ABSTRACT

Title of Document: **CHANGES IN eIF2α PHOSPHORYLATION IN RESPONSE TO NUTRIENT DEFICIENCY AND OTHER STRESSORS IN FISH**

Chieh-lun Liu, Doctor of Philosophy, 2015

Directed By: Associate Professor Rosemary Jagus, University of Maryland Center Environmental Sciences

The present study investigates the response of two teleost species to stressors as measured by phosphorylation of the α-subunit of the translational initiation factor, eIF2. The phosphorylation of the translational initiation factor, eIF2, on its α-subunit is an adaptive response to a variety of stressors in eukaryotes from protists to vertebrates. There are four eIF2α-specific kinases in most vertebrates, GCN2, PERK, PKR and HRI, each of which can be activated by different stressors. Two of these eIF2α-kinases, GCN2 and PERK, respond to changes in nutritional status. It was of particular interest to determine whether eIF2α phosphorylation can be used as an early marker to evaluate diets in fish.

However, eIF2α phosphorylation could also be of use in monitoring other stressors of fish in aquaculture. The increase in global aquaculture production of fish has increased interest in the optimization of fish diets and health to increase production and sustainability. Studies were initiated in a zebrafish *Danio rerio* cell line, ZFL cells, to lay the groundwork for looking at eIF2α phosphorylation in fish and in species of more interest to aquaculture. All the eIF2α-kinases are present in the zebrafish genome and are expressed in ZFL cells. Two forms of eIF2α are expressed in ZFL cells, eIF2α-a and eIF2α-b, with eIF2α-b transcripts ~5-fold higher than those of eIF2α-a. The two gene products are 96 % identical at the amino acid level and are identical at the phosphorylation and kinase docking sites. Phosphorylation of eIF2α in ZFL cells is increased by a variety of agents/conditions; starvation, leucinol, endoplasmic reticulum stress, poly(I)poly(C) and N-methylprotoporphyrin, consistent with activation of the eIF2α kinases GCN2, PERK, PKR and HRI, respectively. Application of the same analyses to a new cell line from a marine fish, cobia *Rachycentron canadum*, shows that these cells are also responsive to activators of GCN2 and PERK. Cloning of eIF2α cDNA from cobia has shown close identity to the zebrafish eIF2αs. Although cobia has two eIF2α transcripts, the coding sequence of each is identical. Preliminary studies have shown that in cobia juveniles, diet, probiotics and water temperature all affect the phosphorylation state of eIF2α.

CHANGES IN eIF2α PHOSPHORYLATION IN RESPONSE TO NUTRIENT DEFICIENCY AND OTHER STRESSORS IN FISH

By

Chieh-lun Liu

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Advisory Committee:
Associate Professor Rosemary Jagus, Chair
Professor Allen R. Place, co-advisor
Associate Professor Shaojun Du
Assistant Professor Eric J. Schott
Dr. José Antonio Fernández Robledo

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Dedication

This work is dedicated to my family, for all of their love and support of my journey in science. To my mother, Yu-Ying Huang, for giving me the strength to move thousands miles away from home to chase my dreams. To the memories of my father, Sin-Yang Liu. His constant words of encouragement and support have allowed me to work through challenges all my life.

To my elder sister, Kay, for her encouragement to continually achieve no matter the endeavor. And to my younger sister, Jamie, for helping to take care of my mother and always supporting me. To my daughter, Phoebe, for helping me to improve my English and understand US culture through the wide open mind of a child. To my son, Jayden, through preparing formula for you during the night while I still need to work on my dissertation. And finally to my wife, Hui-wei, still remembering who I am, always there cheering me on and standing by me through the good times and bad. Thank you for everything you did, I would not have made it this far without you.

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in data analysis and fish sampling. Grant Jones, Kate Gillespie and Erica Dasi for all of their assistance in lab work.

Statement of Contribution

Dr. Aaron Watson performed the fish diet trials and measured taurine levels. Dr. Eunseok Choi developed the zebra fish eIF2α-bS51A constructs. Dr. Yu-Hsuan Kai developed CM cell line in Dr. Shau-Chi Chi's laboratory. Ms. Erica Dasi performed the *in vitro* phosphorylation of zebrafish eIF2α. Michelle Price (Plant Sensory Systems) performed the amino acid analysis of fish diets (Waters Corporation, Milford MA USA).

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List of Abbreviations

ADO	cysteamine dioxygenase
ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
BiP	Immunoglobulin binding protein or glucose regulated protein 78
CDO	cysteine dioxygenase
CHOP	C/EBP homologous protein (also called GADD-153 growth arrest DNA damage-inducible protein)
CRISPR/Cas	Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated genes
CSD/CSAD	cysteinesulfinate decarboxylase/cysteine sulfinic acid decarboxylase
DNA	deoxyribose nucleic acid
DTT	dithiothreitol
eIF2α	alpha-subunit of eukaryotic initiation factor-2
eIF2B	eukaryotic initiation factor 2B (guanine nucleotide exchange factor)
EIF2AK1	eukaryotic translation initiation factor 2-alpha kinase 1 (HRI)
EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2 (PKR)
EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3 (PERK)
EIF2AK4	eukaryotic translation initiation factor 2-alpha kinase 4 (GCN2)
ER	endoplasmic reticulum
FAO	Food and Agriculture Organization of the United Nations
FBS	fetal bovine serum
FCR	feed conversion ratio
FLD	fatty liver disease
FM	fish meal base diet
GCN2	general control non-derepressible-2
GMO	genetically modified organism
GFP	green fluorescent protein
GRP-78	glucose-regulated protein
GTP	guanosine triphosphate
HRI	heme-regulated inhibitor kinase
IACUC	Institutional Animal Care and Use Committee
IκB	inhibitor of NF-κB
IMET	Institute for Marine and Environmental Technology
IPNV	Infectious Pancreatic Necrosis Virus
IRES	internal ribosome entry site
LCMS	liquid chromatography mass spectrophotometer

LMRCSC	Living Marine Resources Cooperative Science Center (NOAA-EPP)
ISR	integrated stress response
MBH	mediobasal hypothalamus
Met-tRNA^{iMet}	methionylated initiator tRNA
NF-κB	nuclear factor κB
NOS	nitric oxide synthase
NVV	nervous necrosis virus
OB fold	oligonucleotide-binding fold
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEPCK	phosphoenol pyruvate carboxykinase
PERK	endoplasmic resident PKR-like or pancreatic eIF2α kinase
PKR	protein kinase double-stranded RNA-dependent
Poly (I:C)	polyinosinic:polycytidylic acid
PP	plant protein base diet
PUFA	polyunsaturated fatty acids
RACE	Rapid amplification of cDNA ends
RNA	ribonucleic nucleic acid
RT-PCR	reverse transcriptase – PCR
RT-qPCR	reverse transcriptase – quantitative PCR
SERCA	sarcoendoplasmic reticulum (SR) calcium transport ATPase
SGR	specific growth rate
TAUT	taurine transporter
TBT	tributyltin
TGD	teleost specific whole genome duplication
Tg	thapsigargin
TILLING	targeting induced local lesions in genomes
tRNA	transfer RNA
USDA	United States Department of Agriculture
uORF	upstream open reading frames
UPR	unfolded protein response
UV	ultraviolet
VSIEF	vertical slab isoelectric focusing
WGD	whole-genome duplication
WISH	whole-mount in situ hybridization
WRS	Wolcott-Rallison syndrome

Chapter 1: General Introduction

Regulation of protein synthesis at the level of translation is a rapid and effective means for the cell to respond to many different stressors, including those affecting nutrition, such as deficiencies of amino acids or glucose and high-fat, as well as the accumulation of misfolded proteins in the endoplasmic reticulum (reviewed, 2). A central mechanism for translational control involves phosphorylation of the α-subunit of eukaryotic initiation factor 2, eIF2, which represses the initiation phase of protein synthesis, allowing cells to conserve resources while a new gene expression program is activated to prevent stress damage (reviewed, 2)

1.1. What is eIF2?

Eukaryotic initiation factor 2 (eIF2) is an essential eukaryotic translational initiation factor that brings the initiator tRNA to the small ribosomal subunit and participates in the ribosomal selection of the start codon (reviewed, 3-5). eIF2 is a heterotrimer consisting of three subunits; the α-subunit which is the target of regulatory kinases (at serine 51), the β-subunit which recognizes the initiation codon and mediates the binding to eIF2B, and the γ-subunit, the catalytic subunit that binds GTP and Met-tRNAi (6-9). The subunits of eIF2 are conserved between Archaea and eukaryotes (10-12). The first step of Met-tRNAi binding to the small ribosomal subunit is the formation of a stable ternary complex composed of the charged initiator Met-tRNAi molecule, GTP, and eIF2. In association with other initiation factors, this pre-initiation complex binds mRNA 5′-

untranslated regions near the mRNA cap and scans downstream until it encounters an initiation AUG codon (reviewed, 5, 7, 13, 14). The function of eIF2.GTP-bound form is not only to align the initiation codon in the partial P-site on the 40 S ribosomal subunit, but also to hydrolyze the bound GTP, and to release Met-tRNA, into the P-site of the small ribosomal subunit allowing the final steps of the initiation process to occur (5, 8, 15). Prior to 60 S subunit joining to form the elongation-competent 80S ribosome with tRNAi with the start codon base paired in the 40S P-site (16), the eIF2-bound GTP is hydrolyzed and eIF2 is released from the ribosome bound to GDP as an inactive binary complex (5, 7). To participate in another round of translation initiation, this GDP must be exchanged for GTP. eIF2 has a higher affinity for GDP than GTP and exchange requires the guanine nucleotide exchange factor, eIF2B, to regenerate active eIF2.GTP (17-20) (Figure 1.1).

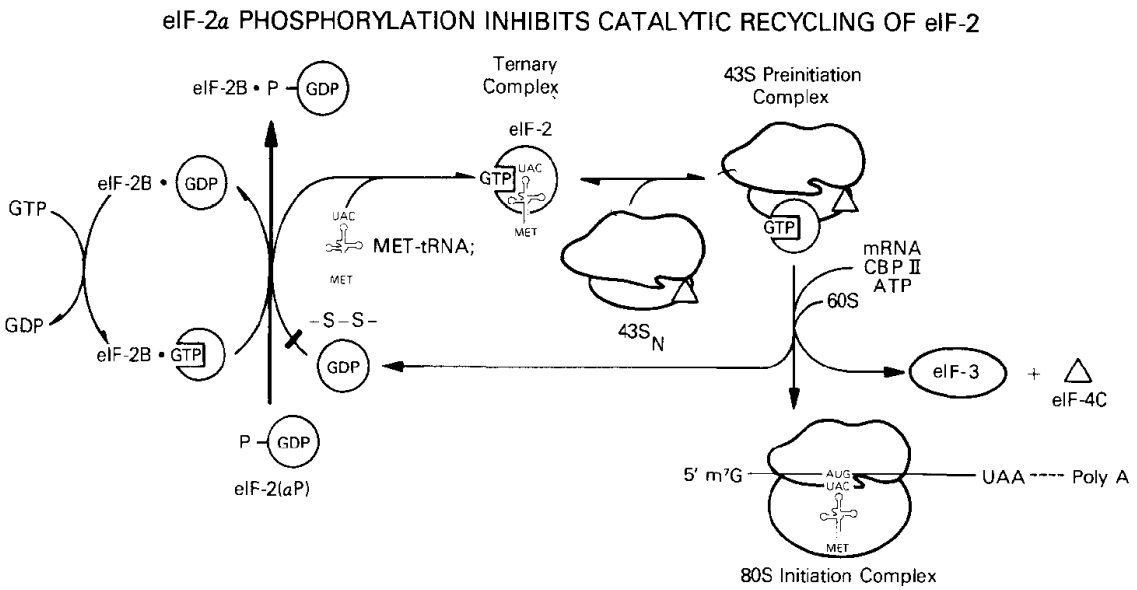

Figure 1.1. eIF2α phosphorylation inhibits catalytic recycling of eIF2B
This series of discrete steps begins with assembly of the 43S preinitiation complex. The 43S PIC is then loaded onto an activated messenger RNA (mRNA)–protein complex near the 5′ cap. Subsequent scanning of the mRNA is accompanied by GTP hydrolysis by the TC without release of phosphate (Pi) from eIF2-GDP. Recognition of the start codon triggers downstream steps in the pathway; Pi release from eIF2; and conversion to the closed, scanning-arrested conformation of the PIC. eIF5B in its GTP-bound form promotes joining of the 60S subunit to the PIC, accompanied by release of eIF5B-GDP form the 80S initiation complex (IC), ready for the elongation phase of protein synthesis. eIF2-GDP, released after subunit joining, is then recycled back to eIF2-GTP by the exchange factor eIF2B; this reaction is impeded by eIF2α phosphorylation. Adopted from (21).

1.2. What is the role of eIF2α phosphorylation?

The activity of eIF2 is regulated by phosphorylation of its α-subunit (reviewed (3, 22, 23). When the α-subunit of eIF2α is phosphorylated at Ser51, it binds with a higher affinity to eIF2B than nonphosphorylated eIF2α, changing from a substrate to an inhibitor of the guanine nucleotide exchange factor, eIF2B. This leads to the inhibition of protein synthesis initiation (3, 17, 20). While the relative amounts of eIF2 and eIF2B vary among different tissues and organisms, eIF2 is always present in excess of eIF2B (21). This means that a small level of phosphorylated eIF2α can severely reduce GDP-GTP exchange activity of eIF2B

preventing eIF2 from returning to its active (GTP-bound) state. Reducing the circulation number of eIF2B in the cell effectively inhibits the translation process. The ensuing reduction in eIF2–GTP levels lowers general translation, allowing cells sufficient time to correct the stress damage, and selectively enhance gene-specific translation that is important for stress remediation. By limiting protein synthesis during stress, this adaptation conserves energy and spares limited resources (Figure 1.2).

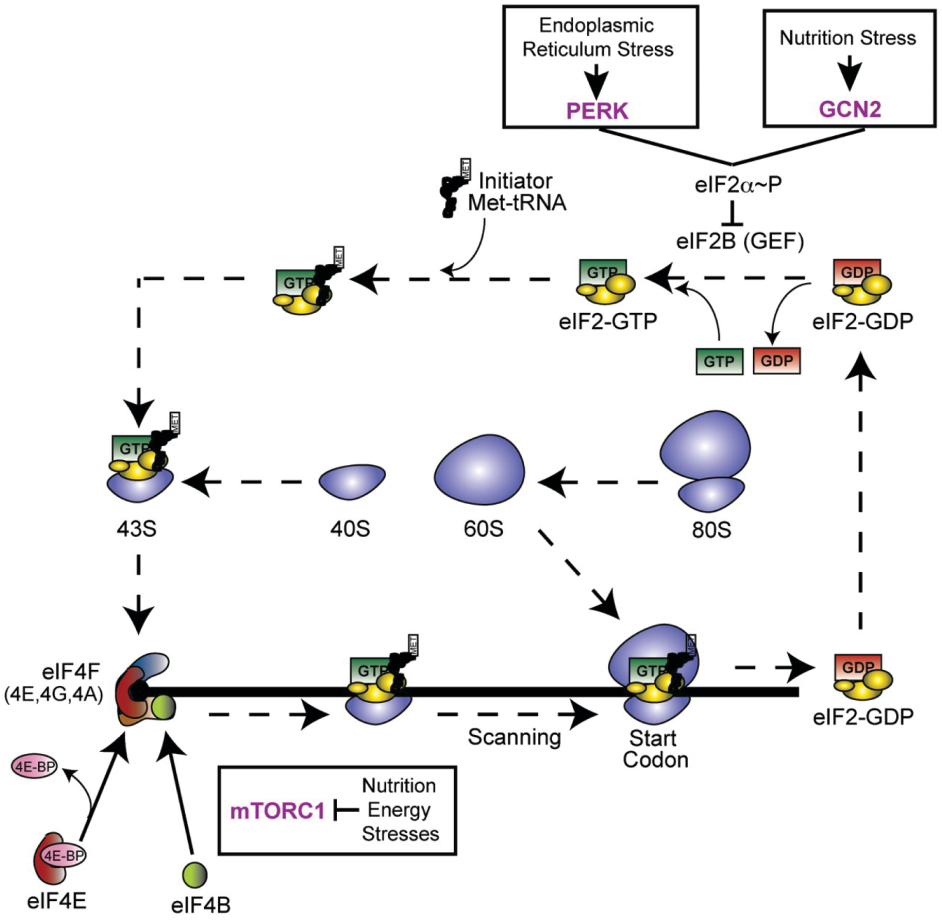

Figure 1.2. Regulation of translation initiation is a rapid means for coupling nutrient deprivation and other stress conditions with levels of protein synthesis. This illustration shows the dissociation of the 80S ribosome complex into the individual 40S and 60S ribosomal subunits, which participate in translation initiation in conjunction with additional translation factors to initiate protein synthesis. Adopted from (2).

Along with the global translational inhibition, eIF2α-P selectively enhances the translation of mRNAs with upstream open reading frames (uORFs). A well investigated example of a uORF-containing mRNA regulated by eIF2α phosphorylation is that encoding activating transcription factor 4, ATF4, a transcriptional activator of genes. Translational regulation of ATF4 involves a delayed reinitiation mechanism featuring the two upstream ORFs (uORFs) in the 5′ leader of the ATF4 mRNA (2, 24, 25) (Figure 1.3). The 5′ proximal region to uORF1 is a positive-acting element that facilitates ribosome scanning and reinitiation at downstream coding regions in the ATF4 mRNA. During non-stressed conditions, when eIF2α-P is low and there is abundant eIF2/GTP/Met-tRNAiMet complex, the 40 S ribosomal subunit can easily scan downstream of uORF1 to reinitiate at uORF2. uORF2 does not have an appropriate context around its termination codon to maintain the ribosome on the mRNA, so if the ribosome initiates at this position, the ribosome falls off after encountering the stop codon and rarely reaches the authentic initiation codon, thus blocking ATF4 expression (reviewed, 26). During stress conditions, phosphorylation of eIF2α and the accompanying reduction in the levels of eIF2-GTP lead to lower levels of eIF2α.GTP.Met-tRNAi and increases the time required for the scanning ribosome to become competent to reinitiate translation. When translation is terminated, the 60 S subunit dissociates at the stop codon of the first uORF, the 40 S subunit remains associated with the mRNA and resumes scanning. However, without eIF2α.GTP.Met-tRNAi the 40 S subunit is not competent to initiate at uORF2. The 40 S scans through the inhibitory uORF2 and instead reinitiates at the ATF4-

authentic initiation codon producing ATF4 (Figure 1.3) (2). This translational control mechanism, first described for the *S. cerevisiae* bZIP transcription factor GCN4 (27), relies on the coordinated interplay between a positive-acting uORF1 and one or more downstream repressing uORFs to sense the levels of eIF2α-P and active eIF2 (2). Such a mechanism results in increased recruitment for a subset of uORF-containing mRNAs that enable cells or animals to respond to a range of stressors (25). Short-term induction of eIF2α phosphorylation has been associated with cell adaptation to survival whereas long-term induction can promote apoptosis (reviewed (28)).

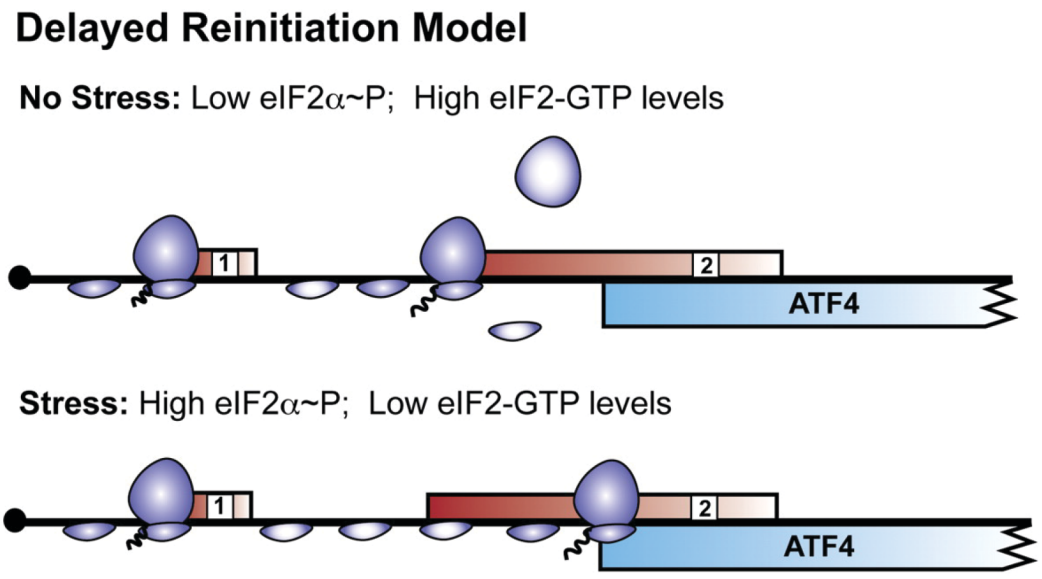

Figure 1.3. **Mechanism of preferential translation during phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2a~P).**

In this delayed translation reinitiation example of ATF4, there are two upstream open reading frames (uORF) (red boxes) in the 5' leader of the ATF4 mRNA. Phosphorylation of eIF2 reduces eIF2–GTP levels and facilitates the bypass of the inhibitory uORF2, thereby enhancing translation of the ATF4-coding region in response to stress conditions. Adopted from (2).

