for GADD34 and CReP, with CReP being uniquely crucial for survival.

Apoptosis

Apoptosis is a routine and highly regulated part of life in which cells are degraded to protect other tissues and maintain proper cell numbers. This process is common and necessary in development to create structures and remove damaged, incompetent, or stressed cells. Dysregulation of apoptotic signaling is involved in the pathogenesis of multiple diseases, including cancers and autoimmune disease when there is inadequate apoptosis, whereas excessive apoptosis can result in or contribute to neurodegenerative diseases, diabetes, immunodeficiency, and atrophy (Oyadomari and Mari, 2004). Apoptosis can be initiated by either intrinsic or extrinsic signals that are activated by various conditions such as DNA damage, excessive stress, hypoxia, low nutrients, improper hormones or factors present for growth, environmental stressors, and viral infection. These signals activate initiator caspases which go on to cleave and activate executioner caspases, both of which are endoproteases that hydrolyze peptide bonds to exert their effects on substrates. Executioner caspases activate DNases, proteases and other executioner caspases and cleave and inactivate cellular proteins to implement cell death.

Apoptosis can be initiated through two distinct genetic programs in response to ER stress, depending on the length of stress exposure (Pyati et al., 2011). As summarized in Figure 2, research by Pyati et al. (2011) found an acute cell death response after 4 hours of ER stress mediated by p63 and puma, and a chronic cell death response after 24 hours of ER stress dependent on CHOP. The upstream signals involved in activating each of these apoptotic

responses have not been identified, and more specifically, the role of eIF2α phosphatases in this process is unknown. Gaining a better understanding of the involvement of GADD34 and CReP in these distinct genetic programs of ER stress-induced cell death served as the central question of this research.

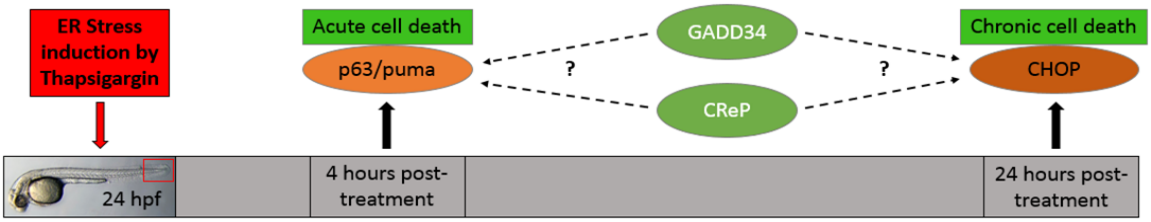

Figure 2: Zebrafish caudal fin epidermal cells undergo two distinct apoptotic responses to ER stress. Studies by Pyati et al. (2011) found that the genetic programs of cell death differ between the acute and chronic apoptotic response to ER stress. The role of eIF2α phosphatases in this is not well characterized.

The UPR in Human Disease

Understanding UPR signaling and the specific roles of GADD34 and CReP has far-reaching applications in human disease. ER stress contributes to the initiation and progression of many human diseases, due to the fact that many factors involved in pathogenesis are also causes of ER stress. Because prolonged UPR activation can induce apoptosis, chronically stressed cells become more susceptible to ER stress-induced apoptosis, contributing further to disease phenotypes. Genetic mutations in genes involved in UPR signaling or in proteins processed by the ER are known to cause and contribute to disease, and in some cases are even lethal. Studies have found that GADD34 plays a role in regulating the translation of nearly 900 mRNA transcripts in unstressed conditions, some of which create proteins involved in diseases, further underlying the impact that alterations in UPR signaling can have in contributing to chronic

disease (Reid et al., 2016). The UPR plays various roles in the progression of neurodegenerative, metabolic, autoimmune, and inflammatory diseases and cancers, and characterization of the roles of eIF2α phosphatases in the PERK branch of the UPR may provide insight into the development of novel disease therapeutics.

Neurodegenerative disease progression and age-related diseases are associated with the accumulation of misfolded proteins in neurons and oxidative stress, which activates the ER stress-induced apoptosis of neuron cells. Neuron loss results in cognitive and neural deficits, as seen in diseases like Parkinson's and Alzheimer's. In human and mouse models of Alzheimer's disease, elevated levels of phosphorylated eIF2α, ATF4, and IRE1 have been identified, confirming that UPR pathways are involved (Baleriola et al., 2014). Specific misfolded proteins have been identified in certain diseases, such as amyloid-β peptides in Alzheimer's disease, and α-synuclein in Parkinson's disease. The abnormal accumulation and aggregation of such proteins induces ER stress, neuron loss, and can even exert inhibitory effects on UPR signaling; for example, α-synuclein inhibits ATF6α processing directly through physical interactions and indirectly hinders protein transport from the ER to the Golgi (Credle et al., 2015). Although the UPR is known to be involved in neurodegenerative diseases, specific mutations in UPR pathways that cause neurodegeneration in humans have not yet been identified (Wang et al., 2016).

ER homeostasis helps regulate glucose and lipid metabolism, so ER stress and UPR pathways can contribute to various metabolic diseases such as diabetes and non-alcoholic fatty liver disease (NAFLD) (Wang et al., 2016). Studies have shown that ER stress causes lipids to accumulate in hepatocytes and that the three UPR pathways are essential for limiting and clearing this accumulation (Wang et al., 2012; Zhang et al., 2006; Lee et al., 2008; So et al.,

2012; Rutkowski et al., 2008; Zhang et al., 2011). In type 2 diabetes, insulin resistance causes β-cells to increase insulin synthesis and secretion, making these cells more susceptible to ER-stress induced apoptosis. In type 1 diabetes, inflammation induces ER stress in β-cells, making insulin synthesis and secretion more difficult and also sensitizing cells to apoptosis. The hyperglycemia and hyperlipidemia associated with metabolic disease can induce ER stress in endothelial cells and neurons, adding to disease complications. GADD34 overexpression has been seen to protect against high-fat diet-induced liver steatosis in mice, presumably by suppressing genes involved in lipogenesis (Oyadomari et al., 2008).

ER stress and UPR activation have been found in a variety of human cancers, as reviewed by Wang and Kaufman (2014). Cancers often develop and progress in stressful microenvironment conditions such as hypoxia, nutrient deprivation, and poor vascularization, resulting in ER stress and consequent UPR activation to survive. Tumors may also use the UPR to survive increased demands in protein synthesis needed for oncogenic transformation, tumor growth or malignant progression. Since the PERK-eIF2α pathway of the UPR can either promote survival or apoptosis, the context of the cancer likely determines the impact of this pathway on tumor progression. Current studies support this paradoxical role for PERK in tumorigenesis. An anti-proliferative role for the PERK pathway was found in normal mammary epithelial cells, where PERK was essential for responding to adhesion-regulated signals to prevent mammary tumor formation (Sequeria et al., 2007). Conversely, a pro-survival function has also been supported for PERK-dependent signaling; in animal models of mammary carcinoma, PERK helps to maintain redox homeostasis and prevent the activation of the oxidative DNA damage checkpoint, facilitating tumor growth (Bobrovnikova-Marjo et al., 2010). In extreme hypoxia, signaling by PERK-eIF2α-P promotes the tolerance of cancer cells

to these conditions, and inactivation of the PERK pathway can impair survival of these cells (Bi et al., 2005; Rouschop et al., 2013). Similarly, the role that CHOP plays in tumorigenesis is also divergent, although the predominant amount of evidence supports a pro-apoptotic role for CHOP-induced apoptosis in stress. For example, in a mouse model of K-ras^{G12V}-driven lung cancer, CHOP appears to control early stages of tumor progression by inducing apoptosis in response to microenvironmental stress (Huber et al., 2013). However, some recent studies have indicated tumor-supporting functions of CHOP in certain cells. Thevenot et al. (2014) found that myeloid-derived suppressor cells with immunosuppressive activity within tumors expressed CHOP without fully undergoing apoptosis, suggesting a potential benefit to inhibiting CHOP in certain cancers.

Different approaches surrounding ER stress and UPR signaling are being taken to treat diseases involving ER stress. Some therapies seek to reduce ER stress through the use of small molecule chemical chaperones or protein-specific chaperones, which prevent protein aggregation and facilitate protein folding by stabilizing protein-folding intermediates (Wang et al., 2016). The protein-specific pharmacological chaperones can be designed to promote the folding and transport of misfolded proteins due to genetic mutations (Sawkar et al., 2002). Another therapeutic approach would induce ER stress and UPR activation to promote apoptotic pathways for cancer treatment. However, the opposite approach of inhibiting UPR activation may help sensitize cancerous cells to other therapies and reduce their resistance to hostile microenvironments or chemotherapy (Wang and Kaufman, 2014). This potential for combination therapies make targeting the UPR a promising approach to combat cancer.

Within the PERK-eIF2α signaling pathway, therapeutic agents enhancing the expression of ATF4 improve survival of pancreatic islet β-cells in murine models of diabetes. The proposed

mechanism for this pro-survival effect is through upregulated ATF4 induction of GADD34 and CHOP, promoting recovery of translation and allowing for increased insulin biosynthesis in the setting of ER stress (Yusta et al., 2006). Certain neurodegenerative diseases such as Alzheimer's disease and prion disease also benefit from restoring translation, as reduced PERK-eIF2α-P levels are neuroprotective in mouse models of these diseases (Ma et al., 2013; Moreno et al., 2013). In contrast, mice models of ALS and Parkinson's Disease show a protective effect from inhibiting eIF2α phosphatases by salubrinal treatment (Saxena et al., 2009; Colla et al., 2012). This is attributed to the stalling of UPR progression, allowing clearance of accumulated misfolded ER proteins and potential removal of damaged ER via autophagy. A potential difference in the response of these neurodegenerative diseases to therapies that stall the UPR may reside in the source of UPR activation. In diseases where the misfolded protein aggregation is nonspecific and cytoplasmic, the effects of prolonged UPR signaling will be apoptosis, since the protein aggregation was not specific to the ER (Halliday et al., 2015). However, when there is a specific misfolded protein underlying disease, protein will accumulate in the ER and will thus benefit from extended UPR activation. Certainly, the divergent outcomes of the PERK-eIF2α signaling pathway add more complexity to the development of drug therapies, yet there is remarkable potential in manipulating this pathway to effectively target a wide variety of devastating human diseases.

Zebrafish as a Model Organism

Zebrafish serve as a model vertebrate organism and will be utilized primarily in this research to measure ER stress-induced apoptosis in the caudal fin epidermis. Zebrafish offer many advantages for studying human disease phenotypes. The genome of this model organism is sequenced and can be accessed by an online database, allowing this system to be utilized for

genetic manipulation experiments. With relative ease, gain-of-function and loss-of-function experiments can be designed and performed on zebrafish embryos, such as CRISPR-Cas9 genome editing, morpholino antisense oligonucleotide (MO) knockdown, pharmacological treatments, and mRNA injections. Embryos during early development are also transparent, allowing for the administration of various fluids by microinjection and for clear imaging of tissues.

Because zebrafish are vertebrates, they share a closer structural and physiological relationship to humans compared to invertebrate models, with human and zebrafish genomes being of similar size (Lardelli, 2008). Orthologues of most human genes are found in zebrafish, and the expression of these genes often show similar expression patterns. Orthologous proteins are 70% identical in amino acid residue sequence to human proteins (Lardelli, 2008). Zebrafish appear to have gone through a widespread gene duplication event, resulting in many duplicate orthologue genes. This does not necessarily impair genetic studies on zebrafish, because often knocking out one gene will result in a loss of function that more similarly resembles human disease, but both genes can easily be abolished as well (Lardelli, 2008).

During early development, the zebrafish epidermis has been shown to continuously induce high basal levels of ER stress and UPR signaling due to rapidly growing cells and large amounts of membrane and secreted proteins being used to form the epidermal basement membrane (Webb et al., 2007). This tissue is therefore sensitized to ER stress induction and will initiate apoptosis with excessive ER stress, providing a system in which the effects of manipulating various components of the ER stress pathways can be observed and measured. The caudal fin epidermal cells are utilized in these studies to determine the changes in the apoptotic response as eIF2α phosphatases are inhibited or overexpressed. Previous studies have shown

that these cells undergo an acute cell death mediated by p63 and puma, but when subjected to chronic stress, long-term UPR signaling results in a CHOP-dependent cell death (Pyati et al., 2011). The ability to clearly visualize these cells by confocal microscopy and easily manipulate them genetically and pharmacologically makes them an ideal system to characterize the roles of GADD34 and CReP in these distinct genetic programs of cell death.

For our studies, zebrafish embryos are grown to 24 hours post-fertilization (hpf) before receiving any pharmacological treatment. This time point was selected because most major developmental patterning is complete by 24 hours, which allows for the assessment of tissue-specific cell death (Pyati et al., 2007). Various drugs can be utilized to induce ER stress in zebrafish embryos or inhibit parts of the PERK pathway. Thapsigargin is a potent and highly specific ER calcium pump inhibitor that induces ER stress in mammalian cells (Thastrup et al., 1990). Salubrinal is a drug that specifically blocks serine/threonine phosphatase-dependent dephosphorylation of eIF2α by inhibiting the PP1 complexes that form with both GADD34 and CReP (Boyce et al., 2005).