ATF4 enhances the expression of additional transcription factors, ATF3 and CHOP (CCAAT/enhancer-binding protein homologous protein)/GADD153

(growth arrest and DNA-damage-inducible protein), that assist in the regulation of genes involved in metabolism, the redox status of the cells and apoptosis (24, 29-31). Reduced translation resulting from the phosphorylation of eIF2α can also lead to activation of stress-related transcription factors, such as NF-κB (nuclear factor κB), by lowering the steady-state levels of short-lived regulatory proteins such as IκB (inhibitor of NF-κB) (32, 33). NF-κB coordinates the transcription of genes involved in immune and inflammatory responses, cell growth and apoptosis in response to a range of environmental stresses, including UV irradiation and amino acid depletion (34). While many of the genes induced by eIF2 phosphorylation are shared between different environmental stressors, eIF2α-kinases function in conjunction with other stress-response pathways, such as those regulated by mitogen–activated protein kinases, to elicit gene expression programs that are tailored for the specific stress condition. The idea that ATF4 is a common downstream target that integrates signaling from multiple eIF2α-kinases has led to the eIF2α-P/ATF4 pathway being referred to as the integrated stress response (ISR) (Figure 1.4). Loss of eIF2α-kinase pathways has been demonstrated to have a range of health consequences in humans and mice (2).

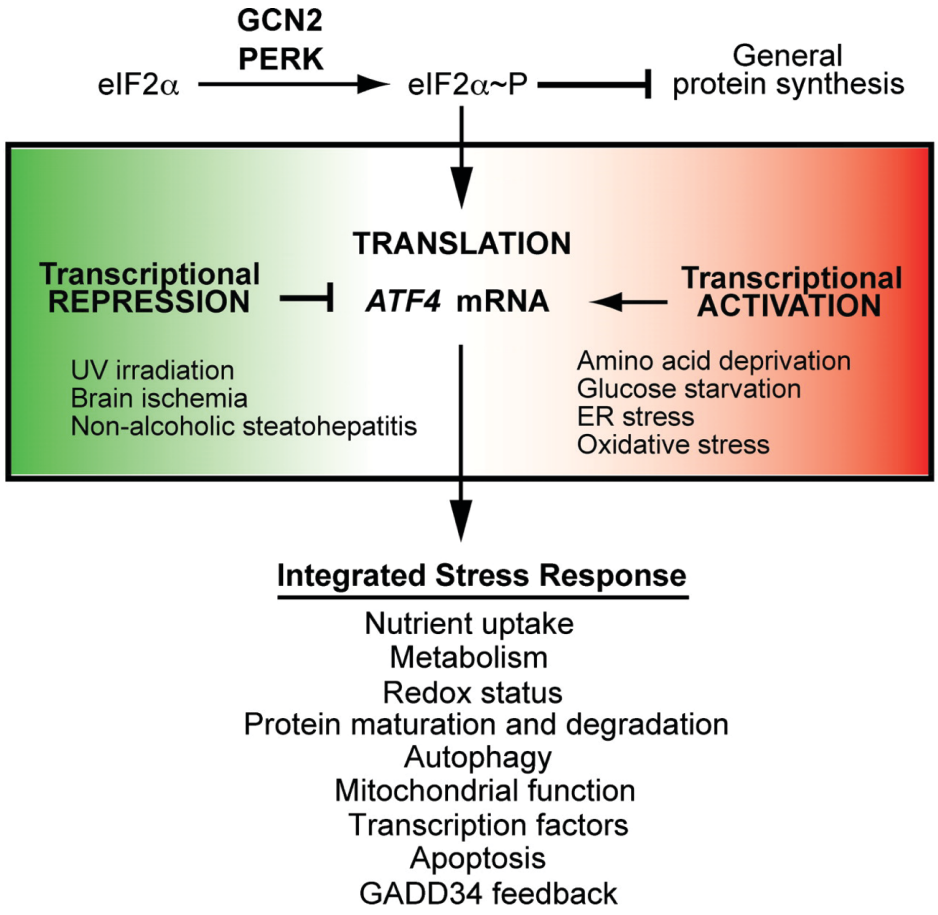

Figure 1.4. **Integrated stress response: transcriptional regulation of ATF4 enables differential expression of integrated stress response (ISR) genes.**
In response to nutritional deprivation and other diverse stress conditions, phosphorylation of eukaryotic initiation factor eIF2α by GCN2 or PERK represses global translation. Adopted from (2).

1.3. eIF2α-kinases:

The eIF2α-kinase family in most vertebrates consists of four members that can respond to different stressors to phosphorylate eIF2α: HRI (EIF2AK1), PKR (EIF2AK2), PERK (EIF2AK3/PEK), and GCN2 (EIF2AK4) (25) (Figure 1.5). These eIF2α-kinases are activated by different cellular stresses while facilitating programs of stress-induced gene expression. There are five eIF2α-specific kinases in vertebrates (five in teleost fish, four in tetrapods) that can phosphorylate eIF2α, each of which is activated by different stressors. In

mammals, two of the eIF2α kinases respond to nutrient deficiencies. HRI (EIF2AK1) is stimulated by heme depletion in erythroid cells and also by oxidative stress, osmotic shock, and heat shock (35, 36). HRI is the only eIF2α kinase activated by arsenite in erythroid cells (36) and is the major eIF2α kinase responsive to heat shock. PKR (EIF2AK2) is stimulated by viral infection (37, 38). PKR also plays a more general role in cellular physiology; it can be activated in response to signals as diverse as oxidative and ER stress (39-41), as well as cytokine and growth factors signaling (42, 43) and has been implicated in the pathology of obesity (44, 45). PERK (EIF2AK3) is an endoplasmic reticulum (ER) transmembrane protein activated by the accumulation of misfolded proteins in the ER, a phenomenon termed the unfolded protein response (UPR) or ER stress (46). Changes in Ca^{2+} levels within the ER negatively affect the ability of the chaperone protein, binding immunoglobulin protein BiP, (also known as 78 kDa glucose-regulated protein GRP-78), to maintain PERK in its inactive state (47) allowing diverse signals such as inhibition of the SERCA calcium pump, glucose deprivation, and high levels of fatty acids reduce the ER luminal calcium concentration (48). GCN2 (EIF2AK4) is a sensor of amino-acid availability (49). GCN2 is the primary responder to nutritional deprivation and is the only eIF2α-kinase conserved among all metazoans. It is activated by reduced amino acid levels in yeast and mammalian cells (27, 50, 51) and can also be activated by glucose deprivation (52). GCN2 is also involved in a variety of organismal functions in vertebrates such long-term memory formation and feeding behavior (53).

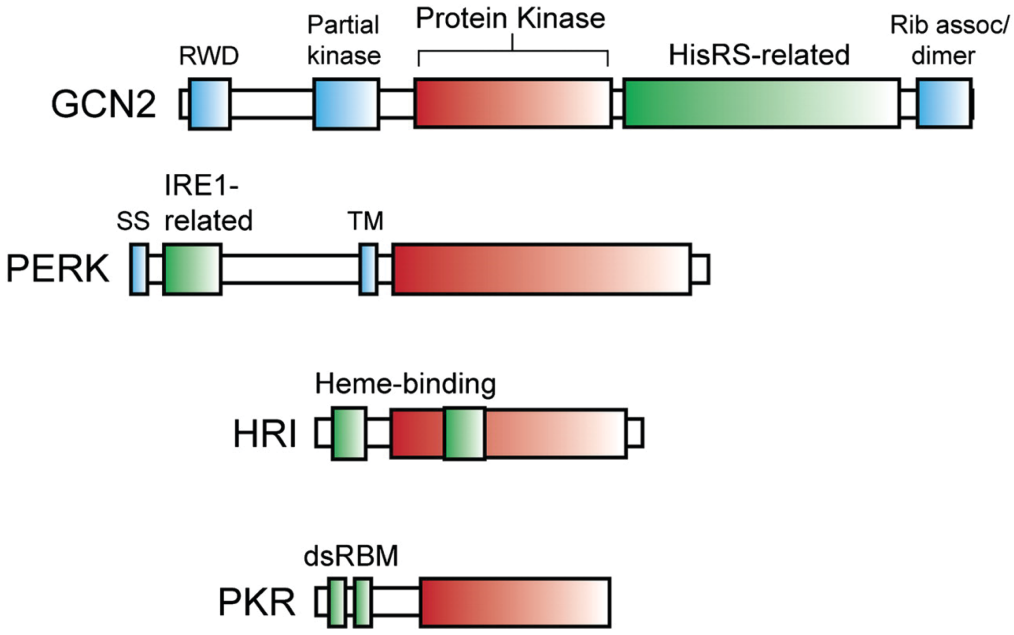

Figure 1.5. **All family members of eukaryotic initiation factor (eIF)2α-kinases are activated in response to diverse stress conditions.**
The red box indicated related protein kinase domain, which facilitate induction of phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α~P) in response to different stress conditions. Adopted from (2).

These four members of the eIF2α kinases share extensive homology in their kinase catalytic domains and phosphorylate eIF2α at the same serine residue (Ser51) (reviewed, 54). Teleost fish have a PKR-like kinase, PKZ, that is more closely related to the kinase domain of PKR compare to other three known eIF2α kinases (55). Phosphorylation of eIF2α on serine 51 or its equivalent reduces the rate of protein synthesis overall. However, it also increases the recruitment of a subset of mRNAs that results in the transient activation of an alternate program of gene expression that enables the cell or organism to respond to a range of stressors (2, 25). Dysfunctions in each of these eIF2α-kinases are linked with pathologies in multiple organs, emphasizing their critical roles in the recognition and alleviation of environmental stress emphasizing their critical role in the recognition and alleviation of stress (2).

1.4. GCN2 and nutrition:

GCN2 is an eIF2α-specific serine/threonine-protein kinase that senses amino acid deficiency through binding to uncharged transfer RNA (tRNA) (50, 56). GCN2 is activated by uncharged tRNA in amino acid-starved mammalian cells, as well as in yeast. In yeast, *Saccharomyces cerevisiae*, GCN2 is the single eukaryotic initiation factor 2 kinase (eIF2α). It phosphorylates eIF2α under conditions of amino acid deprivation, resulting in repression of general protein synthesis whilst allowing selected mRNA such as the uORF-containing GCN4 to be translated (reviewed, 27). During amino acid depletion, accumulating uncharged tRNAs in the cytoplasm bind to a region of GCN2 homologous to histidyl-tRNA synthetases (27, 51, 57). Elevated levels of GCN4 stimulate the expression of amino acid biosynthetic genes in yeast, which code for enzymes required to synthesize all 20 major amino acids (58). Knockout of yeast GCN2 is not lethal, but the Gcn^- strain must be maintained on amino acid containing media (59, 60). Activation of GCN2 involves not only histidine starvation, but also limitations for other essential amino acids as well as some nonessential (2, 27, 57). Furthermore, GCN2 is activated by genetic disruptions of aminoacyl-tRNA synthetases or amino acid transporters and drugs that diminish the uptake or synthesis of amino acids or charging of tRNA (61, 62). In mice, $GCN2^{(-/-)}$ mice are viable, fertile, and exhibit no phenotypic abnormalities under standard growth conditions (63). However, GCN2(-/-) mice are unable to alter the phosphorylation of eIF2α in response to dietary leucine restriction (63). Losing GCN2 activity shifts the normal maintenance of protein mass away from skeletal muscle to

provide substrate for continued hepatic translation (64). Lipid synthesis, which is repressed in livers of wild-type mice during longer periods of leucine starvation, continues unabated in GCN2-deficient mice, ultimately contributing to liver steatosis (44). GCN2 can also be activated by glucose deprivation (52) and exposure to high salt (65) and stresses not directly related to nutrients, such as UV irradiation resulting in a translational arrest through nitric oxide synthase (NOS) leading to arginine depletion irradiation (33, 66). The activation of GCN2 in response to proteasome inhibition has now been noted by several groups (67, 68). The mechanism for this remains to be described; however, it seems plausible that interfering with the ability of the cell to recycle the building blocks of proteins through the proteasome might result in the accumulation of uncharged tRNAs.

GCN2 can also be involved in food choices. To insure an adequate supply of nutrients, omnivores choose among available food sources. This process is exemplified by the well-characterized innate aversion of omnivores to otherwise nutritious foods of imbalanced amino acid content (69). Consumption of a meal of imbalanced amino acid composition selectively elevates levels of phosphorylated eIF2α in anterior piriform neurons, establishing a molecular correlate to the aversive response. GCN2 inactivation diminishes phosphorylated eIF2α levels in the mouse anterior piriform cortex following consumption of an imbalanced meal and impairs this aversive response (53, 69). An ancient intracellular signal transduction pathway responsive to amino acid deficiency thus affects feeding

behavior by activating a neuronal circuit that biases consumption against imbalanced food sources.

1.5. PERK and nutrition:

The other eIF2α-kinase that has a major role in nutrient stress and metabolism is PERK. PERK (also known as PEK, EIF2AK3) is primarily activated by the accumulation of misfolded proteins in the endoplasmic reticulum (ER), a phenomenon termed the unfolded protein response (UPR) or ER stress (46, 70). PERK is an ER transmembrane protein that contains a regulatory region that resides in the lumen of the ER and a cytosolic eIF2α-kinase domain (71-74). PERK blocks the synthesis of new polypeptides, in this manner reducing the entry of nascent polypeptides into the ER lumen. This allows the ER time to refold misfolded proteins and dispose of those that are terminally misfolded, important elements of the cell's "unfolded protein response" (UPR), which seeks to restore ER homeostasis. The levels of PERK in mice are highest in secretory tissues such as pancreas, and salivary gland (75). In mice, it is required for postnatal glucose homeostasis (76) and the episodic synthesis of insulin in pancreatic β-cells (75). Calcium dysregulation, oxidative damage, and increased secretory loads or perturbations in posttranslational modification of proteins can lead to accumulation of misfolded protein that can cause ER stress (70). Fluctuations in glucose levels and high-fat diets are also linked to ER stress (77). In addition to PERK and the ISR, ER stress activates two additional transmembrane proteins, IRE1 (ERN1) and activating transcription factor 6 (ATF6) (70, 78) which collectively induce the UPR. The UPR features

translational control by PERK phosphorylation of eIF2α, which reduces the influx of nascent proteins into the ER. A program of gene expression is activated designed to expand the processing capacity of the ER and enhance ER-associated protein degradation, a mechanism for the clearance and degradation of misfolded proteins from the secretory pathway (29). The consequent UPR initially attempts to re-establish homeostasis via both transcriptional and post-translational mechanisms. However, PERK-dependent phosphorylation of eIF2α on S51 results in a generalized arrest of translation (72). The UPR is linked to the progression and treatment of many disease states, including diabetes and related metabolic disorders, renal disorders, neuropathologies, and cancers (79-81). The importance of PERK in diabetes is highlighted by the discovery that mutations disrupting this eIF2α-kinase result in Wolcott-Rallison syndrome (WRS) in humans, which is characterized by neonatal diabetes, atrophy of the exocrine pancreas, skeletal dysplasia, growth retardation, and hepatic complications resulting in morbidity (82, 83). Activation of PERK is responsible for the increased phosphorylation of eIF2α and inhibition of protein synthesis after transient brain ischemia (84).

1.6. Effects of eIF2α (S51A) knock-in in mouse:

To emphasize the importance of eIF2α phosphorylation in the ER stress response and *in vivo* glucose homeostasis, mice with a homozygous mutation at the eIF2α phosphorylation site (Ser51Ala) died within 18 h after birth due to hypoglycemia associated with defective gluconeogenesis (severe hypoglycemia ensued 6-9 h after birth) (75, 85). The rate limiting enzyme for gluconeogenesis

is phosphoenol pyruvate carboxykinase (PEPCK). Shortly after birth, the transcription of genes encoding the gluconeogenic enzymes is induced under the control of ATF4. Without eIF2α phosphorylation, there is no increased translation of ATF4 leading to reduced transcription of PEPCK. In addition, homozygous mutant embryos and neonates displayed a deficiency in pancreatic beta cells.

1.7. Why the interest in fish?

With the fast growth of the human population worldwide, there is a rising demand of food, especially the protein resources. Fish is one of the major sources of animal protein around the world, providing about 20 % of the animal protein consumed. Fish is the main source of animal protein in many developing countries, providing over 50 % of the animal protein consumed in some areas (86). People who live on the coast are more likely to eat a lot of fish for protein than those who live inland since it is more readily available and they can catch the fish themselves. Approximately three billion people in the world rely on both wild-caught and farmed seafood as their primary source of protein. The increase demand is for fish and fishery products. Overfishing has significantly affected many fisheries around the world; this is not only causes negative ecological consequence, it also reduces fish production, which further leads to negative social and economic consequences (86). As much as 85 % of the world's fisheries may be over-exploited, depleted, fully exploited or in recovery from exploitation (86). Wild fish not only face overfishing but also eutrophication and climate change. World fisheries have undergone major changes over last 20 years; wild stocks have declined and there has been expansion of global

aquaculture production. Currently, the world fisheries are 148.5 million tones with 47 % from aquaculture (Figure 1.6).

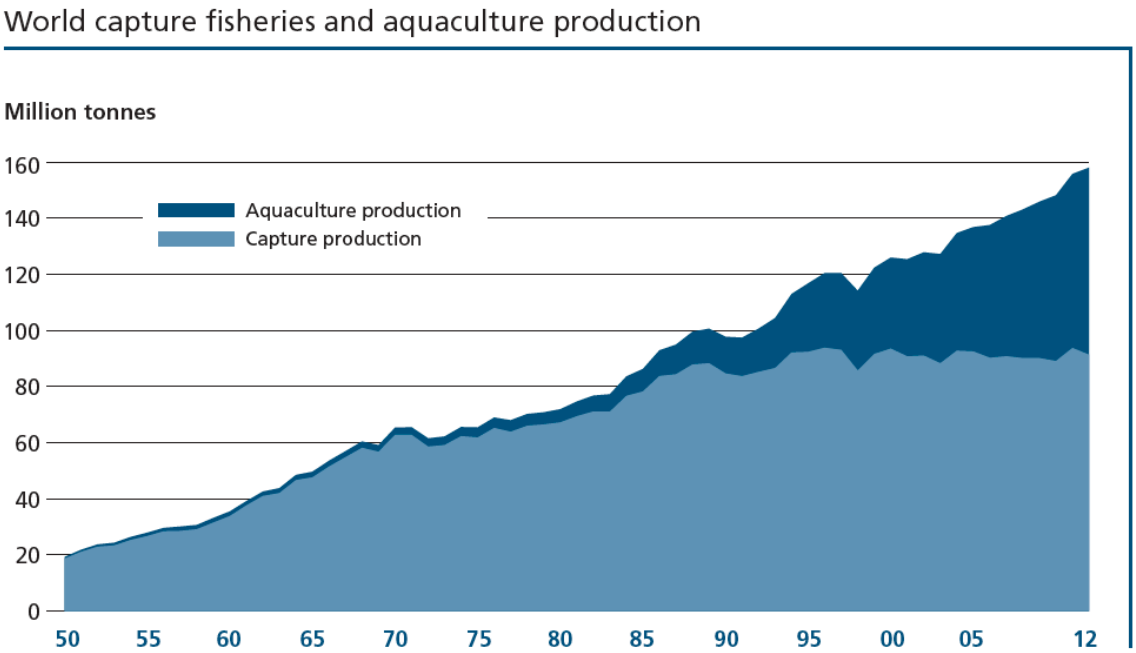

Figure 1.6. Global harvest of aquatic organisms in million tones, 1950–2012, by the FAO.

With continuing decline of wild harvests, aquaculture has become the fastest-growing food sector in the food production. Aquaculture production has continued to show strong growth, increasing at an average annual growth rate of 6.1 percent from 36.8 million tones in 2002 to 66.6 million tones in 2012. The value of farmed food fish production is estimated at USD 137.7 billion in 2012. However, aquaculture has put its own pressure on fisheries stock because it relies heavily on fish meal from forage fish, an unsustainable practice (86). Salmon farming provides a good example of the problems with aquaculture in terms of sustainability (87). Farmed salmon consume more wild fish than they generate as a final product. To produce one pound of farmed salmon, products from five pounds of wild fish are fed to them. Expansion of fish farming requires

the development of alternate diets from sustainable sources and increased interest in the optimization of plant based fish diets to increase production and sustainability. However, the problem with plant based diets have been amino acid imbalance and anti-nutrients. For now, general guidelines for cobia suggest that up to 50 % of dietary protein can be replaced with soybean meal (88-90). An all-plant protein diet has been developed by Dr. Allen Place and Dr. Aaron Watson, supplemented with essential fatty acids and taurine, that gives equal or better performance characteristics to traditional fishmeal diets in cobia and sea bream (91, 92). Diet studies are both time consuming and expensive and could be facilitated by the development of faster indicators of diet quality.

With the known relationship between eIF2α phosphorylation in nutritional status and food choices, it is of interest to determine whether eIF2α phosphorylation can be used as an early marker to evaluate diets in fish. Since eIF2α phosphorylation was first demonstrated in zebrafish (93), and because zebrafish is a tractable model system, studies will be initiated in this species to lay the groundwork for looking at fish of more interest to aquaculture. The usefulness of eIF2α phosphorylation as an indicator for other stressors such as pH, temperature and disease can also be investigated. The outcome of these studies will be applied to the investigation of eIF2α phosphorylation as an indicator of nutritional status and stressors in the aquaculture species cobia, *Rachycentron canadum*. All the eIF2-kinases discussed have been found in the zebrafish genome (Table 1.1).

Table 1.1. eIF2α-kinases in zebrafish genome

eIF2α-kinase	symbol	aa	chromosome	Accession #
HRI	eif2ak1	621	12	NM_001077567
PKR	eif2ak2	682	13	NM_001114470
PERK	eif2ak3	1099	13	NM_001077149
GCN2	eif2ak4	553	17	NM_003336663
PKZ	pkz	511	13	NM_001040376

1.8. Nutrient deficiency in fish

Complete starvation is rare in wild fish, although occasionally a major environmental change, such as the failure of El Niño, can lead to heavy mortalities (94). More common is a reduction in year class numbers or average fish size associated with changes in oceanic or climatic conditions that lower food abundance. Overharvesting of prey species can also impact the condition of carnivores at the top of the food chain and nutritionally compromised diets can increase susceptibility to disease. The main impetus for research on fish nutrition has been the development of the aquaculture industry. Good nutrition is fundamental to the success and sustainability of the aquaculture industry in terms of economics, fish health, high quality product production and minimizing environmental pollution (95). Diet, among other factors, has strong effects on stress tolerance and health, and therefore, for an adequate growth and resistance to stress and disease problems, fish must be fed adequate quantities of diets that meet all their nutrient requirements (96). There is an accumulation of information on the nutrient requirements of most important aquaculture species (97-100). In aquaculture poor feed formulation giving deficiencies in essential

amino acids, essential fatty acids, minerals and vitamins can give rise to poor growth and pathologies (98, 99, 101-103). In general, gross malnutrition is no longer a problem, the more challenging task remains determining the more subtle effects that micronutrients, and their interactions with other dietary components.

In salmonids, lysine deficiencies can give rise to dorsal/caudal fin erosion and increased mortality (104). Tryptophan deficiency can give rise to scoliosis, lordosis, and decreased carcass lipids (105, 106). Dietary imbalances may also arise from the presence of disproportionate levels of specific amino acids; including leucine/isoleucine antagonisms, and to a lesser extent arginine/lysine and cystine/methionine antagonisms (107, 108). Nutritional pathologies may arise from the consumption of feed proteins containing toxic amino acids or their derivatives. In addition, although essential, leucine has been reported to exert a toxic effect in fish when present in dietary excesses (109, 110). When fed experimental diets deficient in essential fatty acids, all aquaculture fish also display reduced growth and poor feed efficiency and pathologies ranging from fatty liver disease (111), reduced spawning efficiency (112), impaired immune responses (101) and increased mortality. In the absence of suitable antioxidant protection lipids rich in polyunsaturated fatty acids (PUFA, including EFA) are highly prone to auto-oxidation on exposure to atmospheric oxygen. Under these conditions, the nutritional benefit of EFA in fact becomes deleterious to the health of the fish. Feedstuffs rich in PUFA which are particularly susceptible to lipid oxidative damage (oxidative rancidity) include fish oils, fish meal, rice bran and expeller oilseed cakes containing little or no natural antioxidant activity. During

the process of lipid auto-oxidation chemical degradation products are formed, including free radicals, peroxides, hydroperoxides, aldehydes and ketones, which in turn react with other dietary ingredients (vitamins, proteins and other lipids) reducing their biological value and availability during digestion. The presence of endogenous anti-nutritional factors within plant feedstuffs can be the largest single factor limiting their use within compounded animal and fish feeds at high dietary levels. These include protease inhibitors, phenols, saponins and toxic amino acids (113).

1.9. Other stressors in fish

Fish are subjected to variable environmental factors such as temperature, salinity, oxygen levels, pH, or parameters related to water composition or quality. Toxins present in water can also be an issue for both wild and aquacultured fish. Other physical, chemical and biological stressors can all evoke stress responses in fish. Stress may alter metabolic scope in fish and affect growth, severe acute stress could cause fish death. Fish kills are most frequently caused by pollution from agricultural runoff or biotoxins arising from algae blooms. Ecological hypoxia is one of the most common natural causes of fish kills. Temperature fluctuations are a much under appreciated stressor of fish. Most tropical freshwater and marine fish do not tolerate temperature changes very well (114, 115).

Under intensive aquaculture conditions, fish are subject to increased stress owing to environmental (nutrient**,** water pollution and oxygen depletion) and health conditions (parasites and infectious diseases) and crowding. All these

factors have negative impacts on fish health and overall performance, with consequent economic losses (116). Though good management practices contribute to reduce stressor effects, stress susceptibility is always high under crowded conditions. Adequate nutrition is essential to avoid deficiency signs, maintain adequate animal performance and sustain normal health. Many studies have shown that optimal diet improves both the overall condition of fish as well as disease resistance (98, 117).

1.10. Responses of fish to a changing world

Since the pre-industrial period atmospheric carbon dioxide concentration ($[CO_2]atm$) has increased from 280 to 379 ppm as of 2005, which exceeds the natural range of 180 to 300 ppm over the last 650,000 years (118). Increased $[CO_2]atm$ poses a two-fold problem for aquatic organisms as CO_2 dissolves in water potentially leading to aqueous hypercapnia (higher than normal aqueous CO_2 levels) and simultaneously acidifying the aqueous environment. The increased $[CO_2]atm$ has decreased oceanic pH by 0.1 with a continued projected decrease up to 0.35 over the 21st century based on the Intergovernmental Panel on Climate Change (IPCC) 2007 report (119). Wootton 2008 reports that coastal pH will fluctuate by 1.5 units based on observations over 8 years period and that model simulations have underestimated the rate of coastal pH decline, indicating oceanic acidification may be worse than anticipated in some areas (120).

Atmospheric change, environmental pollutants, habitat modification and invasive species all effect wild fish from ocean to fresh water (120). Environmental pollutants have the potential not only to kill fish but also have the

potential to affect recruitment. Even atmospherically deposited organic contaminants can accumulate in fish at high elevations, reaching concentrations relevant to human and wildlife health (121). Furthermore, many studies have documented immunosuppressive effects of pesticides, suggesting a role for environmental contaminants in increased pathogen virulence and disease rates (122).

Global climate change and increased pollution can be anticipated to have multiple negative effects on many fish species (123). It is possible that eIF2α phosphorylation could be a useful indicator of stressful environmental conditions and could be of use in predicting what kind of physiological changes might result. Global warming is driven by excessive levels of carbon dioxide. About 30-40 % of the carbon dioxide released by humans' activity into the atmosphere then dissolves into the oceans (124). This anthropogenic carbon dioxide emission to the atmosphere will result in increases in the average temperature, but also to increases in the acidity of land, sea and air (125). Atmospheric CO_2 dissolves in water to form carbonic acid. Increases in CO_2 atmospheric pressure will increase levels of carbonic acid thus increasing the ocean's "acidity". With sustained global warming, the world oceans will absorb more carbon dioxide leading to an increase in ocean acidification (126). Both increased temperatures and decreased pH can be expected to activate eIF2α-kinases.

1.11. Rationale for studying eIF2α phosphorylation in zebrafish

Over the past decades, zebrafish has become a pre-eminent vertebrate model system for clarification of the roles of specific genes, signaling pathways in

development and especially the identification of new drug targets for human disease. There is a substantial historical database regarding basic developmental biology, toxicology, and gene transfer. Zebrafish can be used in forward genetic screens and reverse genetic techniques; genes can be knocked down with morpholinos (127), or knocked out with high efficiency using CRISPR/Cas technology (128, 129). CRISPR (clustered regularly interspaced short palindromic repeats) coupled with guide RNAs and the Cas9 protein can cut at any desired location in the genome. CRISPR/Cas technology has also made development of transgenic zebrafish much easier (130). The benefits of the use of zebrafish as a model system are not only its transparent and accessible embryos, cost-effective mutagenesis screening, and ease of maintenance and breeding, but also the availability of genomic data, fish mutant strains, and other on-line resources such as ZFIN (131). With the completed genome available, zebrafish has become a powerful model system for clarifying mechanisms in development, differentiation, toxicity, disease, and resistance to infection (131). In addition, zebrafish has been proposed as a model fish for nutritional genomic studies, with the expectation that results from this approach will provide comparative genomic information applicable to aquacultured fish (132-134). As a model organism for aquaculture, zebrafish can help in determining optimal conditions for growth, nutrition, and for formulating treatments for fish disease in farmed settings, as well as in identifying stressors. The outcome of the studies will be applied to the investigation of eIF2α phosphorylation as an indicator of nutritional status and stressors in the

aquaculture species cobia, *Rachycentron canadum*, and will form the basis for studies in other species of interest.

1.12. Focus and objectives

My intent in this study was to investigate the usefulness of monitoring eIF2α phosphorylation in fish with the purpose of evaluating its potential usefulness in aquaculture research. My initial hypothesis was that in response to nutritional deficiencies and other stressors, fish and fish cell lines would show increased eIF2α phosphorylation through activation of the eIF2α kinases common to all vertebrates. My initial analyses were with the zebrafish cell line, ZFL, to see if they respond to known activators of eIF2α-kinases. After establishing the ability of ZFL cells to respond to activators of eIF2α-kinases, I made use of a new cobia cell line, CM, to determine whether cells from marine fish show similar responses. After showing the activation of GCN2 and PERK in CM cells, I assessed whether cobia themselves respond to differences in diet and water temperature at the level of eIF2α. Because of my interest in fish diets, I have also adapted ZFL cells to serum-free medium. This has allowed me to investigate the expression of taurine biosynthetic pathway and taurine transporter genes to increase our understanding of the dietary requirement for taurine but has the potential to allow studies of other aspects of ZFL cell metabolism. The results presented here provide the basis for using eIF2α phosphorylation to investigate the response of fish to different diets and stressors. The adapted ZFL cell line will allow further studies on the responses to taurine but may be useful in a range of toxicological, metabolic and innate immune studies.

Chapter 2: eIF2α phosphorylation in response to nutrient deficiency and other stressors in ZFL cells

2.1. ABSTRACT

The zebrafish (*Danio rerio*) genome contains two copies of the gene for the small subunit of the translational initiation factor eIF2, eif2s1a and eif2s1b found on two different chromosomes, chromosome 17 and 20, respectively. eIF2α is the target of phosphorylation by a family of kinases that respond to a range of physiological stressors. The two gene products, eIF2α-a and eIF2α-b, are 96 % identical at the amino acid level and both have identical sequences to human eIF2α at the phosphorylation site and the kinase docking domain. The present study investigates the roles of these two eIF2α isoforms in the zebrafish adult liver cell line, ZFL. Both forms of eIF2α are expressed in ZFL cells, with eIF2α-b transcripts ~5-fold higher than those for eIF2α-a. Here we show that phosphorylation of eIF2α in ZFL cells is increased by a variety of stressors; nutrient deficiency, leucinol, ER stress, PIC and N-methylprotoporphyrin, suggesting activation of the eIF2α kinases GCN2, PERK, PKR and HRI. Consistent with this, all four eIF2α-kinases are expressed in ZFL cells. Since eIF2α-a and eIF2α-b cannot be distinguished by molecular weight, isoelectric point or by immunoblotting with available antibodies, it is unclear whether phosphorylation of one or both forms occurs in response to the kinase activators. However, both wild-type eIF2α-a and -b, but not the eIF2α Ser51Ala variants, can be phosphorylated *in vitro* by purified GCN2, PERK, PKR and HRI, consistent with both being subject to stress-dependent phosphorylation. The

results presented here are consistent with the ability of eIF2α phosphorylation to provide protection against a range of stressors in fish. Evaluation of eIF2α phosphorylation state could therefore provide a means to monitor stress in fish in response to nutrition, temperature, xenobiotics, oxidative stress and infection.

2.2. INTRODUCTION

Thμe regulation of protein synthesis is a key component of metabolic depression in response to environmental stress for poikilotherms. In the sea urchin, *Lytechinus pictus*, protein synthesis accounts for up to 54± 8 % of metabolic rate in embryos (135). In rainbow trout cells 60-90 % of oxygen consumption can be attributed to protein synthesis (136). Accordingly, protein synthesis is tightly regulated. In mammals and yeast, the dominant mechanism for control of overall protein synthetic rates is phosphorylation/dephosphorylation of translational components (2). Most types of stress cause translation inhibition through phosphorylation of the α-subunit of the translation initiation factor eukaryotic initiation factor 2 (eIF2), which delivers initiator Met-tRNAi to the small 40S ribosomal subunit (4). eIF2 is composed of three subunits termed α, β and γ in order of increasing molecular mass. The primary role of eIF2 in translational initiation is to transfer methionyl-tRNAi (Met-tRNAi) to the 40S ribosomal subunits (reviewed, 5). Phosphorylation of Ser51 on the α-subunit of the translational initiation factor eIF2 is a key cellular response to environmental stresses such as low nutrient levels, hypoxia, endoplasmic reticulum stress and viral infection in eukaryotes from protists to vertebrates (2, 6, 137). The translational initiation factor eIF2, a GTP binding protein, delivers initiator tRNA to the small ribosomal

subunit enabling recognition of the start codon. eIF2 is a heterotrimer consisting of three subunits; the α-subunit which is the target of regulatory kinases (at serine 51), the β-subunit which recognizes the initiation codon and mediates the binding to eIF2B, and the γ-subunit which is the catalytic subunit that binds GTP and Met-tRNAi (6, 9). Prior to 60 S subunit joining, the eIF2-bound GTP is hydrolyzed and eIF2 is released from the ribosome bound to GDP in an inactive state. eIF2 has a higher affinity for GDP than GTP and exchange requires the guanine nucleotide exchange factor, eIF2B, to regenerate active eIF2.GTP. When the α-subunit of eIF2α is phosphorylated at Ser51, it binds with a higher affinity to eIF2B than to nonphosphorylated eIF2α, changing it from a substrate to an inhibitor of the guanine nucleotide exchange factor, eIF2B, which leads to inhibition of protein synthesis initiation (17, 20, 138). Reduction in global translation conserves energy but also allows cells to reprogram gene expression to adapt to and recover from stress (reviewed, 25).

A number of protein kinases belonging to the eIF2α kinase family have been identified and found to down-regulate protein synthesis under certain conditions of environmental stress (reviewed, 2, 25, 54). There are five eIF2α-specific kinases in vertebrates (five in teleost fish, four in tetrapods) that can phosphorylate eIF2α, each of which is activated by different stressors. HRI (EIF2AK1) is stimulated by heme depletion in erythroid cells and also by oxidative stress, osmotic shock, and heat shock (35, 36). HRI is the only eIF2α kinase activated by arsenite in erythroid cells (35) and is the major eIF2α kinase responsive to heat shock. PKR (EIF2AK2) activity is stimulated by viral infection

and interferon (37, 38). However, PKR also plays a more general role in cellular physiology; it can be activated in response to signals as diverse as oxidative and ER stress (39-41), as well as cytokine and growth factors signaling (42, 43) and has been implicated in the pathology of obesity (44, 45). PERK (EIF2AK3) is an endoplasmic reticulum (ER) transmembrane protein activated by the accumulation of misfolded proteins in the ER, a phenomenon termed the unfolded protein response (UPR) or ER stress (46). The N-terminal region of PERK lies inside the ER lumen and includes the regions of the protein important for association with the ER chaperone (BiP) that maintains PERK in the inactive state. Accumulation of misfolded proteins or increased levels of secreted proteins in the endoplasmic reticulum (ER) pulls BiP from PERK allowing activation of the kinase (73). Alternatively changes in Ca^{2+} levels within the ER negatively affect the ability of BiP to maintain PERK in its inactive state (47) allowing diverse signals such as inhibition of the SERCA calcium pump, glucose deprivation, and high levels of fatty acids reduce the ER luminal calcium concentration (48). GCN2 (EIF2AK4) is a sensor of amino-acid availability (49). GCN2 is the primary responder to nutritional deprivation and is the only eIF2α-kinase conserved among all eukaryotes. It is activated by reduced amino acid levels in yeast and mammalian cells (27, 50, 51) and can also be activated by glucose deprivation (52). GCN2 is also involved in a variety of organismal functions in vertebrates such long-term memory formation and feeding behavior (53). These four members of the eIF2α kinases share extensive homology in their kinase catalytic domains and phosphorylate eIF2α at the same serine residue (Ser51) (reviewed,

54). Teleost fish have an additional eIF2α kinase, the PKR-like kinase, PKZ, that is more closely related to the kinase domain of PKR compare to other three known eIF2α kinases (55). Its regulatory domain contains both dsRNA binding and DNA-Z binding motifs. Although it is thought to be involved in response to infection, *in vivo* activators of this kinase have not yet been determined. Although PKZ can inhibit protein synthesis *in vivo*, it seems to do so by an eIF2α-independent mechanism (139).

Although phosphorylation of eIF2α by the eIF2α-kinases reduces overall protein synthetic rates, it also serves to activate new gene expression programs that serve to ameliorate stress damage. This results from increased recruitment of a subset of mRNAs that have upstream open reading frames (2, 29, 140-142). These uORF-containing mRNAs encode transcription factors such as GCN4 and ATF4 as well as other regulatory molecules that activate an alternate program of gene expression enabling the cell or organism to respond to a range of stressors (reviewed, 2, 25, 29). This work demonstrates that the eIF2α–kinases in zebrafish ZFL cells respond to the same range of activators as their counterparts in mammalian cells, implying that eIF2α phosphorylation provides protection against a range of stressors in fish. eIF2α phosphorylation could therefore provide a useful indicator to monitor stress in fish in response to nutrition, temperature, xenobiotics, oxidative stress and infection. Although it was not possible to determine directly whether one or both of the eIF2α cognates found in ZFL cells are regulated by phosphorylation, expression data and the ability of

both eIF2α-a and eIF2α-b to be phosphorylated by purified eIF2α-kinases *in vitro* suggests that both are subject to stress-dependent phosphorylation.

2.3. MATERIALS AND METHODS

2.3.1 Culture of ZFL cells

The zebrafish adult liver cell line, ZFL, was obtained from ATCC (cat # CRL-2643), grown in Leibovitz L-15 medium, 20 mM HEPES-KOH, pH 7, 2 mM sodium pyruvate, 100 units/ml of penicillin and streptomycin, and 10 % fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA) at 28 °C, lacking sodium bicarbonate and CO_2.

2.3.2 Primer design for RT-qPCR analysis of zebrafish eIF2α-a and eIF2α-b

Primers used to amplify the zebrafish eIF2α-a and eIF2α-b cDNAs were designed from published sequences in GenBank by Primer 3 software (143). The primers were designed to span exon-exon junctions. Forward and reverse primers for zebrafish eIF2α-a (accession # NM_00199569.1) (144): 5'-GGAAATAGACAGCAGAGACAACGCC and 5'-TGCTGGCATCTTCACCCGAT generated a 150 bp fragment. Primers used for zebrafish eIF2α-b (accession no. NM_00131800) (93): 5'- CCTGGATACGGGGCCTACGA and 5'-TTGACGGCCTGTGGTGTGAG, generated a 137 bp fragment. (See also Table 2.1)

Table 2.1. Primer pairs used for amplification of zebrafish eIF2α-a, eIF2α-b and eIF2α kinase family members

cDNA	5'-3' forward primer	Tm	5'-3' reverse primer	Tm	GenBank Accession #
D. rerio eIF2α-a	GGAAATAGACAGCAGAGACAACGCC	59.6	TGCTGGCATCTTCACCCGAT	59.3	NM_00199569.1
D. rerio eIF2α-b	CCTGGATACGGGGCCTACGA	60.9	TTGACGGCCTGTGGTGTGAG	60.0	NM_00131800.2
D. rerio EF1A	CTTCAACGCTCAGGTCATCAT	55.0	ACAGCAAAGCGACCAAGAGGA	59.1	BC046042.1
D. rerio GCN2	AACGCAGAGGAAGGTGATTG	55.2	GGTGAAAACTGCCCTTGTGT	56.0	NM_003336663
D. rerio PERK	CAGGACCACCCATTTCCTTA	54.3	TCTGTGCATGAGTCCTTTGC	55.5	NM_001077149.2
D. rerio PKR	CGAAACAAAATGCAGCTCAA	52.2	TCCACTAGGCTCACAGAAGGA	57.2	NM_001114470.1
D. rerio HRI	CACGGAGGGACTCTTGATGT	56.5	TCCACTGGTGTGAGTTGCAT	56.8	NM_001077567.1

Primers used to amplify the zebrafish eIF2α-a and eIF2α-b cDNAs were designed based on published zebrafish sequences in GenBank by Primer 3 software. Primers were designed to cover a specified exon-exon junction and generate amplicons of 130-200 bp.