Zebrafish are useful to this research specifically because their genome contains both GADD34 and CReP homologs, whereas other model organisms such as *Drosophila, C. elegans*, and yeast either only contain one GADD34/CReP homolog or no homolog at all (Malzer et al., 2013; Rojas et al., 2014). The ability to perform gain and loss-of-function experiments allows for manipulation of GADD34 and CReP function individually and together to discover the individual and overlapping functions these proteins play in UPR signaling and ER stress-induced apoptosis. Large quantities of zebrafish embryos are produced in a single round of mating, and embryos are only needed up to 48 hpf for most experiments in this study, allowing for data to be collected on a high number of embryos in a relatively short amount of time. Compared to mouse

models, zebrafish are an extremely advantageous organism for experiments involving time course analyses on a substantial number of individuals.

Project Goals

The central hypothesis of this project is centered on the understanding that GADD34 and CReP are highly regulated regulators of the unfolded protein response, and that by gaining knowledge of their in vivo functions, treatment of multiple diseases might be developed based on inhibition or enhancement of the functions of these proteins. The aim of this research is to characterize the roles of GADD34 and CReP in ER stress-induced apoptosis *in vivo* to explore the potential protective effects of manipulating these proteins. This will be accomplished by studying the ER stress-induced apoptotic response in zebrafish caudal fin epidermal cells.

The inhibition of GADD34 and CReP can be induced genetically and pharmacologically. Loss-of-function experiments will be completed using the drug salubrinal, which inhibits function of both GADD34 and CReP and by the use of splice-blocking morpholinos designed to block splicing of GADD34 and CReP pre-mRNA. Gain-of-function of GADD34 and CReP will be achieved through injection of mRNA synthesized in vitro from the genes for zebrafish GADD34 and CReP cloned into the pCS3 MT vector. The effects of these manipulations on the apoptotic response seen in the caudal fin epidermal cells will be determined by treating the embryos with the known ER stress inducer, thapsigargin, and then measuring the number of apoptotic cells in the caudal fin through acridine orange staining and confocal imaging.

Chapter 2: Materials and Methods

Morpholinos

Morpholinos targeting splice junctions of the pre-mRNA for *ppp1r15a* and *ppp1r15b* were obtained from Genetools. The sequences and pre-mRNA targets are as follows:

Ppp1R15a: i2e3 MO sequence: 5'-ACACACCTGCAAGACAAGAAACAGA-3'

Pre-mRNA target: cgctct[tctgtttcttgtcttgcagGTGTGT]TTCAG

Ppp1r15b: e2i2 MO sequence: 5'-ATTTGTGACTGATCATTACCTTCTT-3'

Pre-mRNA target: CAAATTA[AAGAAGgtaatgatcagtcacaaat]aaacca

Total RNA Isolation

RNA was isolated from zebrafish embryos by first washing the embryos several times with sterile embryo media. Embryo media was then removed and 500 µL Trizol was added and embryo mixture was pipetted vigorously until a homogenous mixture was obtained. An additional 500 µL Trizol was added and sample was mixed by pipetting up and down 6 times. The sample was incubated for 5 minutes at room temperature, then 200 µL Chloroform was added and sample was shaken vigorously for 15 seconds and then incubated 2-3 minutes at room temperature. Sample was then centrifuged 15

minutes at top speed (21130 rcf). The aqueous, upper phase was carefully removed without touching the interphase. 1 volume of isopropanol was then added to the supernatant and mixed by pipetting up and down 6 times. This was then incubated on ice for 10 minutes and then centrifuged 30 minutes at top speed (21130 rcf). The supernatant was removed and discarded, and the pellet was washed with 1 mL freshly prepared 75% Ethanol. Sample was then centrifuged 5 minutes at top speed (21130 rcf), and the supernatant removed and discarded. The

pellet was then allowed to air dry and then resuspended in 50 µL nuclease-free water. RNA concentration was determined by using a UV Spectrophotometer, and the RNA was stored at -80℃.

DNase Treatment

The isolated RNA was then treated with DNase to remove genomic DNA. The reaction was set up according to Table 1 below, with a total volume of 100 µL. The sample was incubated at 37℃ for 10 minutes, then heated to 75℃ for 10-15 minutes to stop the reaction.

Table 1: DNase treatment reaction volumes.

Reagents	**Amount**
RNA	10 µg
10X DNase Reaction Buffer	10 µL
Water	To 100 µL
DNase I	1 µL

Phenol Chloroform Extraction

100 µL of nuclease-free water and 20 µL 3M sodium acetate was added to DNase treated samples or restriction digest samples. In a fume hood, 200 µL phenol was added to each sample, gently inverted a few times, and allowed to sit until sample had separated. The top water layer was transferred to a new tube and 200 µL phenol was added to this sample, again inverted a few times and allowed to separate. The top water layer was again transferred to a new tube and 200

µL chloroform was added. The top water layer was transferred to a new tube, and 2.5 volumes of 100% ethanol was added. Sample was frozen at -20℃ for a minimum of 24 hours before proceeding to the next step.

Next, sample was centrifuged at 21130 rcf for 30 minutes. The supernatant was pipetted off and discarded, and 500 µL 70% ethanol was added. The sample was centrifuged at 21130 rcf for 15 minutes. The supernatant was pipetted off and discarded, and the sample was centrifuged again at 21130 rcf for 30 seconds. Any excess liquid was pipetted off and sample was allowed to air dry. Pellet was then resuspended in 20 µL of nuclease-free water. DNA concentration was determined using a UV Spectrophotometer, and the DNA was stored at -20℃.

Reverse Transcriptase Reaction

In a PCR tube, the following was added to make cDNA from RNA samples: 4 µL of 5X iScript Reverse Transcription Supermix for RT-qPCR (or 5X Supermix with no RT as a negative control), 1 µg of RNA from the Total RNA Isolation (after DNase treatment and phenol chloroform extraction), and nuclease-free water to bring the total reaction volume to 20 µL. Sample was then heated to 25℃ for 5 minutes, 46℃ for 20 minutes, and 95℃ for 1 minute.

Verification of GADD34 and CReP Splice-Blocking Morpholinos by Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was set up according to the volumes listed in Table 2 to a final volume of 25 µL, using the PPP1R15a MO and PPP1R15b MO primers, shown in Table 3. Forward and reverse primers were diluted to a concentration of 10 µM. The reaction was set up according to Table 4. The initial denaturation step was 1 minute at 95℃. Then 30 cycles of the following were performed to amplify the template DNA: denaturation step at 95℃ for 30 seconds, primer annealing step at 55℃ for 1 minute, and extension step at 68℃ for 4

minutes. The final extension step was at 68℃ for 5 minutes. Final PCR products were maintained at 4℃ after cycling and were then stored at -20℃.

Table 2: Reaction set up for PCR of GADD34 and CReP for verification of splice-blocking morpholinos.

Reagents	**Volume**
10X Standard Taq Buffer (NEB)	2.5 µL
dNTPs	0.5 µL
Forward Primer	0.5 µL
Reverse Primer	0.5 µL
Template DNA	1.0 µL
Water	20 µL
Taq DNA Polymerase	0.5 µL

Table 3: Sequences of primers used in RT-PCR.

Gene Name	**Primer Sequence**
PPP1R15a MO F	5’ TCTGTCAGCTCCAGAACGAA 3’
PPP1R15a MO R	5’ AACTCTCCTCCTGAAACGC 3’
PPP1R15b MO F	5’ AAGCTCATCTGTGACTCCAG 3’
PPP1R15b MO R	5’ CTCAGTGTCACCGATTCTC 3’
Elongation factor eFIa F	5’ CTGGAGGCCAGCTCAAACAT 3’
Elongation factor eFIa R	5’ TCAAGAAGAGTAGTACCGCTAGCATT 3’
PPP1R15b F (mRNA)	5’ AGAGAATTCAATGGAGAGGAGCGCGTC 3’
PPP1R15b R (mRNA)	5’ GAGACACTGTACAGAAGAAATGACTCG 3’

Table 4: PCR Conditions for amplification of GADD34 and CReP for verification of splice-blocking morpholinos.

Temperature	**Time**
95℃	1 minute
95℃	30 seconds
55℃	1 minute
68℃	4 minutes
68℃	5 minutes
10℃	Infinite

Amplification by Polymerase Chain Reaction

To clone the zebrafish CReP gene for use in overexpression studies, the gene was amplified using PCR from a GFP zCReP plasmid. The reaction was set up according to the volumes listed in Table 5 to a final volume of 50 µL, using the PPP1R15b primers and the elongation factor primers as a positive control (primer sequences listed in Table 3). Forward and reverse primers were diluted to a concentration of 10 µM. The reaction was set up according to Table 6. The initial denaturation step was 1 minute at 95℃. Then 30 cycles of the following were performed to amplify the template DNA: denaturation step at 95℃ for 30 seconds, primer annealing step at 55℃ for 1 minute, and extension step at 68℃ for 2 minutes. The final

extension step was at 68℃ for 5 minutes. Final PCR products were maintained at 4℃ after cycling and were then stored at -20℃.

Table 5: Reaction set up for PCR for amplification of *zCReP* for cloning of CReP.

Reagents	**Volume**
10X Accuprime pfx Reaction Mix	5 µL
Forward Primer (FzCRePRI)	1.25 µL
Reverse Primer (RzCRePXHOI)	1.25 µL
Template DNA (GFP zCReP)	1.0 µL
Water	40.5 µL
Accuprime pfx Polymerase	1.0 µL

Table 6: PCR Conditions for amplification of *zCReP* for cloning of CReP.

Temperature	Time
95℃	1 minute
95℃	30 seconds
55℃	1 minute
68℃	2 minutes
68℃	5 minutes
10℃	Infinite

Silica Isolation of DNA

DNA was extracted from 1% agarose gel using the protocol from Boyle, J.S. and Lew, A.M. (1995). The correct band was cut from the gel and weighed. 2 mL per gram of 6M NaI was added to the gel slice and melted at 55℃ for approximately 10 minutes. After complete melting of the gel, the tube was allowed to cool to room temperature for 1 minute, then 15 µL of silica suspension was added and tube was placed on a rocker for 7 minutes. The sample was then centrifuged for 30 seconds at maximum speed and the supernatant was removed and discarded. The silica pellet was then resuspended in 500 µL of Wash solution (50 mM NaCl; 10 mM Tris pH 7.6; 2.5 mM EDTA; and 50% EtOH) and centrifuged 30 seconds at maximum speed. The supernatant was removed and discarded, and this Wash solution step was repeated. The sample was then allowed to air dry for approximately 5 minutes. The silica pellet was then

resuspended in 20 µL of Elution Buffer (10 mM Tris 8.0) and this was heated for 7 minutes at 55℃. The sample was then centrifuged for 30 seconds at maximum speed and 19 µL of the supernatant was transferred to a new microcentrifuge tube. DNA was stored at -20℃.

Double Restriction Digest

To successfully clone the CReP gene into the pCS3 MT plasmid, shown in Figure 3 below, similar overhang ends were created using two restriction enzymes. PPP1R15b and pCS3 MT were double digested using EcoR1 and XHO1 restriction digest enzymes. The reaction was prepared according to Table 7 below, with a total volume of 20 µL. The reaction was incubated at 37℃ for 2-3 hours.

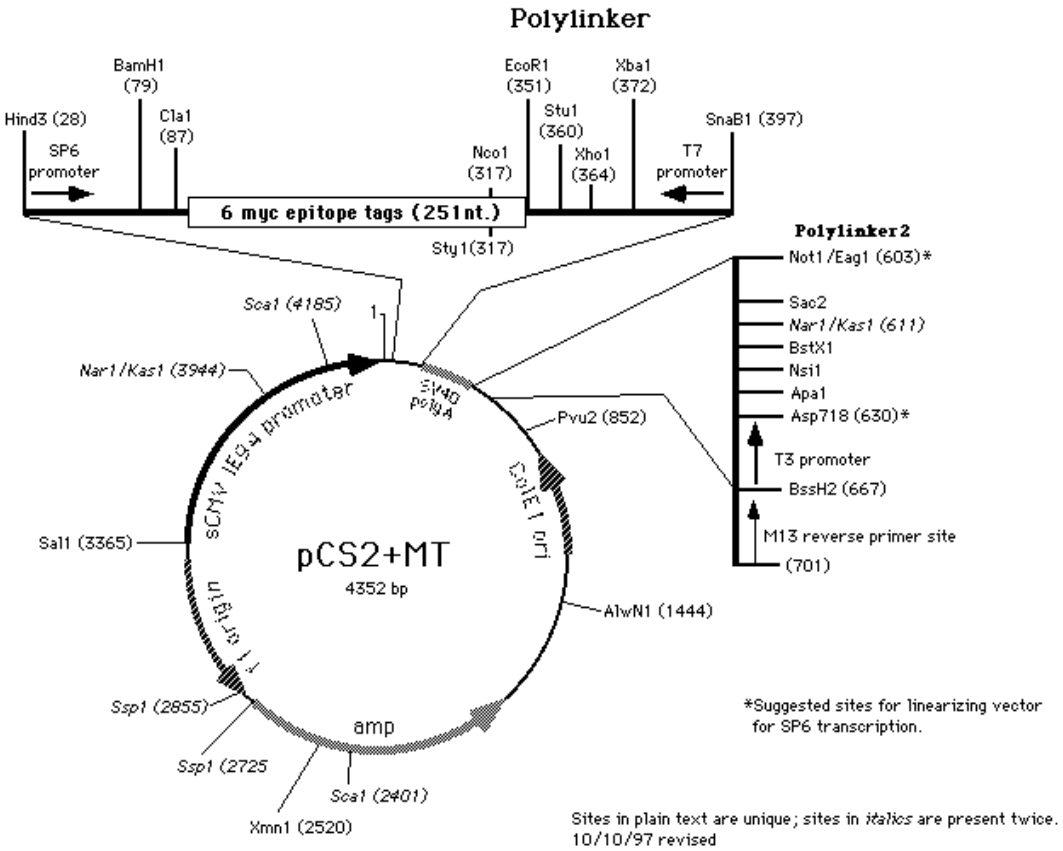

Figure 3: Vector map of pCS3 MT.