2.3.3 Preparation of RNA from ZFL cells, cDNA synthesis and RT-qPCR analysis

RNA was prepared from ZFL cells using the Ambion PureLink total RNA extraction mini kit (Life Technologies Corporation, Carlsbad, CA, USA) following the manufacturer's protocol. The RNA extract was spectrally analyzed for concentration at 260 nm, and for purity at both 260/280 and 260/230 ratios by Nanodrop ND-1000 spectrophotometry and by automated electrophoresis using the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA). Values of >2 for 260/280 and 260/230 ratios were considered to be of sufficient purity for planned uses. Amplification of cDNA fragments of the predicted size was confirmed by end-point RT-PCR. cDNA was synthesized using Revertaid™ M-MULV reverse transcriptase (Fermentas GmbH, St. Leon-Roth, Germany) and random hexamer primers (Qiagen, Valencia, CA, USA) to produce first stand cDNA. The cDNA produced was diluted to represent that produced from 12.5 ng/µl RNA for qPCR

analysis and stored at -80 ^{o}C. The quality of all cDNA preparations was assessed by end point PCR amplification using the primers described above. Amplification of the cDNA for elongation factor 1A (EF1A) was used as a reference for both endpoint and qPCR analysis. Real time PCR was performed on a 7500 Real Time PCR System (Applied Biosystems) with SYBR green fluorescent label. Reactions included Taqman™ Universal master mix, 1:100 SYBR green (100 U stock), 5 μM of each primer. Each 20-μl sample was run in optically clear 96-well plates (Applied Biosystems). Cycling parameters were as follows: 50 °C × 10 min, 95 °C × 2 min, followed by 40 cycles of 95 °C× 15 sec/60 °C × 30 sec. The dissociation step was performed at the end of the amplification phase to verify a single, specific melting temperature for each primer set, reflecting a single product. Data generated by real-time PCR were compiled using SDS v1.4 software (Applied Biosystems). Zebrafish eIF2α-a, eIF2α-b transcript expression was normalized by the 2−ΔCt method against large ribosomal protein L13a.

2.3.4 Zebrafish eIF2α-a and eIF2α-b constructs

The cDNA for zebrafish eIF2α-b, generated from RNA prepared from ZFL cells, was cloned into pGEMT and pCITE, as reported (93). For cloning of eIF2α-a, RNA was prepared from ZFL cells and primers were designed for zebrafish eIF2α-a based on the reported sequence (144, 145). cDNA was prepared from RNA isolated from zebrafish muscle using random hexamers and cloned into pGEMT. All primers sets were designed to provide Nde I and Bam HI recognition sites at the 5'- and 3'-ends. eIF2α-a sequence was verified from five primary transformants. The open reading frame of eIF2α-a was excised from

pGEMT/eIF2α-a using the NdeI and BamHI restriction sites and transferred into similarly digested pCITE4a. The pCITE4a constructs were used as templates for coupled transcription–translation system, using the rabbit reticulocyte TNTQuick system (Promega, Madison, WI, USA).

Generation of eIF2α-bS51A: cDNA containing the S51 to A51 mutation was synthesized by GenScript and cloned into pCITE4a with Nde I and BamHI recognition sites at the 5'- and 3'-ends. eIF2α-aS51A was provided by Dr. Eunseok Choi, a Jagus lab postdoctoral trainee.

2.3.5 Preparation of cell extracts for analysis of eIF2α phosphorylation

ZFL cells (~10^7) in monolayers were rinsed once with ice cold PBS containing 25 mM sodium molybdate, 10 mM β-glycerophosphate, and 100 mM NaF prior to lysis in 500 µl 25 mM HEPES–KOH, pH 7.2, 5 mM EDTA, 100 mM KCl, 0.5 % Elugent (Calbiochem), 10 % glycerol, 1 mM DTT, 0.5 µM microcystin, and 1 tablet/10 ml protease inhibitor pill (Boeringer–Mannheim) (93). Lysates were collected and clarified by centrifugation (10,000 x g for 5 min at 4 °C) prior to snap-freezing and storage in liquid N_2. In general, extracts contained approximately 2–3 mg/ml of protein.

2.3.6 Immunoblot analysis to detect eIF2α phosphorylation

Samples equivalent to 30,000 cells per 20 µl were fractionated by 15 % SDS-PAGE, electrotransferred to PVDF membranes, and subjected to immunoblot analysis using polyclonal antibody against mammalian eIF2α phosphorylated at Ser51 (CellSignaling, cat#3597, Danvers, MA, USA) followed

by goat anti-rabbit secondary antibody coupled to HRP and coupled with chemiluminescence. Chemiluminescence was detected using the ProteinSimple Fluorochem E with quantification using ImageJ. Total eIF2α levels were determined by reprobing the stripped blot with rabbit antibody that recognizes equally the phosphorylated and unphosphorylated forms of eIF2α (CellSignaling, cat#9722, Danvers, MA, USA). Values obtained with the anti-eIF2α[P] antibody were normalized for the total amount of eIF2α present in the sample.

2.3.7 *In vitro* phosphorylation of zebrafish eIF2α-a and eIF2α-b

^{35}S-radiolabeled eIF2α-a and eIF2α-b and their nonphosphorylatable variants were produced in the reticulocyte TnT cell-free transcription/translation system by incubation at 30 ^{o}C for 30 min, after which they were supplemented with 8 ng/μl (specific activity is 53 nmol/min/mg) of purified mammalian recombinant HRI (EIF2AK1), PKR (EIF2AK2), PERK (EIF2AK3) or GCN2 (EIF2AK4) followed by incubation at 30 ^{o}C for an additional 10 min. Microcystin (0.5 μM) was included to inhibit phosphatase activity. Each sample was diluted in 10 volumes isoelectric focusing sample buffer (9.5 M urea, 1 % Pharmalyte, pH 4.5–5.4, 1 % Pharmalyte, pH 5–6, 5%CHAPS (Fluka), 50 mM DTT) prior to analysis. Samples were subjected to vertical slab isoelectric focusing (VSIEF) (146), using a narrow pH range of 4.5–6 in the presence of 8.8 M urea, 3 % acrylamide, 1 % CHAPS, 5 % Pharmalyte (pH 4.5–6), and 50 mM DTT. Proteins were electrophoresed at 2 mA/gel with a 1200 voltage limit for 16 h, using reverse polarity with 0.01 M glutamic acid at the anode and 0.05 M histidine at the cathode. Proteins were transferred to PVDF and ^{35}S-labeled protein was

visualized using a Storage Phosphor screen (Molecular Dynamics, GE Healthcare Life Sciences, Pittsburgh, PA, USA) and imaged with a Typhoon 9410 Variable Mode Imager (GE Healthcare, Healthcare Life Sciences, Pittsburgh, PA, USA).

2.3.8 Transfection using Fugene 6

For transfection with FuGene 6 (Roche, Indianapolis, IN, USA), cells were seeded into the 6-well plates, 8×10^4 cells per well to achieve 50~60 % confluence after 24 h growth. Transfection efficiency was determined using the expression construct pmaxGFP. Positive control vector pmaxGFP^{TM} encodes the green fluorescent protein (GFP) from the copepod *Pontellina* sps. Reagent-DNA complexes were prepared according to the manufacturer's protocol with different ratios. The transfection efficiency and cell survival rate were analyzed after 24 h. Transfection efficiency was determined by the proportion of GFP positive cells and expressed as a percentage of total cells. Cell viability was assessed by trypan blue dye exclusion.

2.3.9 Transfection using the Neon® Transfection System

For transfection using the Neon® Transfection System, ZFL cells were passaged 2-3 days before electroporation. Cells were grown to 70-85 % confluency before harvest. The cells were harvested by trypsinization and plated at $1\sim5\times10^6$ cells per transfection. Using Neon™ Tip, 10 µl cells in buffers suggested by the manufacturer were placed in the Neon® Transfection System device (Invitrogen Inc., Carlsbad, CA, USA), and electroporation was performed according to the manufacturer's protocol. After transfection, cells were incubated

in 500 µl prewarmed culture medium and removed to 6-wells plate immediately. After 24 h at 28 °C, unattached cells were removed by rinsing in PBS and observed using fluorescence microscopy. Transfection efficiency was determined by the proportion of GFP positive cells and expressed as a percentage of total cells. Cell viability was assessed by trypan blue dye exclusion.

2.4. RESULTS

2.4.1 Zebrafish has two eIF2α genes

There are two eIF2α genes in the zebrafish genome, encoding eIF2α-a (accession # NM_00199569) and eIF2α-b (accession no. NM_00131800), found on chromosomes 17 and 20, respectively (93, 144). Table 2.2 shows the percent identities in amino acid composition, predicted molecular weights and isoelectric points, as well as chromosome localization of eIF2α-a and eIF2α-b.

Table 2.2. Characteristics of zebrafish eIF2α

eIF2α	Gene	ID#	#aas	%ID	MW(Da)	pI	Chrom	Location	Accession #
D. rerio eIF2α-a	eif2s1a	321807	315	94	36.13	4.97	17	NC_007128.6	NM_199569.1
D. rerio eIF2α-b	eif2s1b	321564	315	94	36.17	4.98	20	NC_007131.6	NM_131800.2
H. sapiens eIF2α	EIF2S1	1969	315	100	36.1	5.01	14	14q23.3	NM_004094.4

Their gene products are 96 % identical at the amino acid level and both show 94 % identity to human eIF2α. The predicted molecular weights of the two zebrafish eIF2αs, 36.16 kDa for eIF2α-a and 36.17 kDa for eIF2α-b, are in good agreement with those of human eIF2α (36.1 kDa). The deduced isoelectric points are also very similar, 4.97 and 4.95 for eIF2α-a and eIF2α-b, respectively, compared to 5.02 for human eIF2α. Figure 2.1 shows the multiple alignment of the N-terminal

120 amino acids of zebrafish eIF2α-a and eIF2α-b amino acid sequences compared to that of human eIF2α.

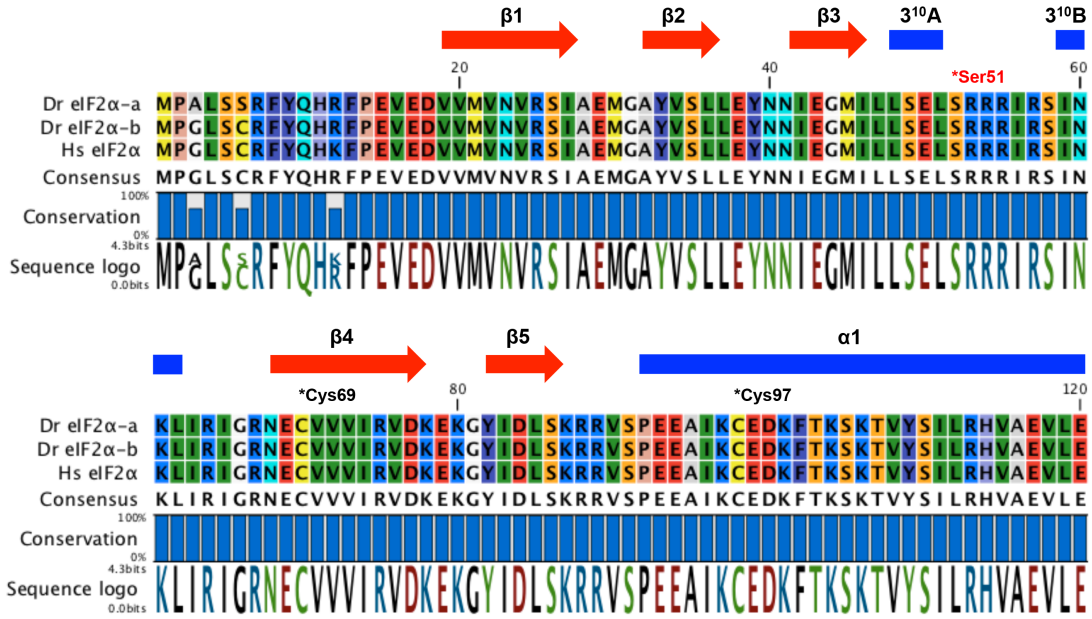

Figure 2.1. Alignment of eIF2α-a and eIF2α-b from zebrafish, *Danio rerio*

Multiple alignment of the N-terminal 120 residues of eIF2α-a and eIF2α-b from zebrafish, *Danio rerio*, with human eIF2α. Secondary structural elements (red arrow, β-sheet; blue box, α-helix) are shown above the sequences. The red asterisk indicates the Ser51 phosphorylation site in eIF2α, and black asterisk shows Cys-69 in β4 and Cys-97 in α1 involved in the disulfide bridge between the OB fold (β 1-5) with the helical domains of the N-terminal region.

This covers the phosphorylation site and kinase docking domain. (A multiple alignment of the complete amino acid sequences are provided as Figure 2.2). In fact, the two forms only differ in 2 residues in this region. Residues in the phosphorylation loop region surrounding the eIF2α phosphorylation site (Ser-51 in human eIF2α) are identical in the two zebrafish eIF2αs and human eIF2α. Similarly, residues in the PKR docking site (residues 79–83) of zebrafish eIF2α-a and eIF2α-b are identical to those in the human sequence, suggesting that both forms are likely to be phosphorylated by eIF2α-specific protein kinases. In

addition, Ser-48, Ile-55, Leu-84, Arg-88 and Val-89, residues critical for interaction with the α-, β-, and δ-subunits of the guanine nucleotide exchange factor, eIF2B (147), are present in both zebrafish eIF2αs indicating that the interaction between both eIF2αs and eIF2B is also conserved.

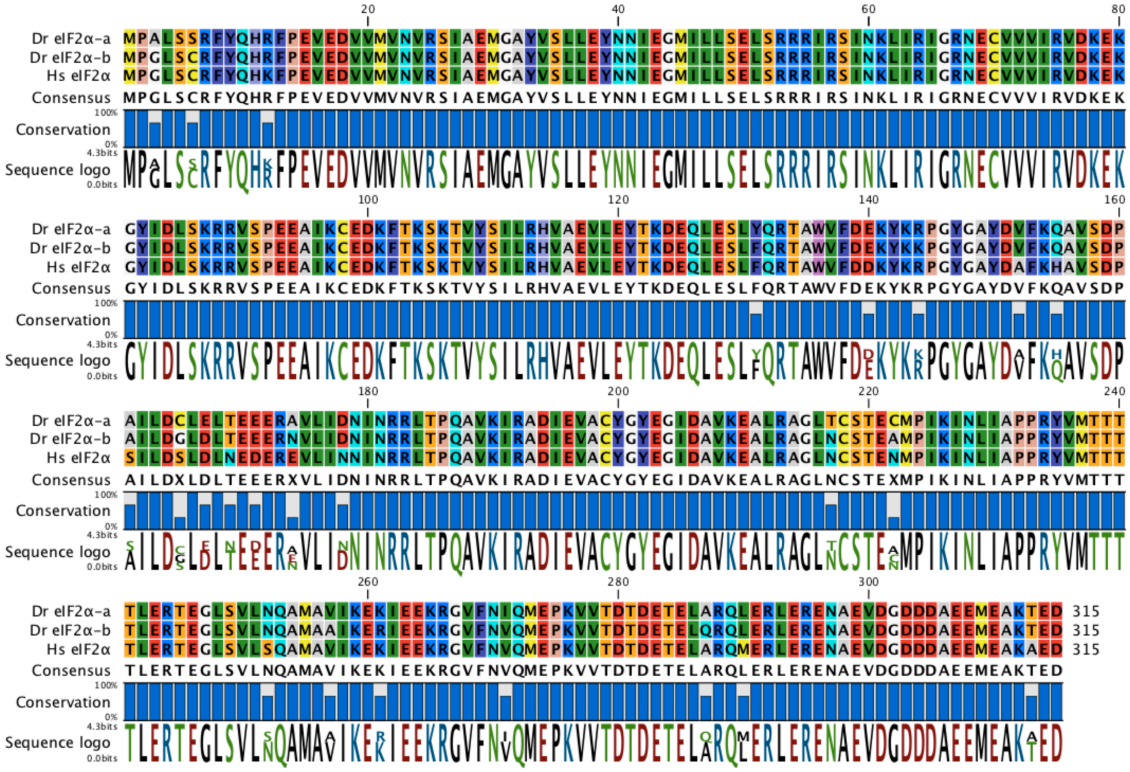

Figure 2.2. Multiple alignment of zebrafish eIF2α-a and eIF2α-b with *H. sapiens* eIF2α

Multiple alignment of complete amino acid sequences (315 amino acids) of eIF2α-a and eIF2α-b from zebrafish, *Danio rerio*, in comparison with human eIF2α by the ClustalW program. Amino acid numbering is shown above the sequences.

The structure of the N-terminal region of eIF2α, considered to be residues 1-182, consists of an oligonucleotide-binding fold (OB fold) (148) (residues 3–90) opposite to the site of association with an α-helical subdomain (residues 91–175). The OB-fold consists of a five-strand β barrel (denoted β1 to β5) interrupted by a helical insert between β strands 3 and 4. In *S. cerevisiae* and human eIF2α,

the helical insert consists of two single-turn 3^{10} helices (denoted 3^{10}A and 3^{10}B) separated by a seven residues linker containing the Ser51 phosphoregulatory site (149, 150). The first α-helix (91-118) of human eIF2α is involved in interactions that fix the orientation of the helical domain with the β-barrel. In mammalian eIF2α, the OB and helical domains of the N-terminal region are linked by a disulfide bridge between Cys-69 in β4 and Cys-97 in α1. The zebrafish eIF2αs also have cysteines at both these sites. This residue is not found in non-vertebrate eIF2αs and appears to be a characteristic of vertebrate eIF2αs. The overall prediction is that both zebrafish eIF2αs will function as eIF2 subunits and both will be substrates for the eIF2α kinases.

2.4.2 Both eIF2α-a and eIF2α-b are expressed in ZFL cells and zebrafish muscle

To determine whether both forms of eIF2α, eIF2α-a, eIF2α-b, are expressed in ZFL cells, relative transcript expression levels were determined by RT-qPCR. Transcript levels in zebrafish muscle were also determined. Figure 2.3 shows the relative levels of eIF2α-a and eIF2α-b transcripts. Both eIF2α-a and eIF2α-b transcripts are expressed, although the expression levels of eIF2α-b is ~2-fold higher than eIF2α-a in ZFL cells, although expression levels of eIF2α-a is ~5-fold higher than eIF2α-b in skeletal muscle. The relative levels of eIF2α-a and eIF2α-b at the protein level cannot be determined since both are recognized by the antibodies available. The amino acid sequences are too similar for eIF2α-a and eIF2α-b specific antibodies to be developed and their physical characteristics are too close to separate by SDS-PAGE or isoelectric focusing.

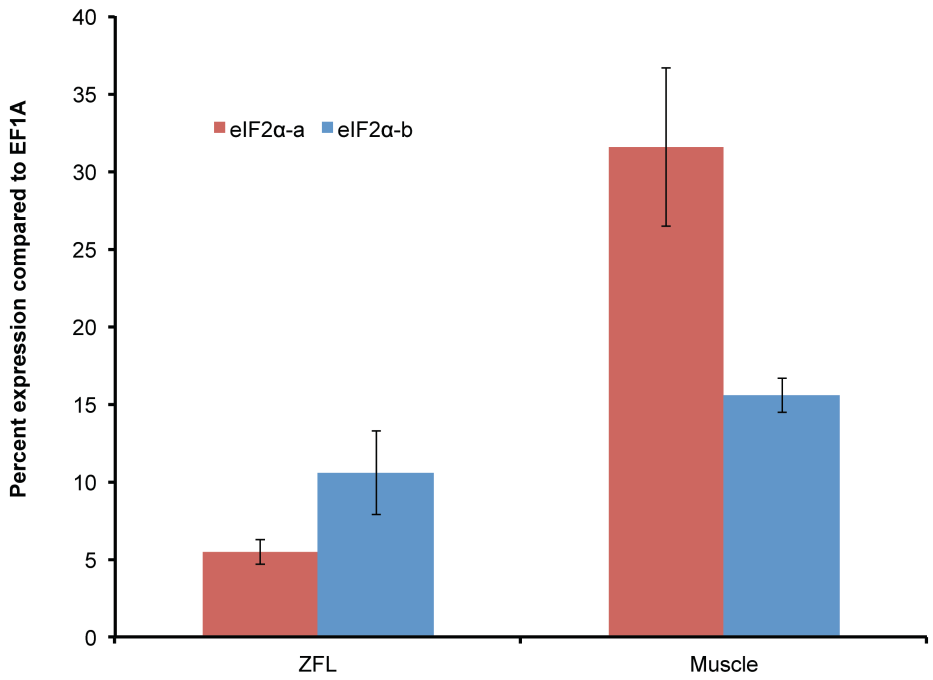

Figure 2.3. Transcript levels of eIF2α-a and eIF2α-b in ZFL cells and muscle

Quantitative RT-PCR was performed using cDNA from 10 ng RNA and primers given in Table 2.1. Transcript levels of DreIF2α-a (blue) and DreIF2α-b (red) are expressed as a percentage of EF1A transcript abundance in ZFL cells and zebrafish muscle. Data are presented as the mean ± S.D. (n = 3 replicates).