Table 7: Reaction set up for restriction digest of pCS3 MT vector and insert.

Contents	**Volume**
DNA	10 μL
Buffer 3.1	2 μL
Water	6 μL
EcoR1	1 μL
XHO1	1 μL

The entire digest samples of the PPP1R15b PCR product and pCS3 MT plasmid were then run on a 1% agarose gel. The gene and plasmid bands were cut from the gel by visualization on a transilluminator.

Ligation

The digested gene and plasmid DNA removed and saved from the 1% agarose gel after the double restriction digest were purified via silica gel isolation. This DNA was then used for a ligation reaction, according to Table 8 below (the negative control ligation reaction is shown in Table 9). A negative control ligation reaction was also performed to control for undigested vectors, and this reaction set up is also shown below. The reaction was set up on ice and allowed to proceed overnight on ice as the ice melted. The 10X T4 DNA Ligase Buffer was warmed to above room temperature before adding to the reaction. Before adding the T4 DNA ligase, the

other contents of the reaction were allowed to sit on ice for 5 minutes. The completed ligation reaction contents were stored at -20℃.

Table 8: Reaction set up for ligation reaction of pCS3 MT vector and PPP1R15b insert.

Contents	**Volume**
10X T4 DNA Ligase Buffer	2 μL
Cut pCS3 MT Vector	2 μL
DNA insert	10 μL
Water	5 μL
T4 DNA Ligase	1 μL

Table 9: Reaction set up for negative control ligation reaction.

Contents	**Volume**
10X T4 DNA Ligase Buffer	2 μL
Cut pCS3 MT Vector	2 μL
Water	15 μL

Mini Prep

An overnight culture was prepared using single colonies from bacterial transformation plates to inoculate 5 mL LB containing 5 µL of 1000X ampicillin in a 14 mL culture tube. Culture was incubated 16 hours at 37ºC in a shaking incubator at 200 rpm. The plasmid DNA was isolated using the E.Z.N.A.® Plasmid Mini Kit I (Omega Bio-tek).

Using the Spin Protocol, 1.5 mL of the overnight culture was transferred to a microcentrifuge tube and centrifuged at 10,000 x g for 1 minute. The supernatant was discarded and this step was repeated until all of the culture was transferred to the microcentrifuge tube and centrifuged. To the pellet of bacterial cells, 250 µL of Solution I/RNase A was added, and the cells were thoroughly resuspended by pipetting and vortexing. Next, 250 µL of Solution II was added and the tube was gently rotated 8-10 times until a cleared lysate formed, with a 2-3 minute incubation. 350 µL Solution III was then added and the tube was inverted and gently rotated until a flocculent white precipitate formed, with a 2-3 minute incubation with occasional mixing. This mixture was then centrifuged at maximum speed (21,130 x g) for 10 minutes to obtain a compact white pellet. Immediately following this centrifugation, the clear supernatant was carefully pipetted into an HiBind® DNA Mini Column Collection Tube. This column was then centrifuged at maximum speed for 1 minute, and the filtrate was discarded. Next, 500 µL of HBC Buffer (previously diluted with 100% isopropanol) was added, and the column was centrifuged at maximum speed for 1 minute, and the filtrate was discarded. Then 700 µL DNA Wash Buffer (previously diluted with 100% ethanol) was added to the column and centrifuged at maximum speed for 1 minute and the filtrate discarded, with this step repeated to be performed twice in total. The column was then centrifuged at maximum speed for 2 minutes to dry the column matrix, and the column was then transferred to a clean, nuclease-free 1.5 mL

microcentrifuge tube. 30 µL of Elution Buffer was added directly to the center of the column matrix and allowed to sit for 1 minute at room temperature. The column was then centrifuged at maximum speed for 1 minute to collect the DNA. DNA concentration was determined using a UV Spectrophotometer, and the Mini Prep DNA was stored at -20℃.

DNA Gel Electrophoresis

1.0 gram of agarose in 100 mL of 0.5X TBE was heated in the microwave until fully dissolved to produce a 1% agarose gel. 5 µL ethidium bromide was then added to the solution, and 25-50 mL was poured to make the gel. The solidified gel was then placed in a gel electrophoresis chamber filled with 0.5X TBE. 5 µL of 1 kb ladder was loaded into the gel, along with the DNA of interest being combined with DNA loading dye. Samples were run at 94-105 volts for 30 minutes to 1 hour. The gel was then visualized using the UVP GelDoc-It®*e* Imaging System.

Sequencing

1500 ng of mini and midi prep samples were sent to Sequetech for sequencing to verify the correct DNA sequence was present in pCS3 MT plasmids for GADD34 and CReP overexpression studies.

Bacterial Transformation

E. Coli competent cells were thawed on ice for 10 minutes, then 0.5-2 µL of DNA was added to 75 µL competent cells and gently stirred with pipette to mix. Mixture was incubated on ice for 20 minutes. Cells were then heat-shocked in a 42ºC water bath for 30 seconds and then immediately placed on ice for 1 minute. After, 900 µL LB Broth was added and cells were incubated at 37ºC in a shaking incubator at 200 rpm for 1 hour. 100 µL of cell transformations

were then plated along with 500 µL LB Broth onto pre-warmed agar plates containing 100 µg/mL ampicillin, and plates were incubated overnight at 37ºC.

Midi Prep

An overnight culture was prepared using single colonies from the bacterial transformation plates to inoculate 50 mL LB containing 50 µL of 1000X carbenicillin (25 µL ampicillin was used for CReP overexpression experiments) in a 250 mL Erlenmeyer flask. Culture was incubated 16 hours at 37ºC in a shaking incubator at 200 rpm. The plasmid DNA was isolated using the E.Z.N.A.® Plasmid Midi Kit Centrifugation Protocol (Omega Bio-tek).

Using this protocol, overnight cultures were transferred to a 50 mL conical tube and centrifuged at 3,000 x g for 10 minutes at room temperature. The supernatant was discarded, and 2.25 mL Solution 1/RNase A was added, and cells were resuspended by pipetting. Next, 2.25 mL Solution II was added and the tube was gently rotated 8-10 times until a cleared lysate formed, with a 2-3 minute incubation. 3.2 mL Solution III was then added and the tube was inverted and gently rotated until flocculent white precipitates formed, with a 2-3 minute incubation with occasional mixing. This mixture was then centrifuged at 15,000 x g for 10 minutes at 23℃ to obtain a compact white pellet. Immediately following centrifugation, the clear supernatant was carefully transferred to a clean 50 mL conical. 3.5 mL was added to a HiBind® DNA Midi Column Collection Tube and centrifuged at 3,000 x g for 5 minutes, with the filtrate being discarded. This was repeated until all of the cleared supernatant was transferred to the Column Collection tube and centrifuged. Next, 3 mL of HBC Buffer (previously diluted with 100% isopropanol) was added to the column and centrifuged at 3,000 x g for 3 minutes, with the filtrate discarded. Then 3.5 mL DNA Wash Buffer (previously diluted with 100% ethanol) was added to the column and centrifuged at 3,000 x g for 3 minutes and the filtrate

discarded, with this step repeated to be performed twice in total. The column was then centrifuged at 3,000 x g for 10 minutes to dry the column matrix, and the column was then transferred to a clean, nuclease-free 15 mL centrifuge tube. 0.5 mL of Elution Buffer was added directly to the center of the column matrix and allowed to sit for 3 minutes at room temperature. This was then centrifuged at 3,000 x g for 5 minutes to collect the DNA. DNA concentration was determined using a UV Spectrophotometer, and the Midi Prep DNA was stored at -20℃.

Restriction Digest

Midi prep DNA was then digested with the Not1 restriction enzyme. The reaction was prepared according to Table 10 below, with a total volume of 100 µL, and the reaction was incubated at 37℃ overnight. The digest was confirmed by DNA gel electrophoresis. The DNA was then recovered from the digest sample by phenol chloroform extraction.

Table 10: Reaction set up for restriction digest of pCS3 MT vector and insert.

Contents	**Amount**
DNA	5 µg
Buffer 3.1	10 µL
Water	To 100 µL
Not1	1 µL

RNA Transcription

Using DNA purified by phenol chloroform extraction, mRNA was synthesized *in vitro* using the mMESSAGE mMACHINE ® kit (ThermoFisher Scientific) protocol using Lithium Chloride precipitation. Following this protocol, the transcription reaction was assembled at room temperature according to Table 11 below with a total volume of 20 µL. 10 µL 2X NTP/CAP and nuclease-free water to bring the total volume to 20µL were combined. Then 2 µL 10X Reaction Buffer, 1 µg of the linear template DNA, and 2 µL of the Enzyme Mix were added and the contents were gently pipetted to mix thoroughly. The reaction was incubated for 2 hours at 37℃. Next, 1 µL TURBO DNase was added to the reaction and incubated 15 minutes at 37℃. Following this incubation, 0.5 µL of the sample was saved and mixed with 2 µL RNA loading dye to run an RNA check gel. To the rest of the sample, the reaction was stopped by adding 30 µL of nuclease-free water and 30 µL of Lithium Chloride Precipitation Solution. This was mixed thoroughly and placed at -20℃ overnight. The next day, the sample was centrifuged at max speed (21130 rcf) for 15 minutes to pellet the RNA. The supernatant was carefully removed and the pellet was washed once with 1 mL 70% ethanol and centrifuged again at max speed for 15 minutes. The ethanol was then removed and pellet was allowed to air dry. Pellet was then resuspended in 30 µL nuclease-free water. RNA concentration was determined using a UV Spectrophotometer, and the RNA was stored at -80℃.

Table 11: Reaction set up for in vitro mRNA synthesis.

Contents	**Amount**
2X NTP/CAP	10 µL
Nuclease-free water	To 20 µL
10X Reaction Buffer	2 µL
Linear template DNA	1 µg
Enzyme Mix	2 µL

RNA Gel Electrophoresis

The gel electrophoresis chamber, plate, and comb were filled with ~5 mL 20% SDS, ~1 mL HCl, and DI water and allowed to soak for 5 minutes. The materials were then thoroughly rinsed and dried. 1.0 gram of agarose in 100 mL of 0.5X TBE was heated in the microwave until fully dissolved to produce a 1% agarose gel. 5 µL ethidium bromide was then added to the solution, and 25-50 mL was poured to make the gel. The solidified gel was then placed in a gel electrophoresis chamber filled with 0.5X TBE. 5 µL of 1 kb ladder was loaded into the gel, along with the RNA of interest being combined with RNA loading dye. Samples were run at 194-205 volts for 10 to 15 minutes. The gel was then visualized using the UVP GelDoc-It®*e* Imaging System.

Fish Maintenance

Zebrafish were kept in 9.5 liter water tanks in a circulating system maintained at 28℃. Males and females were kept in separate tanks and fish were placed on a light/dark cycle for breeding purposes. Fresh water was prepared in volumes of 20 gallons DI water containing 4.5 g Instant Ocean, 1.5 g $CaCl_2$, and 5 g $NaHCO_3$. The water was replaced once per week to prevent waste product accumulation. Fish were fed twice daily with a variety of adult zebrafish irradiated diet, brine shrimp, and tropical flakes.

Fish Breeding

Zebrafish breeding tanks with a divider were filled with fish water and 2-4 males and females were placed on separate sides of the tanks in the late afternoon after feeding. After males and females were separated overnight, the divider was removed the next morning and fish were allowed to breed for 20 minutes or until embryos were present in the tank. Embryos were collected using a strainer and rinsed in E3 embryo media. Embryos were then either placed in a 28℃ incubator or prepared for microinjection. Adult fish were separated by sex and returned to their regular tanks.

Zebrafish Microinjection

Embryos were incubated at room temperature in pronase for 7 minutes in a glass petri dish. 1 liter of E3 embryo media was then used to wash embryos. 1-cell stage embryos were then selected and placed on an agar plate designed for microinjection. Zebrafish embryos were injected with the indicated concentration of morpholino, mRNA, or CRISPR components at the one-cell stage with 1 nl of fluid. Concentrations are listed in Table 12 below. Embryos were kept in E3 media with 0.0001% methylene blue at 28ºC until the desired stage of development.

Table 12: Concentrations used for microinjection of morpholinos and mRNA.

Injection Component	**Concentration**
GADD34 Morpholino	5 ng/nL
CReP Morpholino	5 ng/nL
GADD34 + CReP Morpholinos	5 ng/nL each
GADD34 mRNA	200 ng/nL
CReP mRNA	200 ng/nL

Drug Treatments

In a 24-well plate, 3-5 zebrafish embryos at 24 dpf were placed per well and as much liquid as possible was removed. 500 µL of the appropriate solution was added to each well and fish were incubated at 28.5℃ for either 4 h or 24 h. For drug treatments not involving the use of microinjection, embryos were treated with pronase for approximately 10 minutes at 24 hpf to remove chorions, and then washed with 1 L of E3 embryo media. Drugs were used at the following concentrations:1 µM thapsigargin in E3 embryo media, and 50 µM salubrinal in E3 embryo media. E3 embryo media was used as a control.

Acridine Orange Staining

After the appropriate incubation time for drug treatments, 4-5 zebrafish embryos were placed in microcentrifuge tubes and the drug/E3 embryo media was removed. Fish were incubated in 500 µL 1X Acridine Orange for 10 minutes at room temperature. The Acridine Orange was then removed and discarded and was replaced with 500 µL of E3 embryo media.

Confocal Microscopy

Images were taken using the Leica DMIRE2, Yokogawa CSU XI confocal scanner. The 491 nm laser was used to obtain images stained with acridine orange. Images were viewed at 5X magnification, and images were saved using color combine mode for 491 nm.

Statistical Analysis

Statistical significance was estimated using a 1 factor ANOVA and Holm-Šídák Posthoc comparisons with Daniel's XL Toolbox version 7.2.13. A P value of < 0.05 was considered statistically significant.

Chapter 3: Results

Pharmacological Inhibition of eIF2α Phosphatases Using Salubrinal

The drug salubrinal is an eIF2α phosphatase inhibitor that blocks both GADD34 and CReP function, however its mechanism of action is not currently known. We sought to determine if this inhibition of the assembly of the eIF2α phosphatase complex affected stress-induced apoptosis in the zebrafish caudal fin epidermal cells during both the acute and chronic apoptotic response to ER stress (Pyati et al., 2011). For these experiments, zebrafish embryos were raised to 24 hpf and treated for two hours with salubrinal [50 μM] (controls remained in E3 embryo media). At 26 hpf, both salubrinal pre-treated and control embryos were then treated with thapsigargin [1 μM] to induce ER stress for 4 or 24 hours. After the appropriate time, embryos were assayed for apoptosis by Acridine Orange staining and confocal imaging. First, it was shown that treatment with salubrinal alone for 4 or 24 hours does not increase apoptosis in the caudal fin epidermal cells (Figure 4).

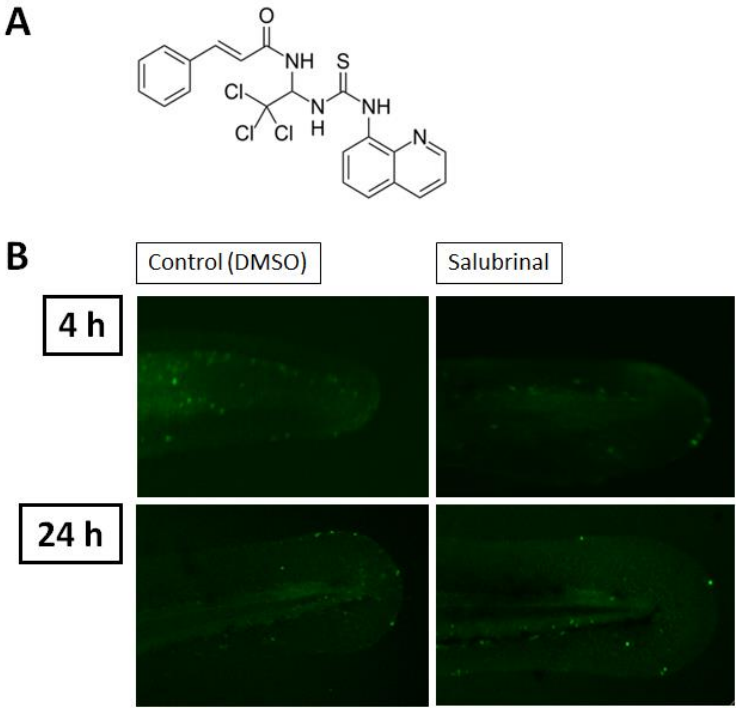

Figure 4: Salubrinal is a pharmacological inhibitor of GADD34 and CReP function that blocks the assembly of the eIF2α phosphatase complex. **A.** Salubrinal chemical structure. **B.** Salubrinal treatment alone does not increase apoptosis in the caudal fin.

Embryos pre-treated with salubrinal before ER stress induction showed a significant decrease in stress-induced apoptosis after both 4 and 24 hours of thapsigargin treatment, compared to controls (4 h: $F_{1, 249} = 6.1$, $p = 0.014$; 24 h: $F_{1, 543} = 190$, $p = 3.4E\text{-}37$) (Figure 5). This data suggests that inhibition of both GADD34 and CReP through blocking the assembly of the eIF2α phosphatase complex has a protective effect on cells experiencing ER stress, during both the acute and chronic apoptotic responses to ER stress.

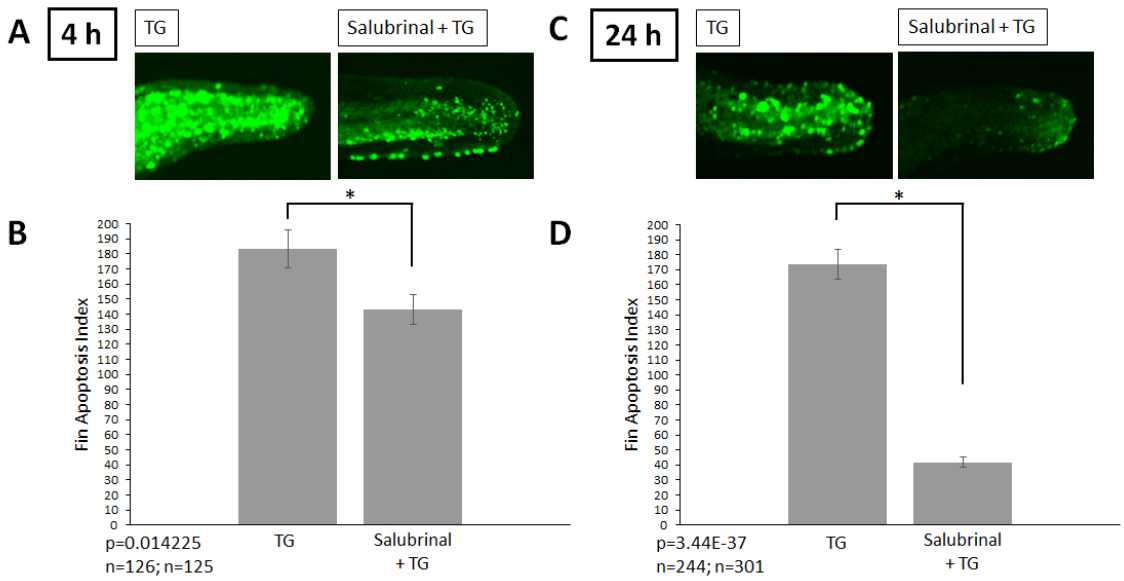

Figure 5: Stress-induced apoptosis levels in zebrafish caudal fin epidermal cells pre-treated with salubrinal at 4 and 24 post-stress induction. Embryos were treated with 50 µM salubrinal for 2 hours starting at 24 hpf. At 26 hpf, salubrinal-treated or untreated embryos were then treated with 1 µM Thapsigargin for the indicated treatment time. Apoptosis was then assayed by acridine orange staining, and tails were visualized by confocal microscopy at 491 nm. Cells undergoing apoptosis were quantified by counting the number of fluorescent cells in a defined region of the caudal fin, and bar graphs represent the mean number of cells found within each treatment group, the fin apoptosis index. Error bars represent the standard error of the mean. Significant differences between treatment groups were detected using a 1 factor ANOVA and Holm-Šídák Posthoc comparisons, the * denotes significant differences when $p < 0.05$. Zebrafish caudal fins are shown within each treatment group representing the average number of cells undergoing apoptosis. **A.** Caudal fins of zebrafish pre-treated with salubrinal compared to WT after 4 hours TG treatment. **B.** Mean values of apoptotic cells in the caudal fins of zebrafish pre-treated with salubrinal compared to WT after 4 hours TG treatment. **C.** Caudal fins of zebrafish pre-treated with salubrinal compared to WT after 24 hours TG treatment. **D.** Mean values of apoptotic cells in the caudal fins of zebrafish pre-treated with salubrinal compared to WT after 24 hours TG treatment.

Morpholino Knockdown of GADD34 and CReP

The protective effect of salubrinal against cell death prompted further investigation into the roles of GADD34 and CReP individually during the apoptotic responses to ER stress.

Previous work by Pyati et al. (2011) showed that at 4 hours of exposure to ER stress, caudal fin epidermal cells undergo an acute-phase apoptosis driven by puma-p63, but with 24 hours of ER stress, these cells undergo a chronic-phase apoptosis driven by the effects of the transcription factor CHOP, a target of ATF4 in the PERK branch of the UPR. To target GADD34 and CReP individually, morpholino oligos were designed to block specific splice junctions of GADD34 and CReP pre-mRNA to knock down expression of these proteins (Table 13). The loss-of-function phenotypes were studied in embryos injected with morpholinos targeting GADD34 alone, CReP alone, and both GADD34 and CReP together. For these experiments, embryos were injected with ~1 nl of the morpholino [5 ng/nl] at the one-cell stage. ER stress was induced at 24 hpf in morpholino-injected and non-injected embryos with thapsigargin treatment [1 μM] for either 4 or 24 hours. Following thapsigargin treatment, acridine orange staining and confocal microscopy were used to assay for apoptosis in the caudal fin epidermis.

Table 13: Morpholino oligo sequences used to target specific splice junctions of GADD34 and CReP pre-mRNA. The pre-mRNA target is shown with the brackets showing the mRNA sequence complementary to the morpholino sequence.

Morpholino	**GADD34**	**CReP**
Splice Junction Target	i2e3	e2i2
Morpholino sequence	ACACACCTGCAAGACAAGAAACAGA	ATTTGTGACTGATCATTACCTTCTT
Pre-mRNA Target	cgctct[tctgtttcttgtcttgcagGTGTGT]TTCAG	CAAATTA[AAGAAGgtaatgatcagtcacaaat]aaacca

Embryos with loss-of-function of GADD34 had significantly fewer cells undergoing apoptosis after 24 hours of exposure to ER stress compared to non-injected embryos, while no significant difference in apoptosis was seen after 4 hours of ER stress, (4 h: $p > 0.05$; 24 h: $F_{1, 205} = 10.3$, $p = 0.0016$) (Figure 6). Embryos with CReP loss-of-function did not show any significant differences in the number of apoptotic cells compared to non-injected embryos after either time point (4 h: $p > 0.05$; 24 h: $p > 0.05$) (Figure 7).

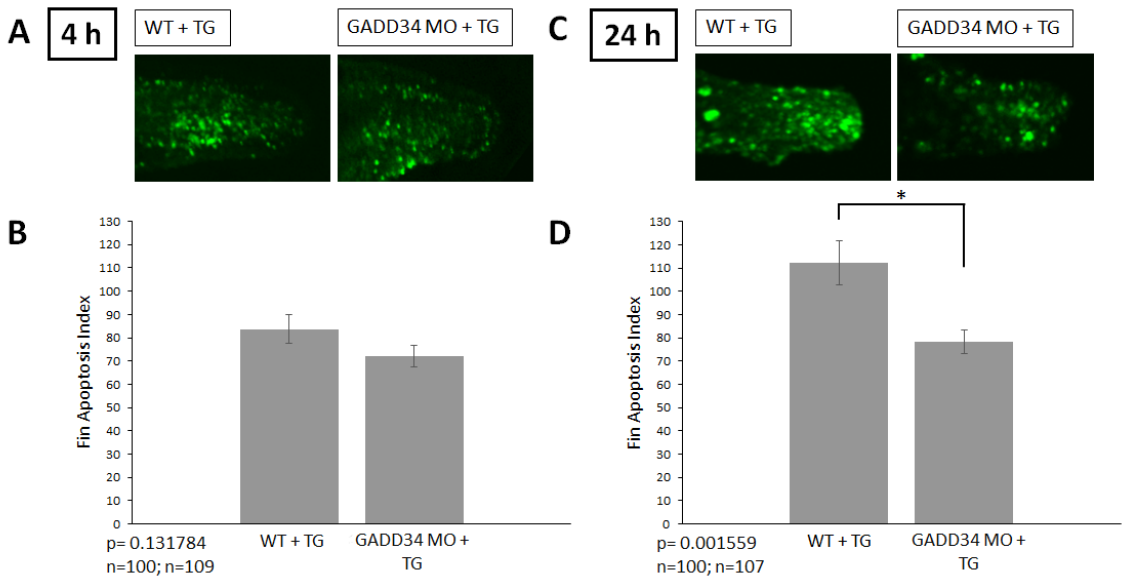

Figure 6: Stress-induced apoptosis levels at 4 and 24 post-stress induction in zebrafish caudal fin epidermal cells with loss-of-function of GADD34 via morpholino knockdown. Embryos were injected with ~1 nl of 5 ng/nl GADD34 morpholino at the one-cell stage and then treated at 24 hpf with 1 µM Thapsigargin for the indicated treatment time. Apoptosis was then assayed by acridine orange staining, and tails were visualized by confocal microscopy at 491 nm. Cells undergoing apoptosis were quantified by counting the number of fluorescent cells in a defined region of the caudal fin, and bar graphs represent the mean number of cells found within each treatment group, the fin apoptosis index. Error bars represent the standard error of the mean. Significant differences between treatment groups were detected using a 1 factor ANOVA and Holm-Šídák Posthoc comparisons, the * denotes significant differences when $p < 0.05$. Zebrafish caudal fins are shown within each treatment group representing the average number of cells undergoing apoptosis. **A.** Caudal fins of zebrafish with morpholino knockdown of GADD34 compared to WT after 4 hours TG treatment. **B.** Mean values of apoptotic cells in the caudal fins of zebrafish with loss-of-function of GADD34 compared to WT after 4 hours TG treatment. **C.** Caudal fins of zebrafish with morpholino knockdown of GADD34 compared to WT after 24 hours TG treatment. **D.** Mean values of apoptotic cells in the caudal fins of zebrafish with loss-of-function of GADD34 compared to WT after 24 hours TG treatment.