2.4.3 Quantification of eIF2α phosphorylation

The phosphorylation status of eIF2α can be determined by western blot analysis probing sequentially with antibody to the phosphorylated form of eIF2α and antibody to eIF2α to measure the total levels of eIF2α. The ability of the method to provide meaningful quantitative results was established in a rabbit reticulocyte cell-free translation system. Incubation with 10 mM EDTA generates 0 % phosphorylation of eIF2α, whereas incubation with purified heme-regulated eIF2α-kinase, EIF2AKI, and 2 μM microcystin gives rise to 100 % phosphorylation of eIF2α, as observed by separation of phosphorylated and nonphosphorylated forms by vertical slab isoelectric focusing (VSIEF) followed

by immunoblotting (Figure 2.4, inset). These samples can be mixed to generate a range of phosphorylation states.

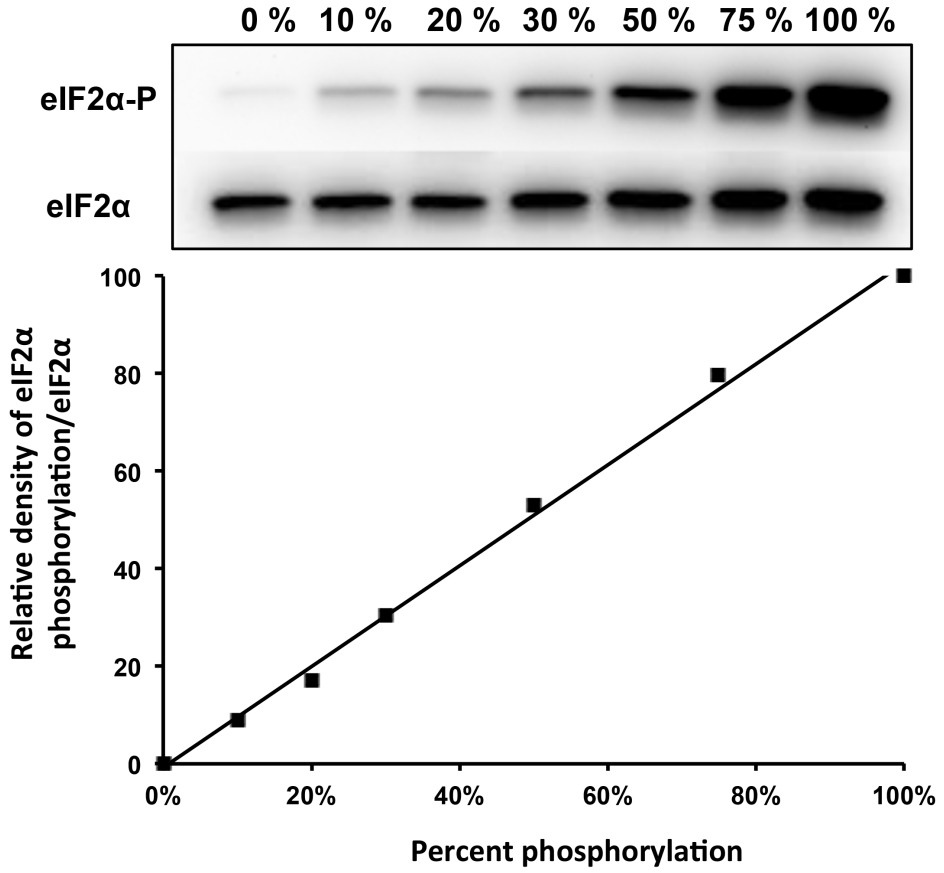

Figure 2.4 Verification of use of antibodies to estimate phosphorylation state of eIF2α

Samples containing eIF2α with a phosphorylation state of 0 % and 100 % were generated by incubation of rabbit reticulocyte lysate with 20 mM EDTA or with purified heme-regulated eIF2α-kinase (EIF2PKI) and 2 μM microcystin. These samples were mixed to generate a range of phosphorylation states from 0-100 %. Panel A shows immunoblot blot of these samples after SDS-PAGE fractionation using antibodies to phosphorylated and (after stripping) antibodies to total eIF2α. eIF2α was visualized by enhanced chemiluminescence following electro-transfer to PVDF. Chemiluminescence was detected and image generated (upper panel) using the ProteinSimple Fluorochem E, with quantification using ImageJ to estimate level of phosphorylated eIF2α (lower panel). Inset shows immunoblots the same samples fractionated by VSIEF to separate nonphosphorylated and phosphorylated forms using antibodies to total eIF2α and processed as above.

Figure 2.4 shows analysis of the same samples using SDS-PAGE fractionation followed by immunoblotting using polyclonal antibody against mammalian eIF2α phosphorylated at Ser51 and subsequently (after stripping) using antibody that

recognizes both phosphorylated and nonphosphorylated forms of eIF2α. Using ImageJ, the relative pixel density for phosphorylated eIF2α compared to that for total eIF2α is compared to the known phosphorylation state and establishes the quantitative usefulness of the assay.

Because chemiluminescence does not have a wide dynamic range, care was taken to establish that the ZFL samples analyzed did not exceed the range of the assay. The ECL signal arising from analysis of increasing numbers of cells from 0.2 x 10^5 to 5 x 10^5 cells was determined. The close identity of human and zebrafish eIF2αs allowed the use of commercial antibodies available for mouse eIF2αs which interact well with zebrafish eIF2α (93). Figure 2.5 shows western blot analysis of total eIF2α using increasing numbers of ZFL cells and demonstrates that the method is effective over a range of 20,000-500,000 cells. Analysis of phosphorylation state was confined to samples equivalent to 3 x 10^5 cells.

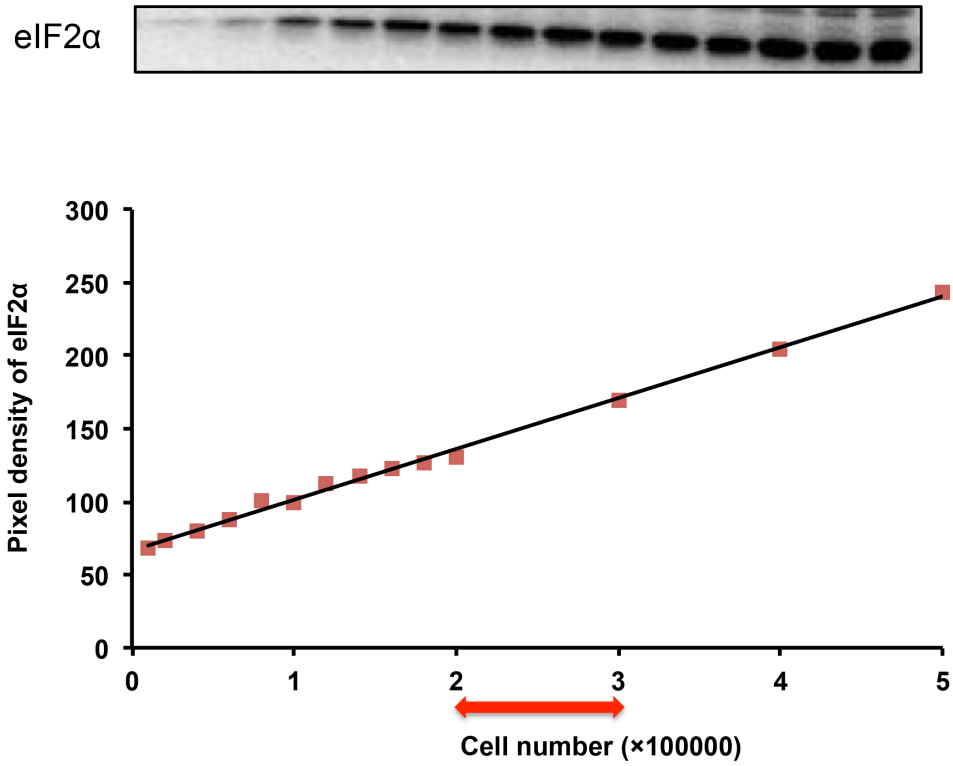

Figure 2.5. Determination of range of cell numbers that can be analyzed to estimate phosphorylation state of eIF2α

Samples were prepared from extracts of ZFL cells to contain the equivalent of 0 - 5 x 10^5 cells. Proteins were fractionated by SDS-PAGE and eIF2α was visualized by enhanced chemiluminescence following electro-transfer to PVDF, and probing with antibody to eIF2α. Chemiluminescence was detected and image generated (upper panel) using the ProteinSimple Fluorochem E, with quantification using ImageJ (lower panel).

2.4.4 ZFL cells respond to known activators of eIF2α kinases

The zebrafish adult liver cell line, ZFL, originally derived from ten normal adult livers, shows typical epithelial morphology and possesses characteristics common to liver parenchymal cells (151). They have been used for a wide range of studies (reviewed, 152), including those related to eIF2α phosphorylation (93). This study uses them to determine potential responses of fish to nutrient and other stressors.

Each of the four eIF2α-kinases, HRI, PKR, PERK and GCN2 can be activated in mammalian cell lines by a variety of stressors. GCN2 can be activated in cultured mammalian cells by nutrient deprivation and leucinol (50, 63). Leucinol, the alcohol formed by total reduction of the carboxylic acid group of leucine, is an inhibitor of leucyl-tRNA synthetase and so can mimic leucine deficiency in cells (153). Thapsigargin is an inhibitor of endoplasmic reticulum Ca^{2+} ATPase that blocks the ability of the cell to pump calcium into the sarcoplasmic and endoplasmic reticulum, leading to depletion of these stores (154) and activation of PERK (72, 74). Polyinosinic:polycytidylic acid (poly I:C) is a synthetic dsRNA that is used experimentally to model viral infections *in vivo* and is used *in vivo* and *in vitro* to activate PKR (reviewed, 155). N-methylprotoporphyrin (NMPP) is a transition-state analogue and potent inhibitor of ferrochelatase, the terminal enzyme of the heme-biosynthetic pathway (156). It is used to induce heme deficiency and has been shown to activate HRI and increase eIF2α phosphorylation (157). Although each of the eIF2α-kinases may play a dominant role in responding to a specific physiological change or stress, each may also contribute to eIF2α phosphorylation signaling in response to a diverse array of perturbations (63).

The response of ZFL cells to these known activators of eIF2α-kinases was examined by incubation in nutrient limiting medium or in the presence of leucinol (4 mM) for activation of GCN2; thapsigargin (1 μM) for activation of PERK; polyinosinic:polycytidylic acid (poly I:C) (50 μg/ml with 200 μg/ml DEAE dextran) for activation of PKR; NMPP (2 μM) for activation of HRI. Cells were harvested at

various time points and analyzed by immunoblotting as described in Materials & Methods.

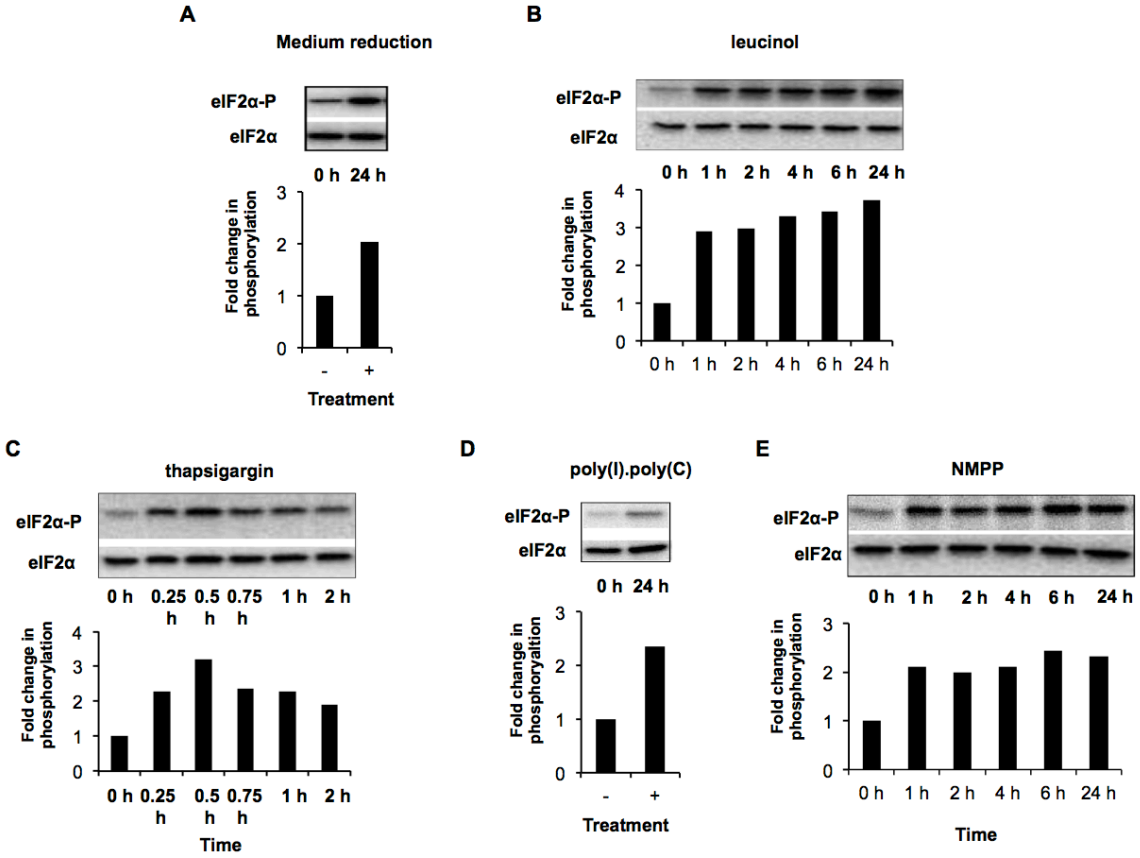

Figure 2.6 Response of ZFL cells to activators of eIF2-kinases

ZFL cells treated with known activators of eIF2α-kinases: (A) Nutrient limited cells were maintained in 30 % L-15 cell culture medium in PBS for 24 h; (B) leucinol (4 mM), for up to 24 h, for activation of GCN2; (C) thapsigargin (1 μM), for up to 2 h, for activation of PERK; (D) PIC (50 μg/ml), for 24 h, for activation of PKR; (E) NMPP (2 μM), for up to 24 h, for activation of HRI. Proteins in cell extracts were fractionated by SDS-PAGE and eIF2α was visualized by enhanced chemiluminescence following electro-transfer to PVDF, and probed with antibody to phosphorylated eIF2α and (after stripping) antibodies to total eIF2α. Phosphorylated and total eIF2α was visualized by enhanced chemiluminescence following electro-transfer to PVDF. Chemiluminescence was detected and image generated (upper panels) using the ProteinSimple Fluorochem E, with quantification using ImageJ to estimate level of phosphorylated eIF2α (lower panels).

2.4.4.1 Effects of nutrient deprivation and leucinol

Reducing the ZFL L-15 culture medium to 30 % in phosphate buffered saline (PBS) for 24 h reduced both amino acid and serum levels. After 24 h, eIF2α phosphorylation was increased (Figure 2.6, Panel A) consistent with the activation of GCN2. Similarly, when leucinol (4 mM) was included in the complete culture medium, an increase in eIF2α phosphorylation could be seen within 1 h and continued to increase over 24 h (Figure 2.6, Panel B). This also strongly suggests activation of GCN2, although not a definitive demonstration. In cultured mouse embryonic stem cells, incubation in leucine-deficient medium increases eIF2α phosphorylation within one hour (63). In contrast *GCN2*$^{-/-}$ ES cells failed to show any induction in eIF2α phosphorylation during the first 3 h of leucine deprivation, demonstrating that the effect of leucine deprivation on eIF2α phosphorylation could be attributed to GCN2 (63).

2.4.4.2 Effects of thapsigargin

Thapsigargin is non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) (158). eIF2α in ZFL cells responds very quickly to incubation with thapsigargin (1 μM). Figure 2.6, Panel C shows that increased eIF2α phosphorylation could be seen as early as 15 min, peaked at 30 min and decreased over 2 h, consistent with activation of PERK.

In situations in which the steady state level of eIF2α phosphorylation have been determined, it has been found that the percent of eIF2α phosphorylated rarely rises above 25 -30 %. To determine whether increases in eIF2α phosphorylation seen with thapsigargin resemble the physiological changes

encountered, we looked at the effect of including microcystin in the culture medium. Microcystins are cyclic nonribosomal peptides and can strongly inhibit protein phosphatases type 1 (PP1) and 2A (PP2A) (159, 160). Microcystin-LR treatment alone resulted in increased eIF2α phosphorylation after a 30-min incubation (Figure 2.7).

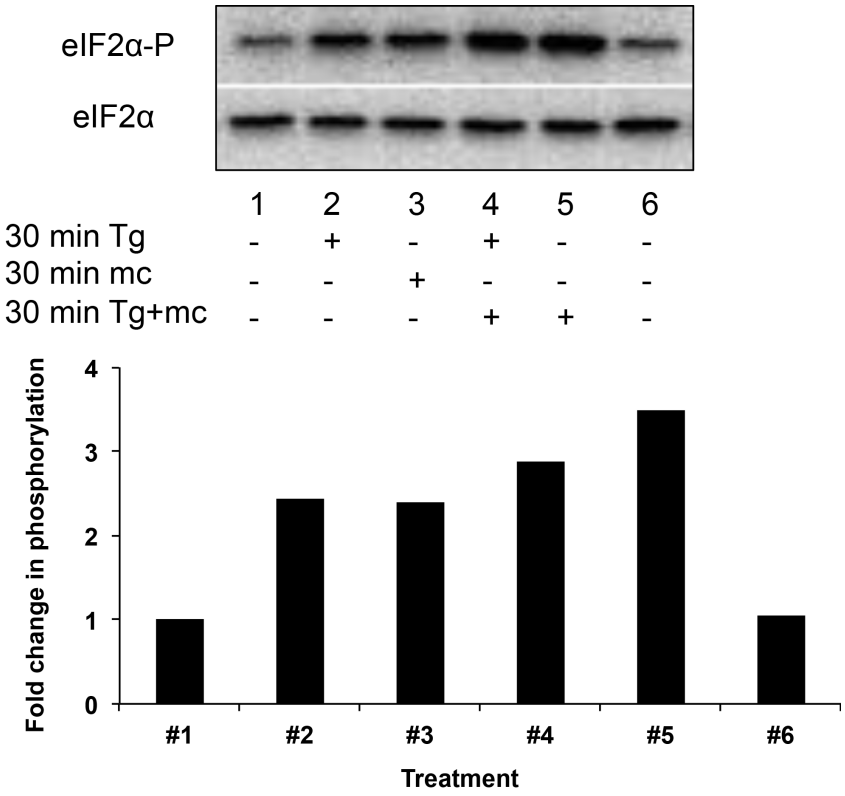

Figure 2.7. Effect of the phosphatase inhibitor, microcystin-LR on phosphorylation of eIF2α in ZFL cells.

ZFL cells were incubated with (Lanes# 2, 4, 5), or without (Lanes# 1, 3, 6) thapsigargin (1 μM) for 30 min, in the presence or absence of microcystin (2.5 μM) from 25-30 min, or 0-30 min. The various lanes are as follows: (1) untreated, 0 min, (2) thapsigargin (1 μM), for 30 min, (3) microcystin (2.5 μM) for 30 min, (4) thapsigargin (1 μM)) for 30 min then microcystin (2.5 μM) for 30 min, (5) thapsigargin (1 μM) and microcystin (2.5 μM) for 30 min, (6) untreated, 30 min. Proteins in cell extracts were fractionated by SDS-PAGE and transferred to PVDF. Phosphorylated and total eIF2α were visualized by enhanced chemiluminescence, using antibody to phosphorylated eIF2α and (after stripping) antibodies to total eIF2α, as described in legend to Figure 2.6. The bar diagrams in lower panels represent the quantification of the blots corresponding to the respective lanes in the upper panel.

Microcystin-LR added at the beginning of thapsigargin treatment or during the last 5 min also resulted in increased eIF2α phosphorylation indicating that the increased eIF2α phosphorylation seen in the presence of thapsigargin is within the scale of normal physiological responses.

2.4.4.3 Effects of poly I:C

ZFL cells were treated with 50 µg/ml poly I:C and 200 µg/ml DEAE dextran for 24 h. Figure 2.6, Panel D, shows that treatment for 24 h showed increased eIF2α phosphorylation. These results are consistent with previous results using poly I:C in ZFL cells and are consistent with PKR activation (93).

2.4.4.4 Effects of N-methylprotoporphyrin

ZFL cells were treated with NMPP (2 µM) for up to 24 h. Figure 2.6, Panel E, shows that increases in eIF2α phosphorylation can be seen as early as 1 h after the beginning of treatment and remain high over 24 h. These results are consistent with activation of HRI in ZFL cells.

2.4.5 ZFL cells express eIF2α kinases

Although the effects of the activators of eIF2α-kinases described above suggest that ZFL cells express the four eIF2α-kinases, it was of interest to determine this. The zebrafish genome contains all four eIF2α-kinases found in tetrapods, HRI (EIF2AK1), PKR (EIF2AK2), PERK (EIF2AK3) and GCN2 (EIF2AK4), shown in Table 2.3, as well as a fish-specific eIF2α-kinase, PKZ (55).