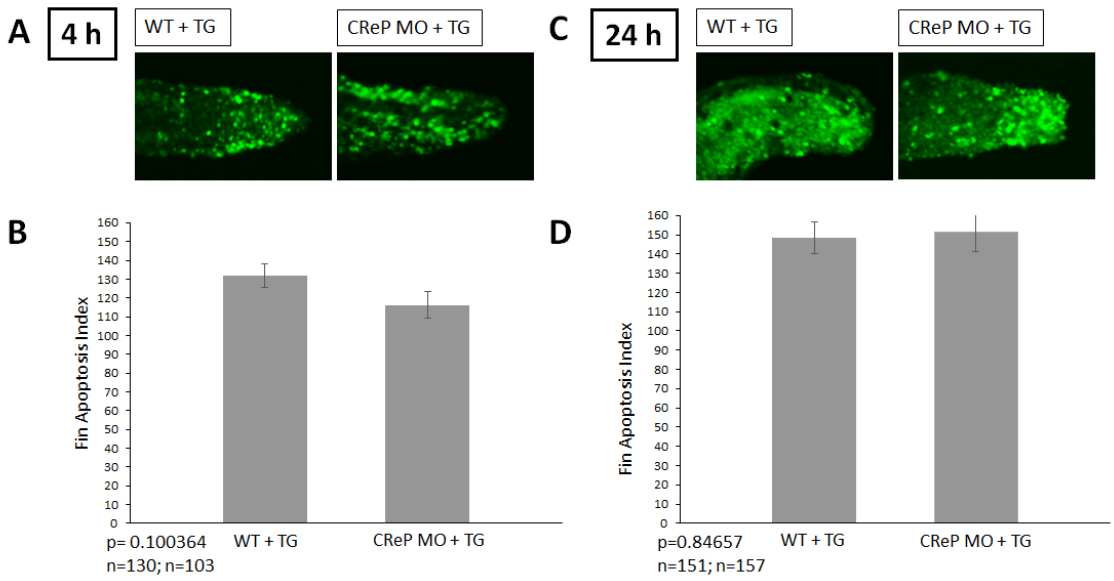

Figure 7: Stress-induced apoptosis levels at 4 and 24 post-stress induction in zebrafish caudal fin epidermal cells with loss-of-function of CReP via morpholino knockdown. Embryos were injected with ~1 nl of 5 ng/nl CReP morpholino at the one-cell stage and then treated at 24 hpf with 1 µM Thapsigargin for the indicated treatment time. Apoptosis was then assayed by acridine orange staining, and tails were visualized by confocal microscopy at 491 nm. Cells undergoing apoptosis were quantified by counting the number of fluorescent cells in a defined region of the caudal fin, and bar graphs represent the mean number of cells found within each treatment group, the fin apoptosis index. Error bars represent the standard error of the mean. Significant differences between treatment groups were detected using a 1 factor ANOVA and Holm-Šídák Posthoc comparisons, the * denotes significant differences when $p < 0.05$. Zebrafish caudal fins are shown within each treatment group representing the average number of cells undergoing apoptosis. **A.** Caudal fins of zebrafish with morpholino knockdown of CReP compared to WT after 4 hours TG treatment. **B.** Mean values of apoptotic cells in the caudal fins of zebrafish with loss-of-function of CReP compared to WT after 4 hours TG treatment. **C.** Caudal fins of zebrafish with morpholino knockdown of CReP compared to WT after 24 hours TG treatment. **D.** Mean values of apoptotic cells in the caudal fins of zebrafish with loss-of-function of CReP compared to WT after 24 hours TG treatment.

Loss-of-function of GADD34 and CReP together resulted in significantly fewer cells undergoing apoptosis after both 4 and 24 hours of thapsigargin treatment compared to non-injected embryos (4 h: $F_{1, 229} = 19.9$, $p = 1.3E\text{-}05$; 24 h: $F_{1, 267} = 23$, $p = 2.7E\text{-}06$) (Figure 8).

These results were consistent with those of the salubrinal pre-treatment experiments, suggesting that a more complete loss of eIF2α phosphatase activity is able to protect cells experiencing ER stress from the acute-phase apoptotic response, although individual knockdown of GADD34 and CReP was not sufficient to significantly reduce apoptosis at this time point. These results also suggest that the protective effects seen with salubrinal pre-treatment and the double knockdown morpholinos after 24 hours of ER stress exposure is primarily through loss-of-function of GADD34.

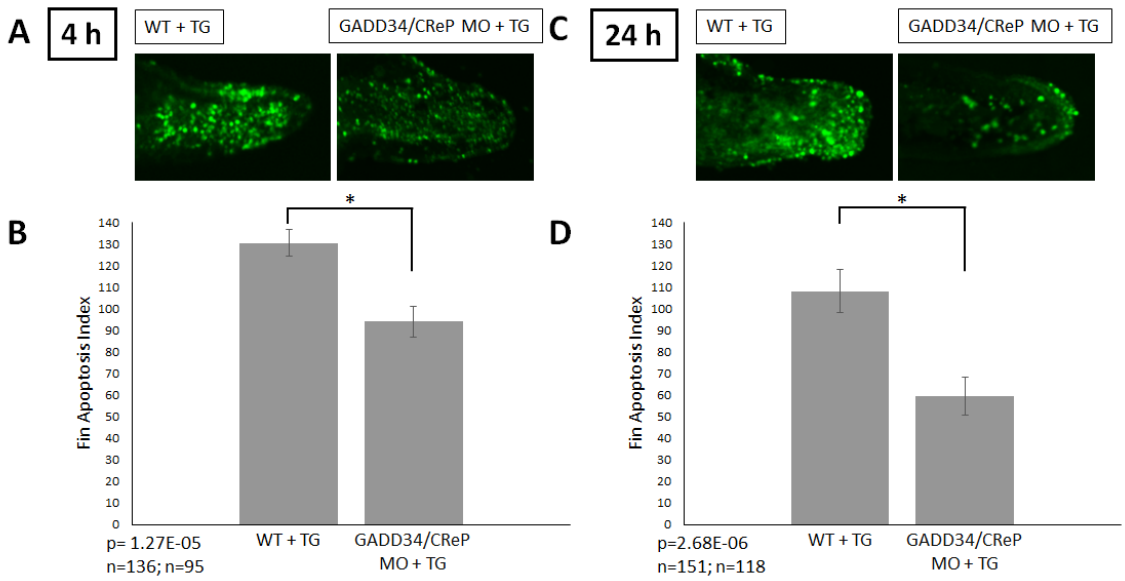

Figure 8: Stress-induced apoptosis levels at 4 and 24 post-stress induction in zebrafish caudal fin epidermal cells with loss-of-function of both GADD34 and CReP via morpholino knockdown. Embryos were injected with ~1 nl of a mixture of 5 ng/nl GADD34 morpholino and 5 ng/nl CReP morpholino at the one-cell stage and then treated at 24 hpf with 1 µM Thapsigargin for the indicated treatment time. Apoptosis was then assayed by acridine orange staining, and tails were visualized by confocal microscopy at 491 nm. Cells undergoing apoptosis were quantified by counting the number of fluorescent cells in a defined region of the caudal fin, and bar graphs represent the mean number of cells found within each treatment group, the fin apoptosis index. Error bars represent the standard error of the mean. Significant differences between treatment groups were detected using a 1 factor ANOVA and Holm-Šídák Posthoc comparisons, the * denotes significant differences when $p < 0.05$. Zebrafish caudal fins are shown within each treatment group representing the average number of cells undergoing apoptosis. **A.** Caudal fins of zebrafish with morpholino knockdown of both GADD34 and CReP compared to WT after 4 hours TG treatment. **B.** Mean values of apoptotic cells in the caudal fins of zebrafish with loss-of-function of both GADD34 and CReP compared to WT after 4 hours TG treatment. **C.** Caudal fins of zebrafish with morpholino knockdown of both GADD34 and CReP compared to WT after 24 hours TG treatment. **D.** Mean values of apoptotic cells in the caudal fins of zebrafish with loss-of-function of both GADD34 and CReP compared to WT after 24 hours TG treatment.

To confirm the efficacy of injected morpholinos, RT-PCR can be utilized to detect successful splice-modifications by designing primers that flank the target splice junction. A successful morpholino that blocks normal splicing results in a PCR product with a larger mass which can be detected by gel electrophoresis by comparing morpholino-injected fish to non-

injected fish. RT-PCR is run on cDNA synthesized from RNA isolated from pooled zebrafish embryos after a 4 hour thapsigargin treatment to induce ER stress (E3 embryo media is used as a control). Thus far, using the primers designed to surround the i2e3 target (Table 14), GADD34 has been detected by RT-PCR in non-injected, stressed fish (Figure 9, lane 2, and Figure 10) by the presence of the expected 234 bp band. CReP has not been successfully detected in non-injected fish using the designed primers, which may be due to low transcription levels even under ER stress conditions. Although attempts have been made, the unspliced GADD34 in morpholino-injected embryos has not yet been detected after RT-PCR (Figure 10). For future attempts, the morpholino concentration injected into the embryos will be increased.

Table 14: Primer sequences designed to amplify the target splice junction of GADD34 and CReP morpholinos using RT-PCR.

Gene/Primer Name	**Primer Sequence**
PPP1R15a MO F	5’ TCTGTCAGCTCCAGAACGAA 3’
PPP1R15a MO R	5’ AACTCTCCTCCTGAAACGC 3’
PPP1R15b MO F	5’ AAGCTCATCTGTGACTCCAG 3’
PPP1R15b MO R	5’ CTCAGTGTCACCGATTCTC 3’

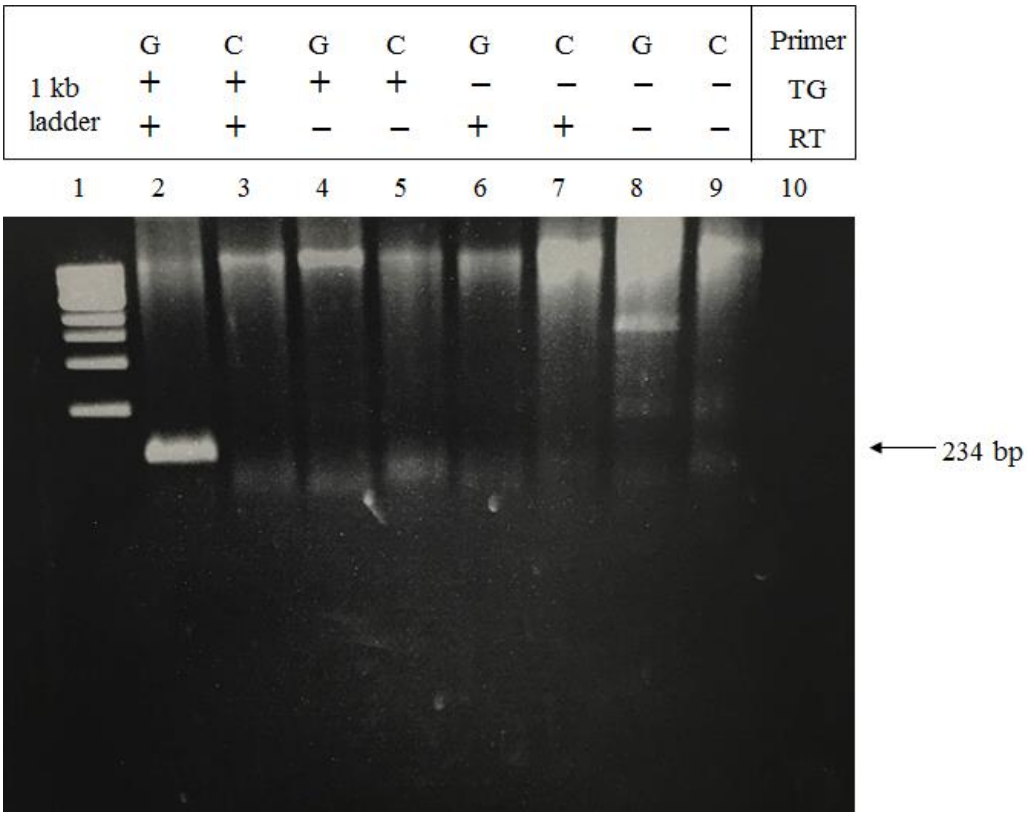

Figure 9: RT-PCR using primers designed to amplify the morpholino splice junction target region of GADD34 (G) and CReP (C) in non-injected zebrafish. 24 hpf embryos were treated for 4 hours with thapsigargin or E3 embryo media (control), then RNA was isolated for cDNA synthesis. "RT" indicates samples that used the reverse transcriptase enzyme for cDNA synthesis, whereas lack of RT indicates negative control samples that lacked the reverse transcriptase enzyme.

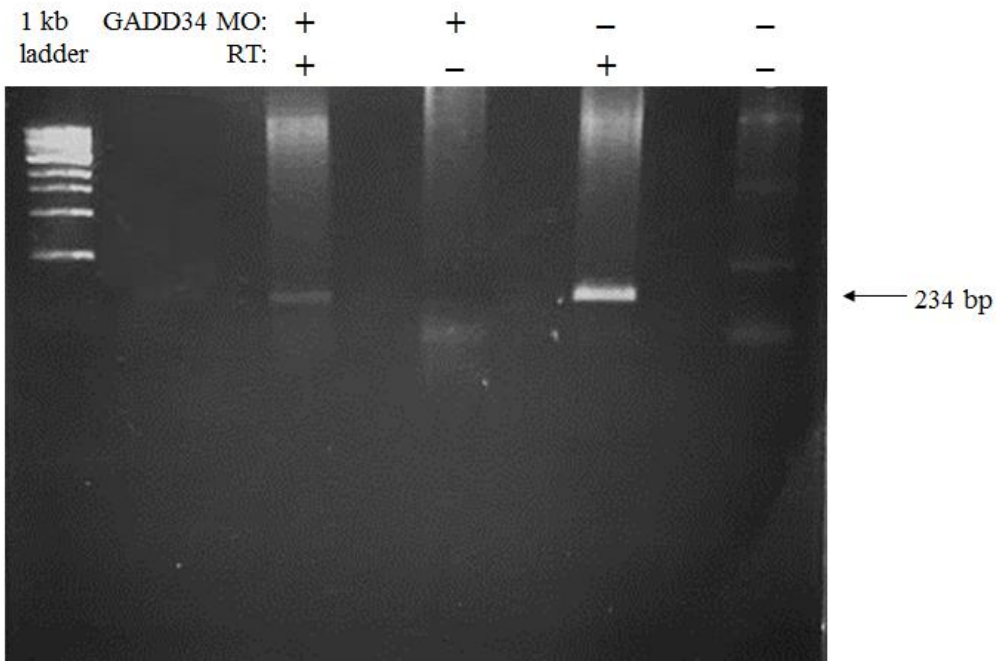

Figure 10: RT-PCR using primers designed to amplify the morpholino splice junction target region of GADD34, in GADD34 MO injected and non-injected zebrafish. 24 hpf embryos were treated for 4 hours with thapsigargin, then RNA was isolated for cDNA synthesis. "RT" indicates samples that used the reverse transcriptase enzyme for cDNA synthesis, whereas lack of RT indicates negative control samples that lacked the reverse transcriptase enzyme.

Overexpression of GADD34 and CReP

After the individual and combined loss-of-function of GADD34 and CReP experiments using morpholinos, we sought to determine the effects of overexpression of these proteins. A previously made zebrafish GADD34 construct was verified to contain the correct gene sequence, so experiments overexpressing GADD34 in zebrafish embryos were able to be completed. The CReP construct did not show the proper sequence and so re-cloning of this gene was attempted, first through cloning into the PCS3 MT vector, and second by TOPO TA cloning. Further investigation and work is required before suitable mRNA can be synthesized from these constructs.

To assess the effects of overexpressing GADD34 on stress-induced apoptosis in the zebrafish caudal fin epidermal cells, zebrafish GADD34 mRNA was synthesized *in vitro* for injection into zebrafish embryos (Figure 11). *E. coli* competent cells were transformed with PCS3 MT plasmids containing zebrafish *ppp1r15a,* and the ampicillin resistance gene contained in this plasmid allowed for the selection of transformed cells to culture for mini preps. Mini prep samples were then sequenced and were confirmed to contain the Danio rerio *ppp1r15a* gene. The plasmid was then linearized by digestion with the Not1 restriction enzyme (Figure 12) and DNA was extracted by phenol chloroform extraction. Capped mRNA was then synthesized *in vitro* with SP6 RNA polymerase using the mMESSAGE mMACHINE SP6 Transcription Kit. Successful transcription of GADD34 mRNA was confirmed by gel electrophoresis by the presence of the expected 1535 bp band (Figure 13). GADD34 mRNA was then prepared at a concentration of 200 pg/nl for 1 nl microinjection into zebrafish embryos at the one-cell stage. ER stress was induced at 24 hpf by incubating the embryos in 1 µM thapsigargin for either 4 or 24 hours prior to Acridine Orange staining and confocal imaging to assay for apoptosis.

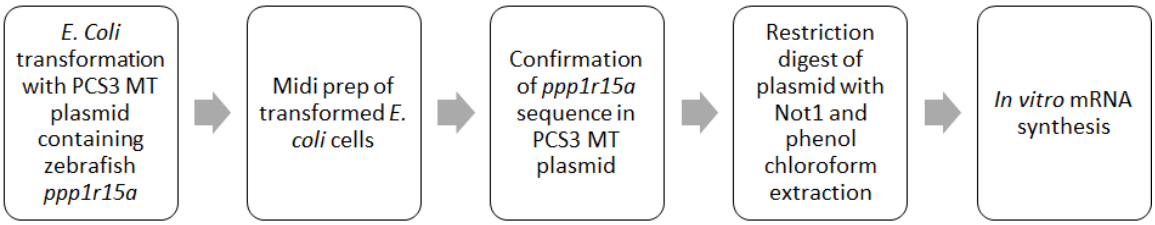

Figure 11: Workflow for *in vitro* synthesis of zebrafish GADD34 mRNA.

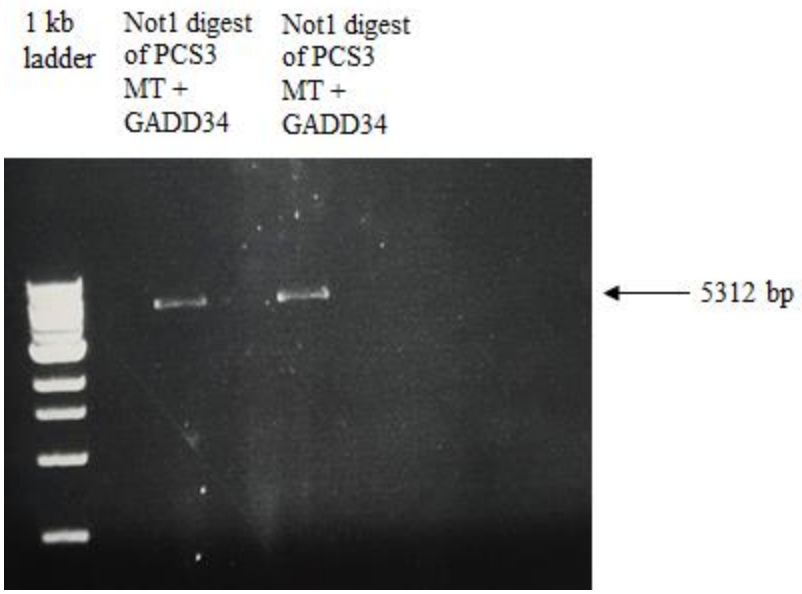

Figure 12: Single digestion of PCS3 MT vector containing zebrafish *ppp1r15a* insert with Not1. The plasmid was linearized by Not1 for *in vitro* transcription of *ppp1r15a*.

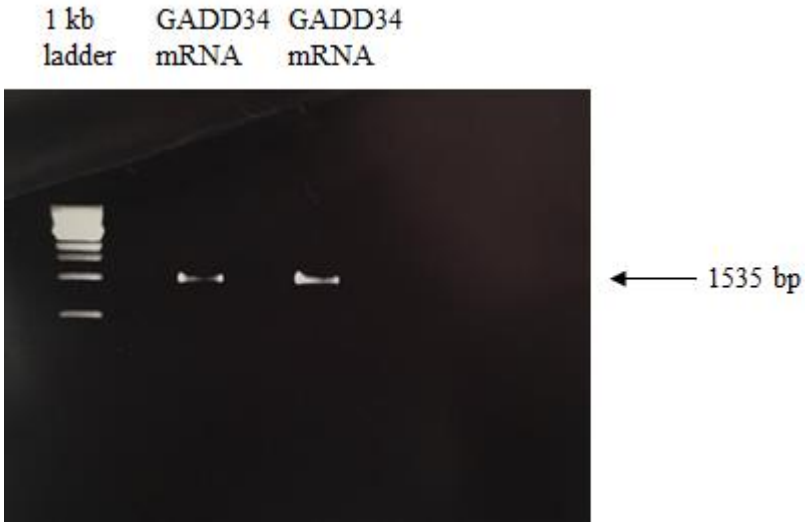

Figure 13: Confirmation gel of GADD34 mRNA. An in vitro transcription reaction was completed to synthesize GADD34 mRNA from the zebrafish GADD34 gene previously inserted into the PCS3 MT vector.

After 4 hours of thapsigargin treatment, embryos injected with GADD34 mRNA surprisingly showed significantly fewer cells undergoing apoptosis than WT embryos ($F_{1, 174}$ = 49, p = 5.1E-11) (Figure 14). However, after 24 hours of thapsigargin treatment, no significant differences were found between the GADD34-overexpressing and non-injected embryos (p > 0.05). Taken with the morpholino results, our studies found that both GADD34 overexpression and GADD34 loss-of-function can protect cells from stress-induced apoptosis, depending on the length of ER stress exposure and therefore the genetic program by which apoptosis is induced. These results indicate that GADD34 may be interacting with the acute-phase apoptotic response to ER stress in a manner independent of its interaction with the chronic-phase apoptotic response.

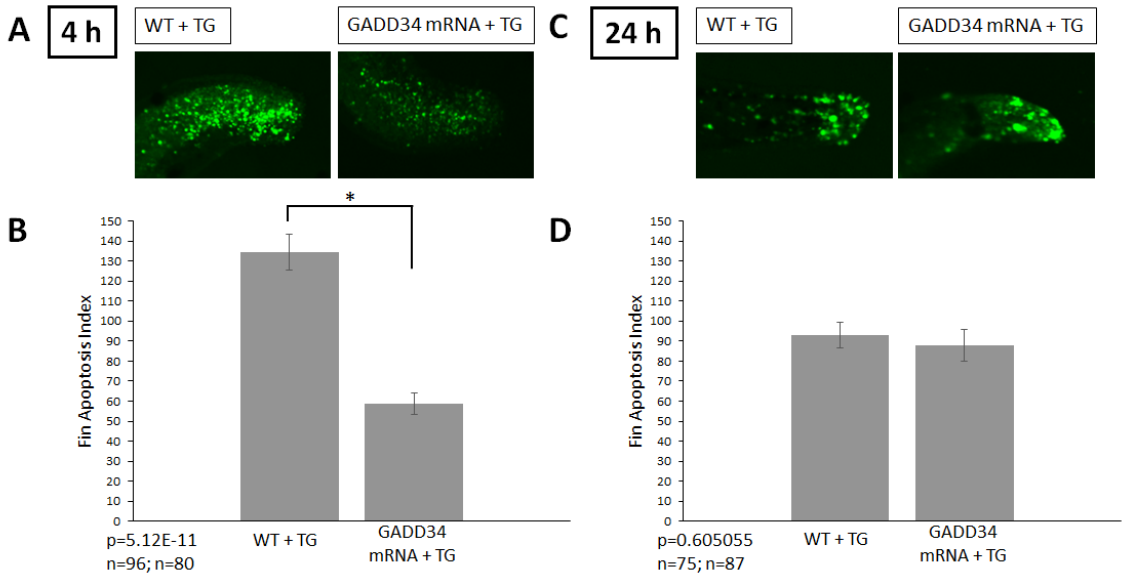

Figure 14: Stress-induced apoptosis levels in zebrafish caudal fin epidermal cells overexpressing GADD34 at 4 and 24 post-stress induction. Embryos were injected with ~1 nl of GADD34 mRNA [200 pg/nl] at the one-cell stage and then treated at 24 hpf with 1 µM Thapsigargin for the indicated treatment time. Apoptosis was then assayed by acridine orange staining, and tails were visualized by confocal microscopy at 491 nm. Cells undergoing apoptosis were quantified by counting the number of fluorescent cells in a defined region of the caudal fin, and bar graphs represent the mean number of cells found within each treatment group, the fin apoptosis index. Error bars represent the standard error of the mean. Significant differences between treatment groups were detected using a 1 factor ANOVA and Holm-Šídák Posthoc comparisons, the * denotes significant differences when $p < 0.05$. Zebrafish caudal fins are shown within each treatment group representing the average number of cells undergoing apoptosis. **A.** Caudal fins of zebrafish overexpressing GADD34 compared to WT after 4 hours TG treatment. **B.** Mean values of apoptotic cells in the caudal fins of zebrafish overexpressing GADD34 compared to WT after 4 hours TG treatment. **C.** Caudal fins of zebrafish overexpressing GADD34 compared to WT after 24 hours TG treatment. **D.** Mean values of apoptotic cells in the caudal fins of zebrafish overexpressing GADD34 compared to WT after 24 hours TG treatment.

Chapter 4: Discussion

GADD34 Overexpression

As previously discussed, two distinct genetic programs for cell death have been found to occur in response to ER stress induction in zebrafish caudal fin epidermal cells (Pyati et al., 2011). Apoptosis occurring at 4 hours post-stress induction was attributed to a p63-puma apoptotic pathway, while apoptosis seen at 24 hours was CHOP-dependent. The results of our studies support a role of eIF2α phosphatases in the promotion of stress-induced apoptosis in chronic ER stress, primarily by GADD34. Additionally, our results indicate a role for GADD34 in the prevention of stress-induced apoptosis in the acute apoptotic response to ER stress. Although these effects of GADD34 may seem paradoxical, the two distinct genetic programs involved in acute and chronic ER stress-induced cell death may allow for the stress-induced protein, GADD34, to exert diverse effects throughout the progression of the UPR to regulate cell death.