Table 2.3: eIF2α-kinases in zebrafish genome

eIF2α-kinase	Symbol	#aas	Chromosome	Accession number
HRI	eif2ak1	621	12	NP_001071035
PKR	eif2ak2	682	13	NP_001107942
PERK	eif2ak3	1099	13	NP_001070617
GCN2	eif2ak4	1552	17	XP_002667149

Because of our interest in eIF2α phosphorylation and fish diets we also looked at eIF2α-kinase expression in zebrafish muscle. RT-PCR amplification of the transcripts of HRI, PKR, PERK and GCN2 using RNA from ZFL cells and zebrafish muscle is shown in Figure 2.8. ZFL cells express all four eIF2α-kinases. Although muscle shows expression of all four eIF2α-kinases, it shows lower expression of GCN2, HRI and PKR than in ZFL cells.

Figure 2.8. Zebrafish eIF2α kinases expressed in different cell types.

RT-PCR amplification of RNA from ZFL and zebrafish muscle using primer pairs specific for HRI (EIF2AK1), PKR (EIF2AK2), PERK (EIF2AK3) and GCN2 (EIF2AK4), and primers given in Table 2.1. Amplicons were fractionated by 1 % agarose gel electrophoresis, visualized by ethidium bromide staining and image generated by FluoroChemE (Proteinsimple) imager.

2.4.6 Both eIF2α-a and -b can be phosphorylated by eIF2α-kinases *in vitro*

Although all activators of eIF2α-kinases tested caused increased eIF2α phosphorylation in ZFL cells, it was not possible to determine whether one or both of these are expressed at the level of protein in ZFL cells or whether one or both of them become phosphorylated. However, the two forms of eIF2α are identical in the phosphorylation site and the kinase docking site (Figure 2.1), so it

seems highly likely that both would serve as good substrates for the eIF2α-kinases. Since it cannot be determined from the above data whether one or both of the two forms respond to activation of the eIF2α-kinases, we took the alternative approach of looking at their ability to be phosphorylated by purified mammalian eIF2α-kinases *in vitro*. ^{35}S-labeled eIF2α-a, eIF2α-a(S51A), eIF2α-b and eIF2α-b(S51A) were synthesized in a reticulocyte translation system to which we later added purified mammalian eIF2α-kinases. Phosphorylated and non-phosphorylated forms were separated by vertical slab gel isoelectric focusing (VSIEF). It should be noted that in the generation of eIF2α-b(S51A), x additional amino acids were added to the eIF2α-b coding sequence giving an isoelectric point of 5.01. This analysis showed that both eIF2α-a and eIF2α -b, but not the S51A variants can be phosphorylated by eIF2α-kinases (Figure 2.9).

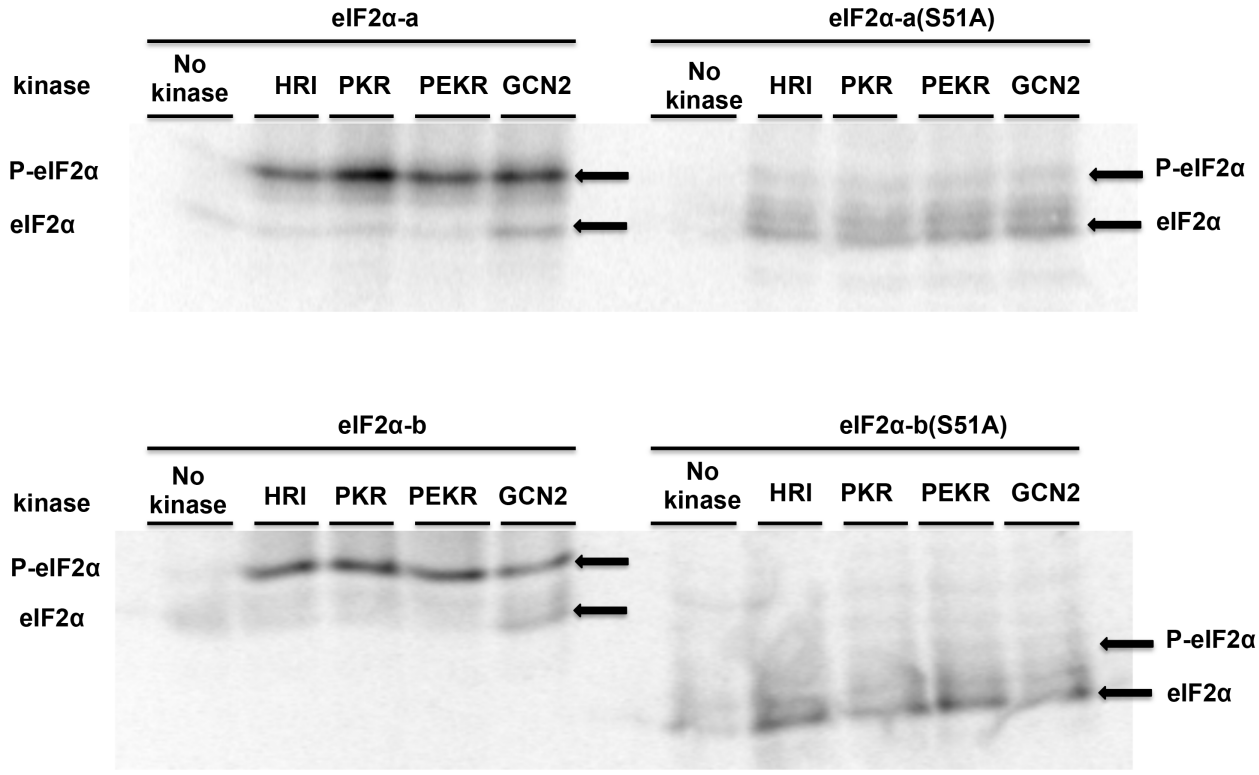

Figure 2.9. ***In vitro*** **phosphorylation of zebrafish eIF2αs by purified recombinant mammalian eIF2α-kinases**

^{35}S-radiolabeled zebrafish eIF2α-a, eIF2α-aS51A, eIF2α-b and eIF2α-bS51A were synthesized in the reticulocyte TnT cell-free transcription/translation system by incubation at 30 ^{o}C for 30 min after which they were supplemented with 8 ng/μl of purified mammalian recombinant HRI (EIF2AK1), PKR (EIF2AK2), PERK (EIF2AK3) or GCN2 (EIF2AK4) followed by incubation at 30 ^{o}C for an additional 10 min. Microcystin (0.5 μM) was included to inhibit phosphatase activity. Each sample was diluted in 40 volumes isoelectric focusing sample buffer prior to analysis. Samples were subjected to vertical slab isoelectric focusing (VSIEF) to separate phosphorylated and non-phosphorylated forms, using a narrow pH range of 4.5–6. Proteins were transferred to PVDF and ^{35}S signal visualized using the Typhoon imager.

2.4.7 Effects of transfection on eIF2α phosphorylation state

Transfection of cells with plasmids encoding a gene of interest, often coupled to a reporter gene is a pivotal technique for introduction and expression of foreign genes. A variety of transfection methods have been developed in mammalian system including chemical (e.g. calcium phosphate co-precipitation), lipid-based methods, physical (e.g. electroporation) and viral (e.g. retrovirus) approaches. These techniques have been easily optimized for use with mammalian cell lines to give transfection with high efficiency. However, when applying the same methods to cell lines from various fish species, usually cultured at lower temperatures, the efficiency is usually well below 10 %. The lack of robust methods to transfect zebrafish cell lines have hindered the application of biochemical and cell-based assays that are frequently used in mammalian studies. Nucleofection is an advanced electroporation technique that varies electrical parameters and buffers to optimize delivery for specific cell types with high efficiency and reproducibility (161). A major advantage of nucleofection is its versatility in transfecting a wide variety of primary dividing and non-dividing cell types. Nucleofector technology has been used to increase transfection efficiency in cultured fish cells up to 90 %, depending on cell type (162, 163).

However, it has been reported that nucleofection increases eIF2α phosphorylation in mammalian cell lines, even in the absence of delivered cDNAs (164).

We compared the optimized nucleofection methods using the Neon Transfection System with FuGene6, a lipid-mediated transfection agent shown to give the highest transfection efficiencies in zebrafish cell lines (163). When ZFL cells were transfected with FuGene 6, our best results were achieved with a reagent:DNA ratio of 3:1. Using transfection with pmaxGFP, this gave a transfection efficiency rate of 6 % and viability of 95 %. In contrast, nucleofection provided a significantly higher efficiency than lipid-mediated transfection. The use of the Neon Transfection System by contrast allowed transfection efficiencies up to 75 % to be achieved with 95 % viability. Depending on the conditions used, transfection efficiency ranged from 5 % to 75 %. However, not only did transfection increase eIF2α phosphorylation, but the eIF2α phosphorylation state correlated directly with transfection efficiency (Figure 2.10).

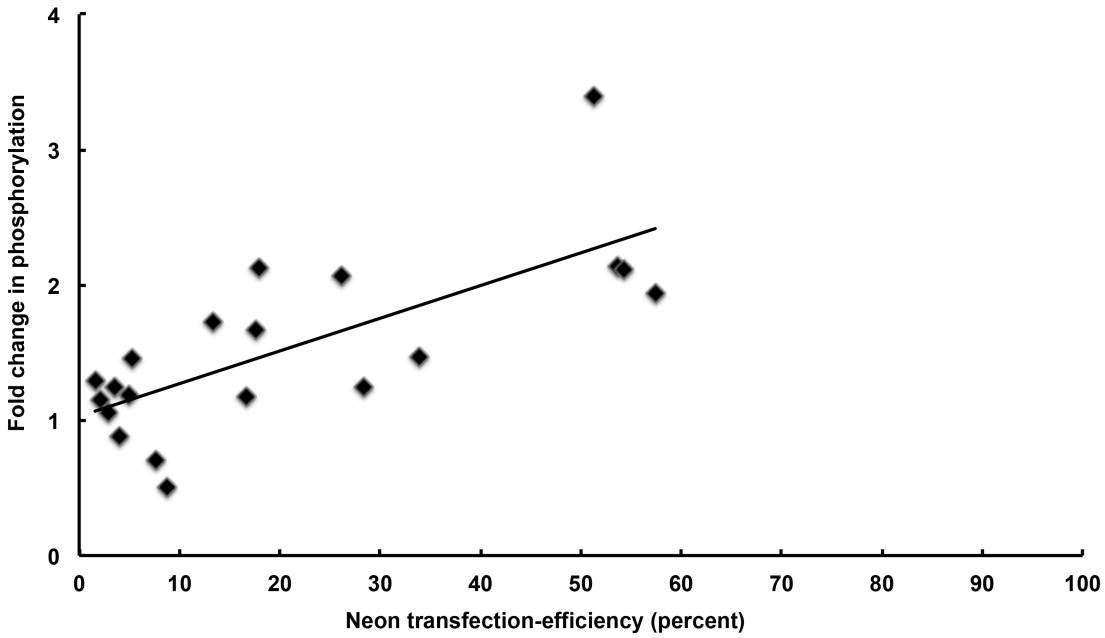

Figure 2.10. Effects of electroporation on eIF2α phosphorylation state in ZFL cells

ZFL were split 3 days before electroporation and grown to 85 % confluency. Cells were trypsinized and 1×10^6 cells were suspended in Neon® Resuspension Buffer R with 2 µg pmaxGFP. Transfection was performed using the Neon® Transfection System according to the manufacturer's protocols, using 24 different conditions. After transfection, cells were either plated for determination of transfection efficiency or processed for analysis of eIF2α phosphorylation (as described in the legend to Figure 2.6). Transfection efficiency was determined by the proportion of GFP positive cells at 24 h and expressed as a percentage of total cells. Cell viability was assessed by trypan blue dye exclusion.

In mammalian cells, increased eIF2α phosphorylation by electroporation has been shown largely to reflect activation of PERK with a smaller contribution from GCN2 (164). So although transfection efficiency of ZFL cells can be significantly improved by nucleofection techniques, the protein synthetic capacity of the cells is compromised. An understanding of the non-specific effects of nucleofection are important for understanding the results obtained with its use.

2.5 DISCUSSION

All living organisms must respond to, and defend against, environmental stressors. These include temperature shock (165), oxygen shock (hypoxia or

oxidative stress) (166), nutrient deprivation (167) and excess (45, 168, 169), DNA damage (170). The ability to respond rapidly to changes in the cellular environment is essential for survival, and numerous strategies for dealing with cell stress have evolved. One of the most commonly used mechanisms for inhibiting global translation is by phosphorylation of the translation initiation factor eIF2 (171). In the present study, the roles of two *Danio rerio* eIF2α cognates (eIF2α-a and eIF2α-b) in the response to stressors through increased eIF2α phosphorylation were explored by looking at the response of ZFL cells to a range of typical activators of eIF2α-kinases; nutrient deprivation, leucinol, thapsigargin, polyI:C and NMPP. Overall the response in ZFL cells mirror the responses seen in mammalian cells and the eIF2α-kinases GCN2 (EIF2AK4), PERK (EIF2AK3), PKR (EIF2AK2), and HRI (EIF2AK1) were all shown to be expressed in ZFL cells. Although it was not possible to pinpoint which eIF2α is phosphorylated in the ZFL cells, the demonstration that both wild-type eIF2α-a and -b, but not the eIF2α Ser51Ala variants, can be phosphorylated *in vitro* by purified GCN2, PERK, PKR and HRI, is consistent with both being subject to stress-dependent phosphorylation.

One question that arises is why zebrafish has two *eif2S1* genes and why the two cognates are so similar. In ray-finned fish (Actinopterygii), whole genome duplication occurred coincident with the radiation of teleost species. This has been termed the teleost specific whole genome duplication (TGD) (172, 173). In addition, there is evidence that two earlier rounds of large-scale gene duplication occurred early in vertebrate evolution (174, 175). In general, while most gene

pairs formed by WGD are subsequently deleted, rapid functional divergence is known to allow duplicate gene retention (176-180). Why the genes encoding two such similar proteins have been retained is unclear. The redundancy may suggestion more control and regulation in the protein translation process.

Although there is a paucity of studies looking at eIF2α phosphorylation in fish, there are increasing reports of eIF2α-kinases in several fish species. PKR has been reported in zebrafish, grass carp, rock bream, green spotted pufferfish, Japanese flounder, olive flounder, goldfish, three-spined stickleback and rare minnow (181-184). The functional analysis of PKR in a couple of fish species has revealed that fish PKR also possesses the ability to phosphorylate eIF2α (181, 182), suggesting that fish PKRs play a similar function as the mammalian homologue in the interferon stimulated defense against virus infection. HRI has been reported in olive flounder (185) and PERK in medaka (186), Chinese rare minnow (187), salmon embryo cells (188) and zebrafish BRF41 fibroblast cells (189). Aquatic birnavirus induces cell death via activation of PERK and eIF2α phosphorylation in salmon embryo cells (188).

Fish are subjected to variable environmental factors such as temperature, salinity, oxygen levels, or parameters related to water composition or quality. In particular, poor water quality seems to compromise the ability to respond to infection and environmental toxins. For instance, chronic hypoxia-induced stress seems to delay and reduce the interferon (IFN) response to infectious pancreatic necrosis virus (IPNV) in salmon (190). Toxic molecules present in water can also present problems for aquacultured salmonids. In a study on exposure to

pollutants such as herbicide pendimethalin, it was found that mortality due to VHSV was accelerated in rainbow trout (191). Similarly, the pro-inflammatory cytokine TNFα alpha was increased in primary cultures of rainbow trout macrophages after copper exposure (192). Tributyltin (TBT) activates PERK and stimulated eIF2α phosphorylation in Chinese rare minnow larvae (187) and in zebrafish BRF41 fibroblast cells (189).

The monitoring of eIF2α phosphorylation could prove useful in assessing the quality of diets in fish grown in aquaculture. The eIF2α kinase most likely to be involved in nutritional responses is GCN2 which has been identified in the genomes of zebrafish and other fish. However, to date, there are no reported investigations of GCN2 in any fish species. In mice, GCN2 is required for adaptation to deprivation of essential amino acids (63). *GCN2*($^{-/-}$) mice are viable, fertile, and exhibit no phenotypic abnormalities under standard growth conditions (63). However, *GCN2*($^{-/-}$) mice are unable to alter the phosphorylation of eIF2α in response to dietary leucine restriction. Losing GCN2 activity shifts the normal maintenance of protein mass away from skeletal muscle to provide substrate for continued hepatic translation. Lipid synthesis, which is repressed in livers of wild-type mice during longer periods of leucine starvation, continues unabated in GCN2-deficient mice, ultimately contributing to liver steatosis (44). GCN2 can also be involved in food choices. To insure an adequate supply of nutrients, omnivores choose among available food sources. This process is exemplified by the well-characterized innate aversion of omnivores to otherwise nutritious foods of imbalanced amino acid content (69, 193). Consumption of a meal of

imbalanced amino acid composition selectively elevates levels of phosphorylated eIF2α in anterior piriform neurons, establishing a molecular correlate to the aversive response (69). GCN2 inactivation diminishes phosphorylated eIF2α levels in the mouse anterior piriform cortex following consumption of an imbalanced meal and impairs this aversive response (69, 193). eIF2α phosphorylation is also involved in the response to the overfed state through PKR (45).

Our findings suggest that eIF2α phosphorylation could be used to monitor response to temperature changes, the effects of acidification, oxidative stress and xenobiotics, such as 4-nonylphenol and tributyltin (TBT) in fish. In addition, tracking eIF2α phosphorylation could prove invaluable for understanding the outcomes of different fish diets in aquaculture species.

Chapter 3: eIF2α phosphorylation in response to nutritional deficiency and stressors in the aquaculture fish, *Rachycentron canadum*

3.1 ABSTRACT

The present study investigates the response of the marine fish cobia, *Rachycentron canadum*, to stressors as measured by phosphorylation of the α-subunit of the translational initiation factor, eIF2. eIF2α is the target of phosphorylation by a family of kinases that respond to a range of physiological stressors. Phosphorylation of eIF2α inhibits overall protein synthesis, but allows cells to reprogram gene expression to adapt to and recover from stress. Here we show that the deduced coding sequence of cobia eIF2α has 94 % identity to both zebrafish (*Danio rerio*) and human eIF2α sequences with phosphorylation and kinase docking sites identical to those found in zebrafish and human eIF2α. The present study uses a newly isolated cobia cell line, CM cells, derived from muscle, to investigate the response of cobia eIF2α to; nutrient deficiency, leucinol, endoplasmic reticulum (ER) stress, poly I:C and N-methylprotoporphyrin, all of which are known to activate eIF2α-kinases. Phosphorylation of eIF2α in CM cells is increased by nutrient deficiency, leucinol, and ER stress, consistent with the activation of GCN2, and PERK, respectively. However, poly I:C and N-methylprotoporphyrin barely affect the phosphorylation state of eIF2α in cobia muscle cells. Since we find that recombinant cobia eIF2α can be phosphorylated *in vitro* by all four eIF2α-kinases, this suggests that CM cells do not express the eIF2α kinases PKR and HRI. Furthermore, preliminary

studies have shown that in cobia juveniles, diet and water temperature affect the phosphorylation state of eIF2α. We conclude that evaluation of eIF2α phosphorylation could have potential as an early marker to evaluate diet, aquaculture stressors and disease in cobia and may be of particular use in the optimization of conditions for raising cobia larvae and juveniles.

3.2 INTRODUCTION

R. canadum is a perciform pelagic fish species found in warm-temperate to tropical waters of the West and East Atlantic Ocean, throughout the Caribbean, and in the Indo-Pacific off India, Australia and Japan. It is eurythermal, tolerating a wide range of temperatures, from 1.6 to 32.2 °C. It is also euryhaline, living at salinities of 5 to 44.5 ppt. Cobia is sold commercially and commands a relatively high price for its firm texture and excellent flavor. However, no designated wild fishery exists because it is a solitary species. It is currently farmed in aquaculture (194, 195) and exhibits rapid growth in both off-shore cage systems as well as marine recirculating aquaculture tanks. Cobia seed stock used for large scale commercial aquaculture production comes exclusively from hatcheries and fingerling production is still an industry-wide bottleneck despite improvements in cobia larviculture (194, 196). Problems exist in larvae/fingerling culture, including high mortality due to stress and disease in the larval stage and the reliance on unsustainable fishmeal diets. Expansion of cobia farming would benefit from the development of sensitive assays to monitor stress during larval culture as well as the optimization of plant-based diets to increase production and sustainability (92). Diet studies are both time consuming

and expensive and could be facilitated by the development of faster indicators of diet quality. Similarly, a sensitive indicator of stress has the potential to assist in optimization of larviculture conditions.

Phosphorylation of Ser51 of the α-subunit of the translational initiation factor eIF2 is a key cellular response to wide range of environmental stresses such as nutrient deficiency, hypoxia, endoplasmic reticulum stress and viral infection in eukaryotes from protists to vertebrates (2, 6, 137). There are five eIF2α-specific kinases in vertebrates (five in teleost fish, four in tetrapods) that can phosphorylate eIF2α, each of which is activated by different stressors. HRI (EIF2AK1) is stimulated by heme depletion in erythroid cells and also by oxidative stress, osmotic shock, and heat shock (35, 36). HRI is the only eIF2α kinase activated by arsenite in erythroid cells (36) and is the major eIF2α kinase responsive to heat shock. PKR (EIF2AK2) is stimulated by viral infection (37, 38). PKR also plays a more general role in cellular physiology; it can be activated in response to signals as diverse as oxidative and ER stress (39-41), as well as cytokine and growth factors signaling (42, 43) and has been implicated in the pathology of obesity (44, 45). PERK (EIF2AK3) is an endoplasmic reticulum (ER) transmembrane protein activated by the accumulation of misfolded proteins in the ER, a phenomenon termed the unfolded protein response (UPR) or ER stress (46). Changes in Ca2+ levels within the ER negatively affect the ability of the chaperone protein, binding immunoglobulin protein (BiP), also known as 78 kDa glucose-regulated protein (GRP-78), to maintain PERK in its inactive state (47). This allows diverse signals such as inhibition of the SERCA calcium pump,

glucose deprivation, and high levels of fatty acids reduce the ER luminal calcium concentration (48). GCN2 (EIF2AK4) is a sensor of amino-acid availability (49). GCN2 is the primary responder to nutritional deprivation and is the only eIF2α-kinase conserved among plants and metazoans. It is activated by reduced amino acid levels in yeast and mammalian cells (27, 50, 51) and can also be activated by glucose deprivation (52). GCN2 is also involved in a variety of organismal functions in vertebrates such as long-term memory formation and feeding behavior (53). These four members of the eIF2α kinases share extensive homology in their kinase catalytic domains and phosphorylate eIF2α at the same serine residue (Ser51) (reviewed (54)). Teleost fish also have a PKR-like kinase, PKZ, that is more closely related to the kinase domain of PKR compared to the other three eIF2α kinases (55). Phosphorylation of eIF2α on serine 51 or its equivalent reduces the rate of protein synthesis overall. However, phosphorylation of eIF2α increases the recruitment of a subset of mRNAs resulting in the transient activation of an alternate program of gene expression that enables the cell or organism to respond to a range of stressors (2, 25).

With the known relationship between eIF2α phosphorylation and nutritional status and food choices in mammals, it was of interest to determine whether eIFα phosphorylation can be used as an early marker to evaluate diets and aquaculture conditions in fish. Since eIF2α phosphorylation was first demonstrated in zebrafish, and since zebrafish is a tractable model system, studies were initiated in this species to lay the groundwork for looking at fish of more interest to aquaculture (Liu et al m/s in preparation). These studies have

shown that zebrafish ZFL cells respond to a variety of stressors that activate the range of eIF2α–kinases. However, it was of more interest to see if cells from a marine species of aquaculture interest, such as cobia, would respond in the same way. A number of cell lines have been described from economically important marine species. These include cell lines from groupers (197, 198), tilapia (199), seabass (200), red sea bream (201), turbot (202), southern bluefin tuna (203), flounder (204, 205) sea perch (206) and gilthead seabream (207, 208). The isolation of two cobia cell lines, Cb and Cf from brain and fin, respectively, was previously reported and characterized, but the lines were subsequently lost (209). In this study we report the establishment and use of a new cell line from cobia muscle, CM cells. To facilitate studies on CM cells and cobia, we have successfully cloned eIF2α cDNA using degenerate primers based on eIF2α sequences from other fish species and completing the coding sequence using 5' & 3' RACE. The current work demonstrates that recombinant cobia eIF2α can be phosphorylated by stress-activated eIF2α-kinases and that CM cells respond to activators of eIF2α-kinases by increasing eIF2α phosphorylation. Furthermore, preliminary studies show that changes in eIF2α phosphorylation can arise from nutritional deficiencies and temperature in cobia juveniles. These results suggest that eIF2α phosphorylation could provide a useful indicator to monitor cobia with regard to aquaculture conditions, as well as response to diets and disease.

3.3 MATERIALS AND METHODS

3.3.1 Establishment and culture of cobia muscle (CM) cell line

A cobia with body weight of 15 g derived from a hatchery farm in Southern Taiwan was used for the primary culture. The fish was anaesthetized by using MS-222 (Sigma) in concentration of 200 mg/ml for 5 min. The fish body surface was sterilized by dipping in 5 % bleach, followed by wiping with 70 % ethanol. Muscle tissue was dissected from the body and washed three times in a washing medium (L-15 plus 400 IU/ml penicillin, 400 µg/ml streptomycin and 10 µg/ml fungizone). The muscle tissue was minced to approximately 2 mm2 pieces, and placed into phosphate buffer containing 0.25% trypsin and 0.2% ethylenediaminetetraacetic acid (EDTA). The tissue fragments in the trypsin solution were slowly agitated with a magnetic stirrer at 25 °C for 15 min, and the cells in the suspension was concentrated by centrifugation at 200 g at 25 °C for 10 min. The cells were resuspended in complete medium (L-15 plus 20% FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml fungizone), and seeded into 25 cm2 tissue culture flasks. The cells were incubated at 28 °C without CO_2 supplementation. When the confluent monolayer formed in primary culture, cells were dislodged from the flask by 0.1 % trypsin solution (0.1 % trypsin and 0.2 % EDTA in phosphate buffer), and subcultured at a ratio of 1:2. The concentration of FBS gradually decreased to 10 % as the subculture times increased to 70. The continuous cell line, named as CM, was then cultured in antibiotic-free medium. The morphology of CM cells is fibroblast-like (Figure 3.1 A–C).

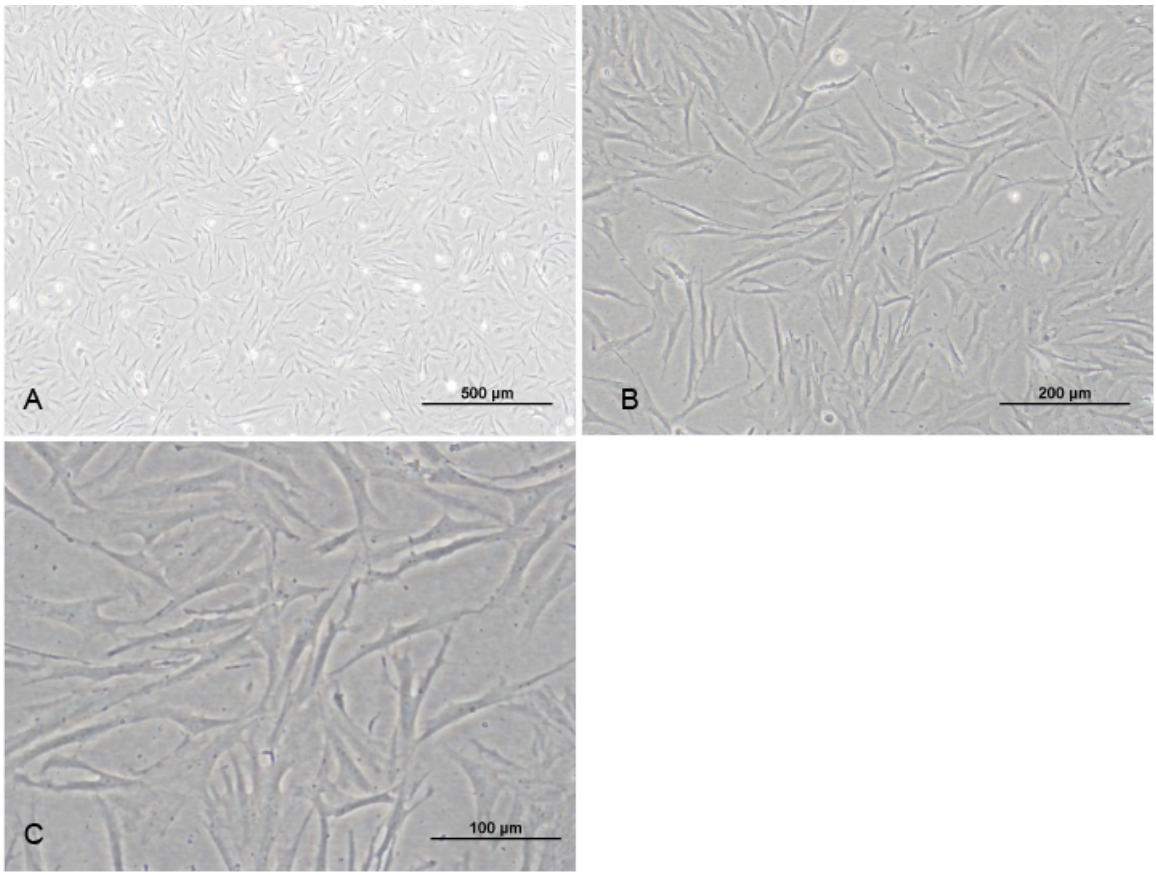

Figure 3.1. Phase-contrast photomicrograph of cobia cell line monolayers.

3.3.2 Identification of CM cell origin

The sequence of mitochondrial cytochrome oxidase subunit I gene (cox I) was used to verify whether the cell lines were actually derived from cobia. RNA was isolated from CM cells and fresh cobia muscle using the Ambion PureLink® RNA Mini Kit (Life Technologies Corporation, Carlsbad, CA, USA) following the manufacturer's protocol. cDNA was synthesized using Revertaid™ M-MULV reverse transcriptase (Fermentas GmbH, St. Leon-Roth, Germany) and random hexamer primers (Qiagen, Valencia, CA, USA) to produce first strand cDNA. Amplification of cDNA fragments of the predicted size was confirmed by end-point PCR amplification using the primers described from FDA, 2011 (210). PCR

products were separated using 1 % TAE (Tris-acetate-EDTA) agarose gel electrophoresis and stained with ethidium bromide. The cDNA products were recovered using a QIAquick PCR purification Kit (Qiagen) followed by sequencing. CLC and The Basic Local Alignment Search Tool (BLAST, NCBI) were used to compare sequence identity (210).

3.3.3 Primer design for analysis of cobia eIF2α

Degenerate primers were designed for PCR from five closely related fish species, zebra fish, catfish, puffer fish, Atlantic salmon and rainbow trout. The RT-PCR products were cloned into the vector, pGEM-T and DNA sequence analysis was carried out on an Applied Biosystems Automated Sequencer. 5' and 3' rapid amplification of cDNA ends (RACE)-PCR was used to construct the full length of cDNA sequence. RACE primers used to amplify the cobia eIF2α cDNAs were designed by Primer 3 software. For a list of primers, see Table 3.1.

Table 3.1. Primer pairs used for eIF2a-RACEprimer, cloning of cobia eIF2α and cobia COX1 gene recognition

Primer	Sequence (5'-3')	Tm
RceIF2α 5'GSP1	CACACTTGATGGCCTCCTCT	56.9
RceIF2α 5'NGSP1	TACTCGGATGACGACCACGC	59.1
RceIF2α 3'GSP2	GGGTCTGTCTGTCCTCAACC	57.3
RceIF2α 3'NGSP	AGAGGCTAGAGCGGGAGAAC	58.4
RceIF2α F1	GAGGTGGAGGATGTGGTGAT	56.4
RceIF2α F2	GAGTACAACAACATCGAGGG	52.9
RceIF2α R1	YTCDGCTTTGGCCTCCAT	55.7
RceIF2α R2	TGACVGCCTGTGGDGTSAG	59.7
RcBaF	GATCCTGACAGAGCGTGG	55.3
RcBaR	AGCACAGTGTTGGCGTACAG	57.9
RcFISHCOILBC_ts	CACGACGTTGTAAAACGACTCAACYAATCAYAAAGATATYGGCAC	64.3
RcFISHCOIHBC_ts	GGATAACAATTTCACACAGGACTTCYGGGTGRCCRAARAATCA	65.9
RcCOX1F	TCAACCAACCACAAAGACATTGGCAC	60.2
RcCOX1R	TAGACTTCTGGGTGGCCAAAGAATCA	59.8

Primers used to amplify the cobia eIF2α cDNAs were designed from the published coding sequences from five close related fish species, zebra fish (NM_131800.2), catfish (GU588091.1), puffer fish (CR685632.2), Atlantic salmon (NM_001140183) and rainbow trout (NM_001124296.1), selected most conserved sequence as target region and we initially amplified a ~900 bp cDNA. The complete coding sequence was assembled by 5' & 3' RACE. The degenerate primers among the above oligonucleotides incorporate a statistical mix of monomers at the positions labeled V (A, C or G), S (C or G), R (A or G), Y (C or T) or D (A, G or T) [in accordance with IUPAC convention].

3.3.4 Preparation of RNA from CM cells and cobia muscle and cDNA synthesis

Total RNA was extracted from cobia cell line, liver and muscle using the PureLink® RNA Mini Kit (Ambion) following the manufacturer's protocol. The recovered RNA was spectrally analyzed for concentration at 260 nm, and for purity at both 260/280 and 260/230 ratios by Nanodrop ND-1000 spectrophotometry and by automated electrophoresis using the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA). Values of >2 for 260/280 and 260/230 ratios were considered to be of sufficient purity for planned uses. Amplification of cDNA fragments of the predicted size was confirmed by end-

point RT-PCR. cDNA was synthesized using Revertaid™ M-MULV reverse transcriptase (Fermentas GmbH, St. Leon-Roth, Germany) and random hexamer primers (Qiagen, Valencia, CA, USA) to produce first stand cDNA. Total RNA (1 µg) was used for first-strand cDNA synthesis with SuperScript™ II Reverse Transcriptase (Invitrogen) and random hexamers (QIAGEN) according to the manufacturer's instructions. The cDNA produced was diluted to represent that produced from 12.5 ng/µl RNA for qPCR analysis and stored at -80 ^{o}C. The quality of all cDNA preparations was assessed by end point PCR amplification using the primers described above.

3.3.5 Cobia eIF2α cloning and generation of eIF2αS51A constructs

Approximately 30 mg of cobia liver tissue was used as starting material for total RNA extraction for cloning. Degenerate primers were designed for RT-PCR from the closely related five fish species, zebra fish, catfish, puffer fish, Atlantic salmon and rainbow trout. The RT-PCR products were cloned into the vector, pGEM-T and DNA sequence analysis was carried out on an Applied Biosystems Automated Sequencer. 5' and 3' rapid amplification of cDNA ends (RACE)-PCR was used to construct the full length of cDNA sequence. The cDNA for cobia eIF2α, generated from RNA prepared from cobia liver and muscle tissue, was cloned into pGEMT and pCITE. For cloning of eIF2α, cDNA was prepared from RNA isolated from cobia muscle tissue, and cDNA prepared using random hexamers. All primers sets were designed to provide NdeI and BamHI recognition sites at the 5'- and 3'-ends (Table 3.1). eIF2α sequence was verified from five primary transformants. The open reading frame of eIF2α was excised

from pGEMT/eIF2α using the NdeI and BamHI restriction sites and transferred into similarly digested pCITE4a. The pCITE4a constructs were used as templates for coupled transcription–translation system, using the rabbit reticulocyte TNTQuick system (Promega, Madison, WI, USA).

Generation of eIF2α-bS51A: cDNA containing the S51 to A51 mutation was synthesized by GenScript and cloned into pCITE4a with Nde I and BamHI recognition sites at the 5'- and 3'-ends.

3.3.6 Preparation of cell extracts for analysis of eIF2α phosphorylation

CM cells (~10^7) in monolayers were rinsed once with ice cold PBS containing 25 mM sodium molybdate, 10 mM β-glycerophosphate, and 100 mM NaF prior to lysis in 500 µl 25 mM HEPES–KOH, pH 7.2, 5 mM EDTA, 100 mM KCl, 0.5 % Elugent (Calbiochem), 10 % glycerol, 1 mM DTT, 0.5 mM microcystin, and 1 tablet/10 ml protease inhibitor pill (Boeringer–Mannheim) (93). Lysates were collected and clarified by centrifugation (10,000 x g for 5 min at 4 °C) prior to snap-freezing and storage in liquid N_2. In general, extracts contained approximately 2–3 mg/ml of protein.

3.3.7 Immunoblot analysis to detect eIF2α phosphorylation

Cobia cell extract samples were fractionated by 15 % SDS-PAGE, electrotransferred to PVDF membranes, and subjected to immunoblot analysis using polyclonal antibody against phosphorylated Ser51 of mammalian eIF2α (CellSignaling, cat#3597, Danvers, MA, USA) followed by goat anti-rabbit secondary antibody coupled to HRP and coupled with chemiluminescence.

Chemiluminescence was detected using the ProteinSimple Fluorochem E with quantification using ImageJ. Total eIF2α levels were determined by reprobing the stripped blot with rabbit antibody that recognizes equally the phosphorylated and unphosphorylated forms of eIF2α (CellSignaling, cat#9722, Danvers, MA, USA). Values obtained with the anti-eIF2α[P] antibody were normalized for the total amount of eIF2α present in the sample (Liu et al. m/s in preparation).

3.3.8 *In vitro* phosphorylation of cobia eIF2α

^{35}S-radiolabeled eIF2α-a and eIF2α-b and their nonphosphorylatable variants were produced in the reticulocyte TnT cell-free transcription/translation system by incubation at 30 ^{o}C for 30 min, after which they were supplemented with 8 ng/μl (specific activity 53 nmol/min/mg) of purified mammalian recombinant HRI (EIF2AK1), PKR (EIF2AK2), PERK (EIF2AK3) or GCN2 (EIF2AK4) followed by incubation at 30 ^{o}C for an additional 10 min. Microcystin (0.5 μM) was included to inhibit phosphatase activity. Each sample was diluted in 10 volumes isoelectric focusing sample buffer (9.5 M urea, 1 % Pharmalyte, pH 4.5–5.4, 1 % Pharmalyte, pH 5–6, 5%CHAPS (Fluka), 50 mM DTT) prior to analysis. Samples were subjected to vertical slab isoelectric focusing (VSIEF) (146), using a narrow pH range of 4.5–6 in the presence of 8.8 M urea, 3 % acrylamide, 1 % CHAPS, 5 % Pharmalyte (pH 4.5–6), and 50 mM DTT. Proteins were electrophoresed at 2 mA/gel with a 1200 voltage limit for 16 h, using reverse polarity with 0.01 M glutamic acid at the anode and 0.05 M histidine at the cathode. Proteins were transferred to PVDF and ^{35}S-labeled protein was visualized using Typhoon.

3.3.9 Experimental fish and systems

This study was carried out in accordance with the guidelines of the International Animal Care and Use Committee of the University of Maryland Medical School (IACUC protocol # 0610015). Juvenile (~2 g) cobia, *R. canadum*, were obtained from the Virginia Agricultural Experiment Station, Virginia Tech, Hampton, VA. Juveniles were maintained at 27 ^{o}C in a marine recirculating system at the Institute of Marine and Environmental Technology's Aquaculture Research Center, Baltimore, MD. Diet formulations are given in Table 3.2.

Table 3.2: Dietary formulations for the fish meal (FM) versus plant protein diets (PP)

Ingredient (g kg^{-1})	FM	PP
Menhaden fish meal	345	0
Soy Protein concentrate	0	269
Corn Protein concentrate	44.3	193.4
Poultry by-product meal	118	0
Wheat Flour	237.7	175.5
Soybean meal, solvent extracted	90	90
Wheat Gluten meal	0	22
Blood meal, spray	39	0
Menhaden fish oil	90	120
Vitamin pre-mix	20	20
Mono-Dical Phosphate	0	42.5
Lecithin	0	20
L-Lysine	0	19.9
Choline CL	6	6
Potassium Chloride	0	5.6
DL-methionine	0	5
Threonine	0	2.8
Sodium Chloride	0	2.8
Stay-C	2	2
Trace mineral pre-mix	1	1
Magnesium Oxid	0	0.5
Mycozorb	2	2
Taurine	5	0
Performance characteristics (extrapolated from two different experiments)		
SGR*	4.72+/-0.02	0.57+/-0.12

3.3.10 Cobia sampling and tissue extraction

Diet trials were being conducted by the Place laboratory, giving me the opportunity to collect three to five fish per condition, randomly selected, for analysis of eIF2α phosphorylation. Fish were anesthetized with tricaine methanosulfonate (MS-222, 70 mg l-1, Finquel, Redmond, WA). Liver samples were snap-frozen in liquid N_2 immediately and stored at -80 °C for later analyses. Tissue extracts using lysing matrix D (MP Biomedicals, Solon, OH) homogenized with lysis buffer (3 lysis buffer: 1 tissue ratio). Tissue was disrupted using FastPrep-24 5G (MP Biomedicals, Solon, OH) for 40 sec, kept on ice for 5 min and microcentrifuged at 12,000 g for 15 min at 4 ^{o}C. Supernatants were snap-frozen in liquid N_2 immediately and stored at -80 °C for later analyses. Phosphorylation of eIF2α was determined by immunoblot analysis of SDS-PAGE fractionated proteins as described in Section 3.3.7 above.

3.4 RESULTS

3.4.1 Identification of CM cell origin

Using each of two primer sets, the PCR-amplified cox 1 cDNA amplicons from CM cells and fresh muscle tissue were of the same predicted sizes ~ 650 bp band after agarose gel electrophoresis. DNA sequencing and comparative analysis demonstrated that cox 1 cDNAs amplified from fresh cobia muscle tissue and from CM cells were identical. Moreover, all cox 1 cDNA sequences amplified from RNA from CM cells or fresh cobia muscle were 99%~100% identical with (GenBank accession # FJ154956.1) and cobia cytochrome oxidase

subunit 1 (COI) gene (GenBank accession # KJ202194.1) (Figure 3.2). These data confirm that the origin of the established CM cells is from cobia.