The decrease in cell death seen when GADD34 protein was overexpressed during the acute apoptotic response suggests that GADD34 may be able to either directly or indirectly affect the p63-puma apoptotic pathway to prevent apoptosis at this stage of the UPR. Direct interaction between GADD34 and p63 or puma has not been reported; however, previous research on the role of p63 in zebrafish development provides a context in which we can better understand the role of ER stress and GADD34 in p63-dependent apoptosis. It has been well established that p63 transcriptional regulation is required for epithelial development in zebrafish and higher vertebrates (Lee et al., 2002; Ellisen et al., 2002; Mills et al., 1999; Yang et al., 1999). p63 is transcribed from two different promoters which produces two different proteins with opposing functions (Lee et al., 2002). One form contains an N-terminal transactivation domain (TAp63)

and is able to activate p53 target genes making it a potent activator of apoptosis, while the other form (ΔNp63) lacks this domain but can act as a dominant-negative factor that blocks the transcription-activating ability of TAp63, p53 or p73 (Lee et al., 2002; Yang et al., 1998). The ΔNp63 form is expressed early in development in the zebrafish epidermis and is necessary for epidermal cell development and proliferation. This proliferation regulated by ΔNp63 is accomplished through inhibiting the transcription of p53 target genes. The first major burst of epidermal growth occurs at around 20 hpf, contributing to the sensitivity of caudal fin epidermal cells to ER stress-induced apoptosis at the time of stress induction in our experiments at 24 hpf (Lee et al., 2002). We hypothesize that acute ER stress exposure disrupts normal ΔNp63 transcriptional regulation, reducing inhibition of TAp63, which would allow for TAp63 activation of apoptotic targets including *puma*. The specific ways in which ΔNp63 transcriptional regulation is affected by the UPR, and specifically by GADD34 overexpression, remains unknown but offers an exciting direction for future research.

The significant protective effect of GADD34 overexpression during the acute cell death response was unexpected but is not a solitary example of GADD34 having pro-survival effects. GADD34 cytoprotection has been noted in relation to the transient activation of autophagy induced between 15 and 75 minutes of ER stress, discovered in HepG2 and HEK293 cells in response to thapsigargin or tunicamycin (Holczer et al., 2016). These researchers found that increased GADD34 levels in response to acute ER stress were highly correlated with a transient mTOR inactivation and induction of autophagy (Holczer et al., 2015). Studies by Kapuy et al. (2014) revealed that cells coordinate autophagy and apoptosis in a sequential and mutually exclusive manner, dependent upon the severity, duration, and nature of ER stress. Autophagy is activated early in the response to ER stress to contribute towards cell survival for a few hours,

even under high stress conditions. If a certain threshold of intolerable ER stress is met, autophagy becomes inactivated and apoptosis is initiated (Kapuy et al., 2014). The transient boost in autophagy seems to improve cell survival and plays an important role in helping cells remain under the threshold value of ER stress required for apoptosis induction (Holczer et al., 2015; Hyrskyluoto et al., 2012). In starvation conditions and in a model of Huntington's disease in PC6.3 cells, it has been shown that GADD34 activates autophagy via the formation of a stable GADD34-TSC complex and TSC2 dephosphorylation to inhibit mTOR activity (Watanabe et al., 2007; Uddin et al., 2011; Hyrskyluoto et al., 2012). In a model of Huntington's disease, GADD34 overexpression was able to enhance and prolong autophagy and increase cell viability in neuronal cells expressing the disease-causing huntingtin protein (Hyrskyluoto et al., 2012).

For our experiments, ER stress is induced during early zebrafish development when the epidermal cells are producing large amounts of membrane and secretory proteins to form the epidermal basement membrane and therefore experience high basal levels of UPR signaling (Webb et al., 2007). It is plausible that the nature of the acute ER stress these cells experience with thapsigargin treatment is characterized by nutritional stress, due to the high volume of protein synthesis required for epidermal development. These particular ER stress conditions might especially benefit from the transient upregulation of autophagy that occurs early in ER stress. GADD34 overexpression may therefore improve survival under these conditions by promoting and prolonging autophagy through GADD34-dependent inhibition of mTOR. These results may have broader applications in disease conditions impacted specifically by high levels of protein synthesis and susceptibility to nutrient deprivation, as autophagy may help restore nutrient availability and keep ER stress under the threshold required for promotion to cell death. It should be noted that a considerable number of studies have reported GADD34 overexpression

to worsen cell viability in response to ER stress in a variety of systems, so it would seem that the protective effects of GADD34 are possible within highly specific conditions of ER stress (Farook et al., 2013; Adler et al., 1999; Otsuka et al., 2016).

It would be beneficial to further test this hypothesis by analyzing the effects of GADD34 inhibition with the drug guanabenz on the acute apoptotic response to ER stress in caudal fin epidermal cells. Studies by Holczer et al. (2016) found that guanabenz pre-treatment of HEK293T cells significantly worsened cell viability with 1-2 hours of ER stress exposure. Similarly, we hypothesize that within the same context of ER stress, inhibition of GADD34 with guanabenz would reduce caudal fin epidermal cell viability during acute ER stress by decreasing autophagy.

During the chronic apoptotic response to ER stress, GADD34 overexpression did not significantly alter apoptosis compared to WT, which seems consistent with GADD34 loss-of-function results. We saw that GADD34 loss, whether alone or in combination with CReP, consistently showed to be cytoprotective, so it is not surprising that GADD34 overexpression at this same time point had no effect on apoptosis levels.

GADD34 Loss-of-Function

GADD34 loss-of-function was found to protect cells from apoptosis after 24 hours of ER stress, during the chronic apoptotic response to ER stress. The dynamics of UPR progression from the early to late stage provide the foundational framework for interpreting GADD34 loss-of-function results. It will be helpful to survey the research done by Reid et al. (2016) that examined the effects of GADD34 knockout on progression of the UPR and ER stress-induced apoptosis.

Studies by Reid et al. suggested that the early UPR is focused on translational suppression and degradation of misfolded proteins, while the late UPR enhances ER protein folding capacity through increased synthesis of chaperones. A normal response to ER stress shows peak levels of eIF2α phosphorylation between 30 minutes and 1 hour of exposure to ER stress, based on studies using WT MEFs. GADD34 protein is induced to significant levels by 2 hours of ER stress exposure and acts to progress the UPR in two main ways, first by resuming protein translation by reducing levels of phosphorylated eIF2α to near-baseline levels by 4 hours, and second by returning previously released mRNAs of ER-targeted proteins back to the ER. In WT MEFs, the translational activation of ATF4 and CHOP began at 30 minutes of ER stress and protein expression increased in a time dependent manner to significantly higher levels by 4 hours.

Reid et al. showed that MEF cells lacking GADD34 made this transition much later, exhibiting elevated eIF2α phosphorylation still 8 hours after ER stress induction with decline in phosphorylation not occurring until 18-24 hours post-stress induction. Without GADD34, prolonged elevation of phosphorylated eIF2α and early UPR signaling is active for approximately 14-20 additional hours compared to WT cells. The MEFs lacking GADD34 function showed a significant reduction in ATF4 and CHOP expression, although mRNA levels were increased in a similar manner as in the WT. By 24 hours, ATF4 and CHOP protein expression reached nearly complete restoration in GADD34-/- MEFs, despite only 16% recovery of global protein translation. This indicates that the translational recovery of the late UPR favors the translation of these UPR stress response proteins. Furthermore, expression of the Xbp-1 protein was impaired, although mRNA levels and splicing were also comparable to WT. The studies found that the ribosomes were still heavily recruited to these UPR target mRNAs in cells

lacking GADD34, suggesting that lack of protein expression was primarily due to phosphorylated eIF2α inhibition of translation. Analysis of the translation of early and late UPR genes showed that loss of GADD34 causes the increase in early UPR target gene expression to continue rather than decline and a significant suppression of late UPR gene expression. This indicates that without GADD34, the UPR translational program is stalled in the early UPR conditions. Consistent with this stalled UPR, MEFs with GADD34 loss-of-function fail to return released mRNAs to the ER over the time period this is accomplished in WT cells. The GADD34 knockout MEFs showed significantly lower levels of apoptosis markers after 30 hours of tunicamycin treatment. This protective effect of GADD34 loss-of-function was also seen in mouse kidneys exposed to ER stress with tunicamycin for 24 hours. After 4 days, GADD34-/- mice showed reduced levels of multiple UPR genes, including ATF4, CHOP, chaperones and co-chaperones, ERAD genes and Ero1β, and also showed protection from renal lesions and cleaved caspase-3, a hallmark of apoptosis (Reid et al., 2016).

The impacts of GADD34 loss-of-function found in these studies strongly indicate a role for GADD34 in progressing the UPR from the early to late stage. Stalling UPR progression seems to underlie the protective effects rendered by GADD34 loss-of-function seen in the studies described above by Reid et al. (2016) and in our morpholino knockdown studies. GADD34 loss-of-function seems to protect cells experiencing chronic ER stress against a CHOP-dependent stress-induced apoptosis. The precise means by which this protection occurs still remains unknown, although we can speculate that the primary protective effects of GADD34 loss-of-function against chronic ER stress-induced apoptosis occurs through sustained eIF2α phosphorylation, prolonging translational inhibition and reducing the synthesis and accumulation of CHOP protein. The downstream effects of resuming protein translation and of CHOP

signaling are multifaceted and complex but offer many possible means by which a cell experiencing chronic ER stress is progressed toward a CHOP-dependent cell death.

First, CHOP is involved in a variety of ways in significantly disrupting redox homeostasis, which contributes to protein misfolding and sensitizes stressed cells to apoptosis (McCullough et al., 2001; Marciniak et al., 2004). Some studies have found that CHOP inhibits transcription of the anti-apoptotic protein Bcl2, upregulates and promotes the effects of the pro-apoptotic proteins Bim and Bax, and contributes to cell growth arrest (McCullough et al., 2001; Wang et al., 2001). However, research by Han et al. (2013) did not find CHOP or ATF4 to activate the expression of pro-apoptotic genes, but rather found that CHOP and ATF4 upregulate genes involved in the UPR functions of autophagy, mRNA translation, protein synthesis, ATP depletion, and oxidative stress. Adding to these findings, the translation studies by Reid et al. (2016) also did not show any differences in the translation of mRNAs encoding pro- or anti-apoptotic proteins between WT and GADD34-/- MEFs. Finally, CHOP is also known to play a role in promoting GADD34 expression, furthering the recovery of protein translation and potentially worsening ER stress in conditions of continuous or severe ER stress (Marciniak et al., 2004). Although it is not yet confirmed whether CHOP directly affects the transcription of apoptotic genes, it is clear that CHOP contributes to the oxidative stress associated with protein synthesis recovery and also enhances this recovery process to further CHOP accumulation. These later-stage effects of the UPR seem to be capable of sensitizing cells to apoptosis, particularly those experiencing chronic ER stress. Thus, it seems that through loss of GADD34 function, the UPR is stalled at an early state characterized by suppressed protein translation and reduced accumulation of CHOP. Without GADD34, this stalled UPR extends far into chronic levels of ER stress, providing the cell more time to clear misfolded proteins in the ER before

protein synthesis is resumed to a normal workload. The stalled UPR would also prolong low CHOP levels and therefore minimize its downstream effects that progress a cell toward apoptosis. The significantly delayed transition into recovery of protein translation with GADD34 loss-of-function, which favors the translation of ATF4 and CHOP first, may offer UPR dynamics better suited to deal with chronic ER stress. While the normal progression of the UPR with functional GADD34 likely exists to transition cells back to normal functions after transient incidents of ER stress, stalling UPR progression by knockdown of GADD34 function may provide significant benefits to cells dealing with chronic ER stress associated with disease.

CReP Loss-of-Function

While current data now supports some degree of CReP regulation by UPR signaling, GADD34 is still consistently shown to be the primary regulator of eIF2α phosphorylation during ER stress. GADD34 is transcriptionally activated by ATF4 in the PERK-eIF2α pathway and is important for negative feedback regulation of eIF2α phosphorylation during the recovery phase of the UPR, while CReP regulation and function during the UPR is still not fully understood (Novoa et al., 2001). Studies on CReP-/- MEFs showed that the translational recovery profile in response to ER stress was very similar to WT cells, and the return of previously released mRNAs to the ER was also unchanged (Reid et al., 2016). It is known that CReP is negatively regulated by RIDD; IREα cleavage of CReP mRNA contributes to elevating eIF2α phosphorylation and translation attenuation independent of PERK (So et al., 2015). This decline in CReP protein from baseline levels is seen as early as at 2 hours of ER stress exposure in Hepa1-6 cells, with a continual decline to undetectable levels by 12-16 hours of ER stress (So et al., 2015). Before this reduction of CReP, the initial decline of phosphorylated eIF2α that occurs prior to GADD34 reaching detectable levels has been attributed to the presence of CReP (Jousse et al., 2003).

Furthermore, CReP mRNA was reported to show a 10-fold increase in GADD34-/- MEFs after 16 hours of ER stress compared to WT. Taken together, it seems that while CReP is regulated by the UPR to enhance the PERK pathway and can also partially compensate for loss of GADD34 function, CReP loss-of-function alone is not sufficient to significantly alter the dynamics of UPR progression. This supports the results of our CReP morpholino knockdown studies, in which no significant changes in ER stress-induced apoptosis were seen after either 4 or 24 hours of ER stress. With GADD34 still functional during morpholino knockdown of CReP, UPR progression from the early to late stage would not be attenuated, thereby abolishing any protective effects of a stalled UPR with chronic ER stress. Furthermore, since CReP is only involved in eIF2α dephosphorylation for a short time at the onset of ER stress, the ability of CReP loss-of-function alone to enhance eIF2α phosphorylation would be transient and fairly inconsequential to the acute apoptotic cell death program.

GADD34 and CReP Loss-of-Function

The inhibition of GADD34 and CReP together consistently protected cells from both acute and chronic ER stress-induced apoptosis in both experimental approaches utilized: morpholino knockdown and pre-treatment with the small molecule drug salubrinal. Although neither GADD34 nor CReP knockdown individually using morpholinos resulted in protection from acute phase of ER stress-induced apoptosis, it is plausible that a more complete abolition of eIF2α phosphatase activity could have a protective effect even against the p63-puma-dependent cell death program. Since caudal fin epidermal cells experience high baseline UPR signaling during early development, the onset of pharmacological ER stress likely causes intolerable levels of ER stress, driving the acute-phase apoptotic response. With loss-of-function of both GADD34 and CReP at the onset of ER stress, no phosphatase activity would be present to

oppose the PERK-eIF2α pathway. This might result in peak levels of phosphorylated eIF2α to be reached earlier in response to ER stress, without the presence of functional CReP in the initial stages of the UPR. The additional lack of GADD34 function may allow for higher phosphorylated eIF2α levels to be reached. The known stall in UPR progression caused by GADD34 loss-of-function would be sustained until other compensatory mechanisms become activated to oppose the PERK pathway, such as upregulation of the PERK inhibitor, AKT, which has been shown to increase transiently after 8 hours of ER stress exposure (Reid et al., 2016). The combined effects of GADD34 and CReP loss-of-function likely results in a faster and more potent increase in phosphorylated eIF2α, resulting in a more rapid attenuation of protein translation and onset of the early UPR. With faster entry into the early UPR, the ER may gain a greater capacity to release mRNAs encoding ER-targeted proteins which occurs during acute stress. The degree of mRNA released from the ER has been found to correlate positively with the increase of eIF2α phosphorylation, with around 50% of mRNAs released from the ER at the peak of eIF2α phosphorylation (Reid et al., 2014). In addition to earlier mRNA release from the ER, earlier attenuation of protein synthesis would also decrease the ER's protein folding load, further reducing acute ER stress. The pro-survival benefits of GADD34 and CReP loss-of-function together against acute phase apoptosis are likely a result of their combined ability to alleviate acute ER stress. Increased survival seen at the chronic apoptotic response is probably primarily driven by the effects of GADD34 loss-of-function: stalled progression of the UPR. Since a more dramatic pro-survival effect is seen with complete eIF2α phosphatase loss-of-function compared to GADD34 alone, the additional loss of CReP function likely enhances eIF2α phosphorylation in the ways discussed above. Total loss of eIF2α phosphatase activity

seems to offer cells an even greater resistance to stress-induced apoptosis caused by chronic levels of ER stress.

Salubrinal inhibition of eIF2α phosphatases. A wide range of research has documented various pro-survival benefits of salubrinal treatment, lending further insight into the mechanisms by which salubrinal specifically may protect cells against ER stress-induced apoptosis. Although it is not known precisely how salubrinal blocks the eIF2α phosphatase complexes and whether it has other direct cellular interactions, the cytoprotective impact of salubrinal is commonly attributed to the effects of sustained eIF2α phosphorylation (as explained above) and suppression of apoptotic signaling pathways. The first research to discover salubrinal found that its ability to protect PC12 cells from ER stress-induced apoptosis by tunicamycin was consistently correlated with its ability to enhance eIF2α phosphorylation (Boyce et al., 2005). Salubrinal was able to protect neurons against chronic ER stress-related disease progression by significantly delaying the UPR in mice models of ALS (Saxena et al., 2009). In rodent models of Parkinson's Disease, salubrinal extended life span in diseased animals and significantly attenuated neurodegeneration caused by chronic ER stress by slowing the rate of disease onset and disease symptoms (Colla et al., 2012). Salubrinal was consistently found to protect cells from damage and apoptosis induced by an extensive selection of xenotoxicants after both acute and chronic levels of exposure (Matsuoka and Komoike, 2015). Overall, the cytoprotection of salubrinal against the majority of xenotoxicants was attributed to the prevention of eIF2α dephosphorylation and enhanced inhibition of global protein synthesis, which alleviate the ER workload and conserve cellular energy (Matsuoka and Komoike, 2015).

Additionally, there is evidence to support a role for salubrinal in suppressing apoptotic signaling. Studies by Boyce et al. (2005) found that salubrinal treatment reduced several

markers of apoptosis, including caspase-7 processing, the activity of caspase-3 and caspase-7 type enzymes, and DNA fragmentation in PC12 cells treated with tunicamycin. Salubrinal also protected HK-2 cells from apoptosis when exposed to ER stress induced by cadmium chloride and reduced caspase-3 cleavage and levels of CHOP protein (Komoike et al., 2012). Decreased apoptosis was seen with salubrinal treatment in studies on ER stress induced by photodamage in murine leukemia cells (Kessel, 2006). Salubrinal protected these cells from apoptosis by preventing inactivation of anti-apoptotic protein Bcl-2 caused by photodamage, and salubrinal was also confirmed to protect Bcl-2 against its non-peptidic antagonist, HA14-1 (Kessel, 2006). A different study in a rat model of poisoning with paraquat found that salubrinal increased Bcl-2 levels and prevented pulmonary damage and apoptosis (Matsuoka and Komoike, 2015). Research on xenotoxicant-induced cellular damage found that salubrinal sometimes inhibited two apoptotic signaling pathways induced by the IRE1 pathway. Although this interaction could be indirect through enhanced PERK signaling, salubrinal may be directly interacting with proteins in the ASK1/JNK-mediated and caspase-12-dependent apoptotic pathways, which are activated by phosphorylated IRE1 (Matsuoka and Komoike, 2015). In summary, these various studies reveal that salubrinal might also interact directly with apoptotic pathways to protect cells from ER stress-induced apoptosis. The inhibition of eIF2α phosphatases likely accounts for a majority of the pro-survival benefits of salubrinal treatment during acute and chronic ER stress in the caudal fin epidermis, but it would be interesting to further investigate whether these other mechanisms of salubrinal action contribute to cell survival during the UPR.

Future Directions

A wide variety of future experiments would further the research in this study and contribute to a deeper level of understanding of the role of eIF2α phosphatases in ER stress and

stress-induced apoptosis, as depicted in Figure 15. The first major goal is to create mutant zebrafish lines lacking GADD34, CReP, and GADD34 and CReP together using the CRISPR-Cas9 genome editing system. This approach disrupts the *ppp1r15a* and *ppp1r15b* genes, allowing for complete knockdown of GADD34 and CReP protein. Established mutant zebrafish lines will allow experiments to be done on large numbers of embryos that already have complete GADD34 and CReP loss-of-function, eliminating the need for morpholino injection. This will streamline many procedures and give our lab a greater capacity to dissect the roles of eIF2α phosphatases in ER stress. While ultimately we hope to have established zebrafish lines with these mutations, experiments can also be done on F0 fish to verify loss-of-function results and even provide preliminary data for other experiments.

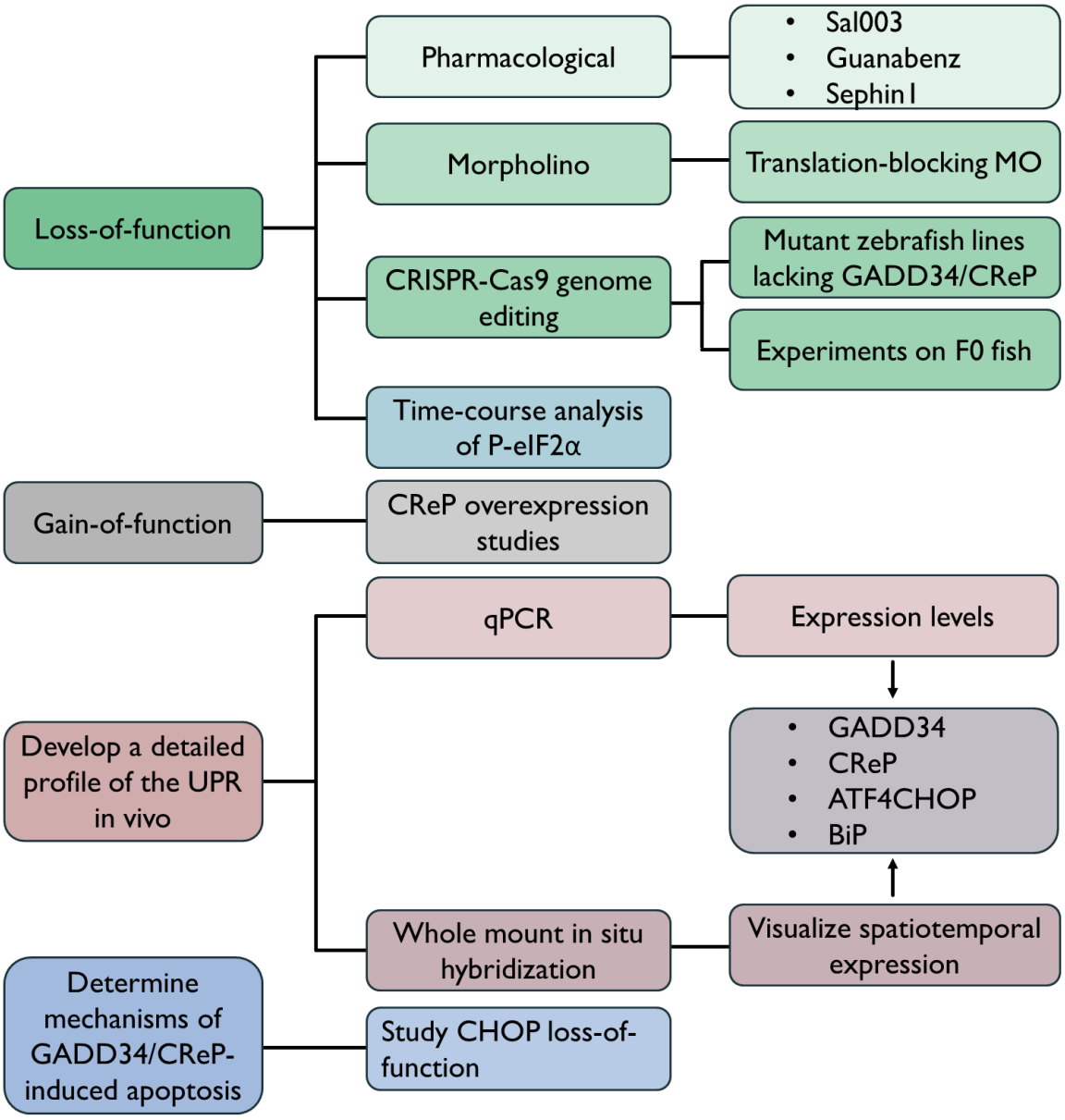

Figure 15: Diagram outlining future research directions.

Further experiments exploring loss-of-function of eIF2α phosphatases can be done to substantiate the results obtained thus far. For pharmacological inhibition of eIF2α phosphatases we would like to run drug treatment experiments using a more potent and soluble salubrinal analog, sal003, as well as with two drugs that inhibit GADD34 only, guanabenz and sephin1. Additionally, we would like to utilize translation blocking morpholinos to confirm results seen using splice blocking morpholinos. We also hope to complete the gain-of-function studies by examining the effects of CReP overexpression on caudal fin stress-induced apoptosis. We are currently analyzing the *ppp1r15b* gene sequence that we have cloned into the PCS3 MT vector and may need to insert the gene into a different vector before it can be used to synthesize mRNA for microinjection. It will be of particular interest whether CReP overexpression exerts the same protective effects as GADD34 at the acute cell death response.

Next, we would like to determine whether GADD34 and CReP contribute to apoptosis in a CHOP-dependent manner. This can be accomplished through injecting morpholinos targeting CHOP into WT, GADD34 mutant, CReP mutant, and double mutant embryos. If GADD34 and CReP protect cells by regulating CHOP, then we hypothesize that CHOP loss-of-function in the WT should show no difference in apoptosis relative to the mutants.

Finally, we would like to measure various metrics of UPR signaling at multiple time points following ER stress induction. This would include the use of quantitative PCR to measure the expression levels of GADD34, CReP, BiP, ATF4, and CHOP at several time points between 1 and 48 hours of ER stress exposure. In coordination with these experiments, we would also perform whole mount in situ hybridization to examine the spatiotemporal expression of these same genes. Furthermore, we would like to perform a detailed in vivo time-course analysis to examine the relative contributions of GADD34 and CReP to eIF2α dephosphorylation during ER

stress. This would be accomplished through western blot analysis of phospho-eIF2α in WT and loss-of-function embryos at multiple time points following ER stress.

Certainly, there is much left to be understood regarding the roles of GADD34 and CReP in the UPR and their role in determining a cell's fate in response to ER stress. Nonetheless, the protective effects seen in inhibiting eIF2α phosphatases and overexpressing GADD34 indicate that treatment options for multiple diseases may be accomplished through manipulation of GADD34 and CReP. In diseases affected by cell death resulting from chronic ER stress and prolonged UPR signaling, therapies that inhibit eIF2α phosphatases may improve disease phenotypes by stalling progression of the UPR. Moreover, diseases or conditions affected primarily by high demands for protein synthesis and intolerable initial levels of ER stress at the acute apoptotic response may benefit from therapeutics that upregulate or increase GADD34 to promote autophagy. Altering the influence of eIF2α phosphatases in the UPR to minimize ER stress-induced apoptosis seems to be a promising future therapeutic for the vast number of diseases and conditions exacerbated by ER stress.

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