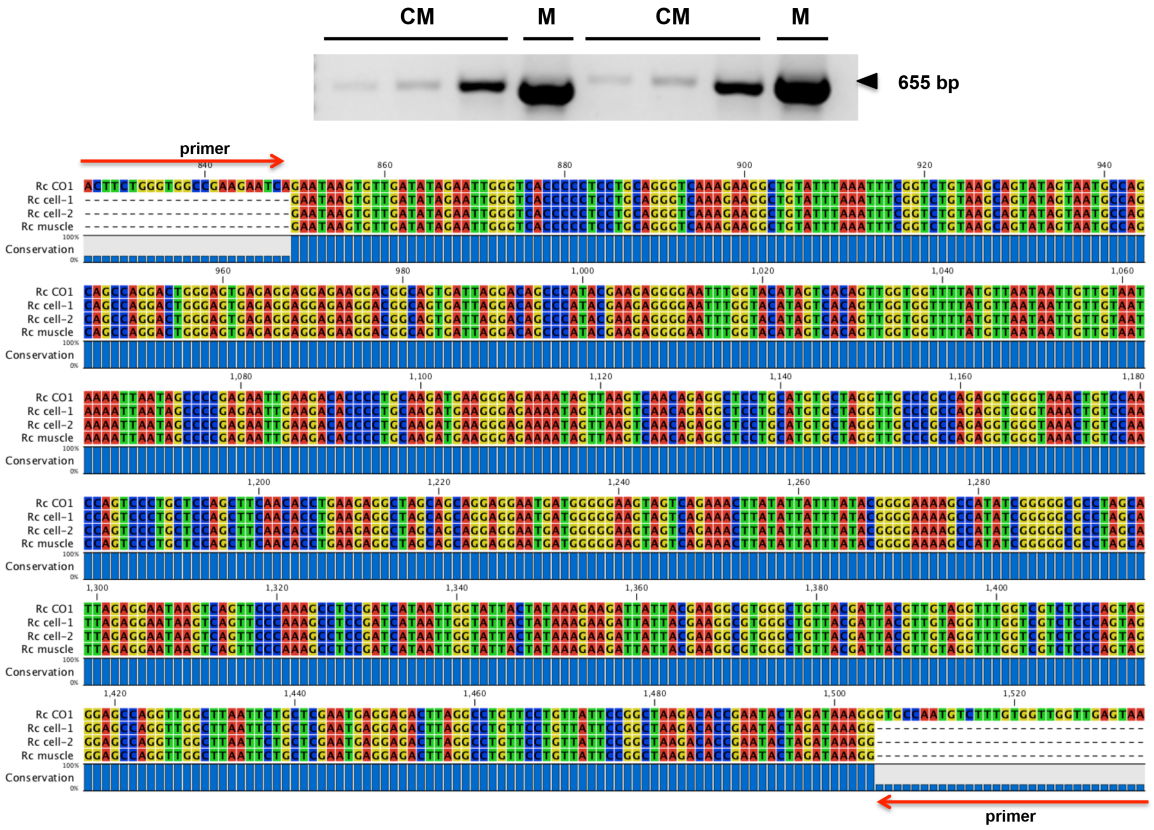

Figure 3.2. Identification of cobia cell origin

Upper panel shows PCR-amplified DNA from cobia muscle cell line (CM) and cobia muscle (M) using primer pairs specific for cobia cytochrome oxidase subunit 1 (COI) gene, and primers given in Supplemental Table S1. Amplicons were fractionated by 1 % agarose gel electrophoresis. Multiple alignments of amplified cobia COI gene from cobia muscle cell line and cobia muscle in comparison with cobia cytochrome oxidase subunit 1 (COI) gene (gene bank accession # KJ202194.1) by the ClustalW program are shown below. The red arrows indicate the position at which the primers anneal.

3.4.2 Cobia have two eIF2α transcripts but identical coding sequences

Two transcripts of eIF2α are found in cobia, although the coding sequence for each is identical (accession # KJ513464). Compared to human and zebrafish eIF2α, the cobia coding sequence is 94 % identical at the amino acid level. Table 3.3 shows the percent identities in amino acid composition, predicted molecular

weights and isoelectric points. The predicted isoelectric point and molecular weight of the eIF2α are in good agreement with those of human and zebrafish eIF2α. The cobia eIF2α is predicted to be 35.97 kDa, respectively, compared with human eIF2α that has a predicted molecular weight of 36.1 kDa.

Table 3.3. Characteristics of cobia eIF2α

eIF2α	Gene	ID#	#aas	%ID	MW(Da)	pI	Chrom	Location	Accession #
R. canadum **eIF2α**	eif2s1		315	94	35.97	5.02			KJ513464
D. rerio **eIF2α-a**	eif2s1a	321807	315	94	36.13	4.97	17	NC_007128.6	NM_199569.1
D. rerio **eIF2α-b**	eif2s1b	321564	315	94	36.17	4.98	20	NC_007131.6	NM_131800.2
H. sapiens **eIF2α**	EIF2S1	1969	315	100	36.1	5.01	14	14q23.3	NM_004094

The deduced isoelectric points are also very similar, 4.84 for cobia eIF2α compared to 5.02 for human eIF2α. Figure 3.3 shows the multiple alignment of the N-terminal 120 amino acids of cobia eIF2α compared to human and zebrafish eIF2αs. This covers the phosphorylation site and kinase docking domain. A multiple alignment of the complete amino acid sequences are provided as Figure 3.4. Residues in the phosphorylation loop region surrounding the eIF2α phosphorylation site (Ser-51 in human eIF2α) are identical in all eIF2αs. Similarly, residues in the PKR docking site (residues 79–83) of cobia eIF2α are identical to those in the human and zebrafish sequences, suggesting it is a good substrate for eIF2α-specific protein kinases. In addition, Ser-48, Ile-55, Leu-84, Arg-88 and Val-89, residues critical for interaction with the α-, β-, and δ-subunits of the guanine nucleotide exchange factor, eIF2B (147), are present in cobia

eIF2α indicating that the interaction between both eIF2αs and eIF2B is also conserved.

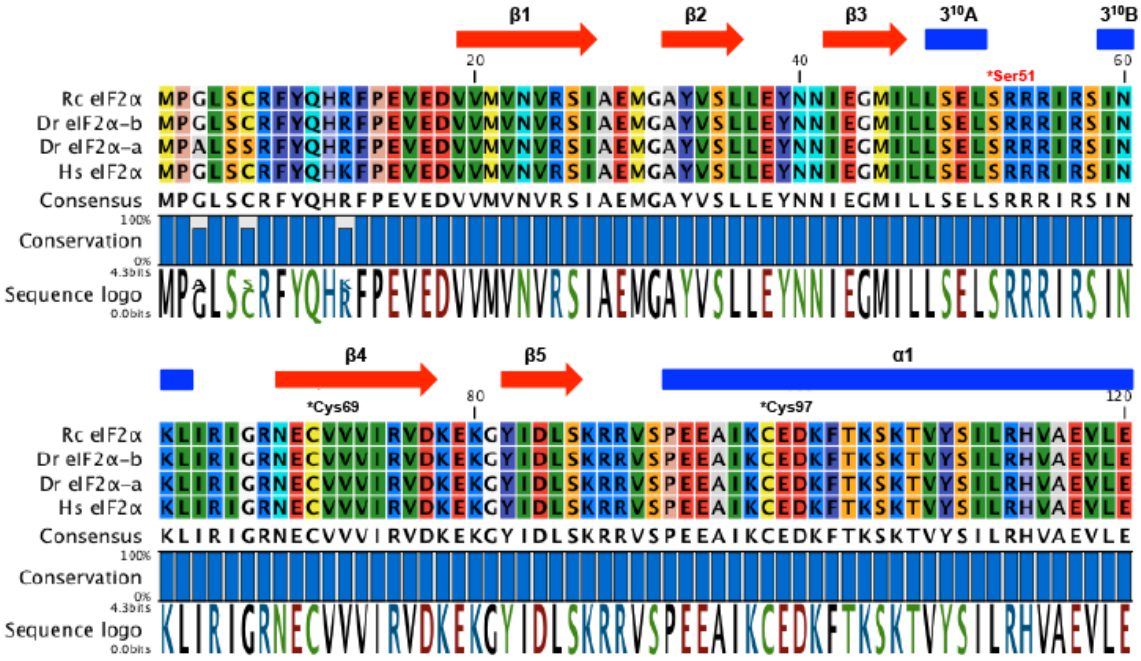

Figure 3.3. Alignment of eIF2α from cobia, zebrafish and human

ClustalW alignment of the N-terminal 120 residues of eIF2α from cobia, *Rachycentrum canadum*, in comparison with zebrafish eIF2αs and human. Secondary structural elements (red arrow, β-sheet; blue box, α-helix) are shown above the sequences for the N terminal domain. The red asterisk indicates the Ser51 phosphorylation site in eIF2α, and black asterisk shows the disulfide bridge between Cys-69 in β4 and Cys-97 in α1.

The first α-helix of human eIF2α is known to be involved in interactions that fix the orientation of the helical domain with the β-barrel (148). In mammalian eIF2α, this region contains a cysteine residue that is involved in a disulfide bridge and is thought to stabilize the interaction of the two domains. This residue is not found in non-vertebrate eIF2αs and appears to be a characteristic of vertebrate eIF2αs.

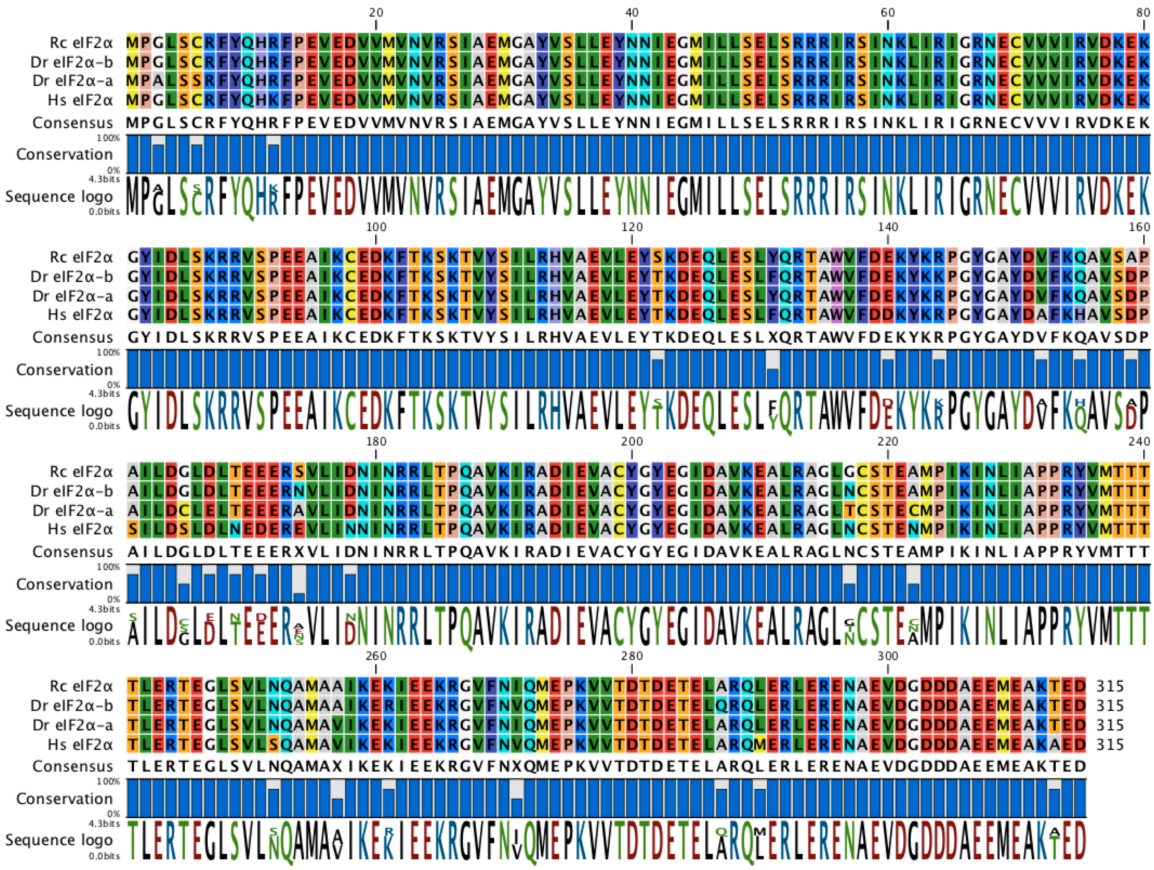

Figure 3.4. Multiple alignment of cobia eIF2α with zebrafish eIF2α, eIF2α-b and *H. sapiens* eIF2a: Multiple alignments of full amino acid sequences (315 aa) from cobia eIF2α in comparison with zebrafish eIF2α-a and eIF2α-b and human eIF2α by the ClustalW program. Amino acid numbering is shown above the sequences.

3.4.3 Cobia muscle cells respond to known activators of eIF2α kinases

Each of the four eIF2α-kinases, HRI, PKR, PERK and GCN2 can be activated in mammalian cells by a variety of stressors. Thapsigargin is an inhibitor of endoplasmic reticulum Ca^{2+} ATPase that blocks the ability of the cell to pump calcium into the sarcoplasmic and endoplasmic reticula which causes these stores to become depleted (154). This causes endoplasmic reticulum (ER) stress and activates the unfolded protein response, including the activation of PERK (72, 74). GCN2 is activated by starvation (49). Leucinol is the alcohol formed by total reduction of the carboxylic acid group of leucine. Leucinol is an

inhibitor of leucyl-tRNA synthetase and so can mimic leucine deficiency in cells (153). N-methylprotoporphyrin (NMPP) is a potent inhibitor of ferrochelatase (Ki~10 nM), the terminal enzyme of the heme-biosyntheic pathway (211, 212) and is able to activate the heme-sensitive kinase HRI (213). Polyinosinic:polycytidylic acid (poly I:C) is a synthetic dsRNA that is used experimentally to model viral infections *in vivo* (reviewed, 155) and is used *in vivo* and *in vitro* to activate PKR.

The response of CM cells to these known activators of eIF2α-kinases was examined, as shown in Figure 3.5 shows the response of CM cells to activators of eIF2α-kinases. CM cells treated with known activators of eIF2α-kinases: A) incubation in nutrient limiting medium or in the presence of leucinol (4 μM) for activation of GCN2; B) thapsigargin (1 μM) for activation of PERK; C) poly I:C (50 μg/ml with 200 μg/ml DEAE dextran) for activation of PKR; D) NMPP (2 μM) for activation of HRI. Cells were harvested at various time points, as determined by preliminary experiments, and analyzed by immunoblotting as described in Section 3.3.7.

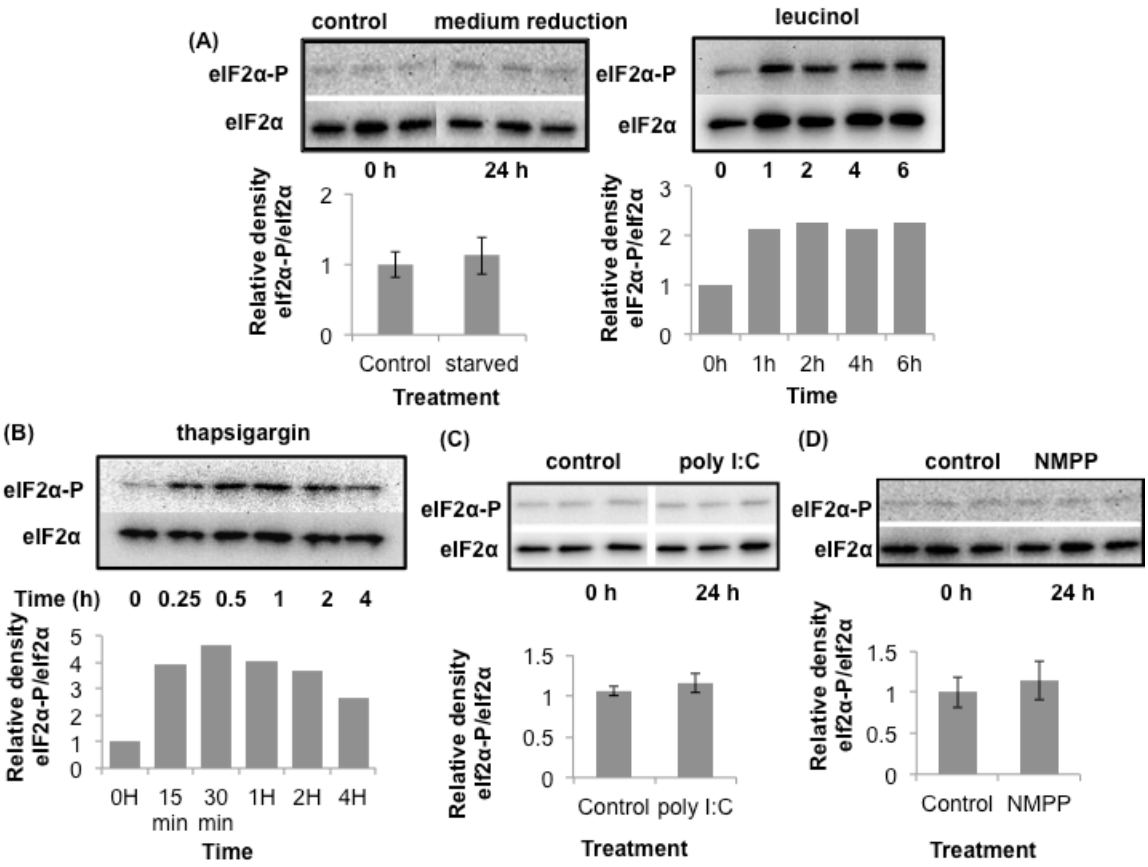

Figure 3.5. Response of cobia muscle cells to activators of eIF2-kinases

Cobia muscle cells treated with known activators of eIF2α-kinases: (A) Medium reduction cells were maintained in 30 % L-15 cell culture medium in PBS for 24 h; (B) leucinol (4 mM), for up to 24 h, for activation of GCN2; (C) thapsigargin (1 μM), for up to 2 h, for activation of PERK; (D) PIC (50 μg/ml), for 24 h, for activation of PKR; (E) NMPP (2 μM), for up to 24 h, for activation of HRI. Proteins in cell extracts were fractionated by SDS-PAGE and eIF2α was visualized by chemiluminescence following electro-transfer to PVDF, and probed with antibody to phosphorylated eIF2α and (after stripping) antibodies to total eIF2α. Phosphorylated and total eIF2α was visualized by chemiluminescence following electro-transfer to PVDF. Chemiluminescence was detected and image generated (upper panels) using the ProteinSimple Fluorochem E, with quantification using ImageJ to estimate level of phosphorylated eIF2α (lower panels).

3.4.4 Effects of nutrient deficiency and leucinol on CM cells

Nutrient deficiency and leucinol treatment were used to determine whether GCN2 can be activated in cobia cells. Reducing the CM cell L-15 culture medium to 30 % in phosphate buffered saline (PBS) for 24 h reduced both amino acid and serum levels. After 24 h, eIF2α phosphorylation was no increased (Figure

3.5, Panel A, left) consistent with the activation of GCN2. When leucinol (4 mM) was included in the complete culture medium, an increase in eIF2α phosphorylation could be seen within 1 h and continued to increase over 24 h (Figure 3.5, Panel A, right). This also strongly suggests activation of GCN2. Mouse ES cells also respond very rapidly to leucinol treatment. However, GCN2$^{(-/-)}$ mouse ES cells failed to show any induction in eIF2α phosphorylation during the first 3 h of leucine deprivation demonstrating that the effect of leucine deprivation on eIF2α phosphorylation could be attributed to GCN2 (63).

3.4.5 Effects of thapsigargin on CM cells

Thapsigargin is non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) (158). As in the case of zebrafish ZFL cells (Liu et al ms in preparation), eIF2α in CM cells respond very quickly to incubation with thapsigargin (1 μM). Figure 3.5, panel B, results indicate that PERK can be activated in cobia cells.

3.4.6 Effects of poly I:C on CM cells

CM cells were treated with 50 μg/ml poly I:C and 200 μg/ml DEAE dextran. Figure 3.5, panel C, shows that treatment for 24 h showed almost no eIF2α phosphorylation level difference between control and treatment group. This is in contrast to the effect in ZFL cells (Liu *et al* ms in preparation). These results could indicate that PKR is not expressed in CM cells, is not activated, or that cobia eIF2α is not a good substrate for PKR.

3.4.7 Effects of N-methylprotoporphyrin on CM cells

N-methylprotoporphyrin (NMPP) is a transition-state analogue and potent inhibitor of ferrochelatase, is used to induce heme deficiency and has been shown to increase eIF2α phosphorylation. Cobia muscle cell line were treated with NMPP (2 μM) for up to 24 h. Unlike the effect of NMPP in zebrafish ZFL cells which respond rapidly to NMPP, Figure 3.5, panel D, shows that no increases in eIF2α phosphorylation over 24 h. These results indicate that HRI may not be expressed in CM cells, is not activated or is not a good substrate for HRI.

3.4.8 Cobia eIF2α can be phosphorylated by eIF2α-kinases *in vitro*

Although CM cells responded to activators of GCN2 and PERK with increased eIF2α phosphorylation, they did not respond to activators of PKR or HRI. We have previously found that recombinant zebrafish eIF2α can be phosphorylated by all vertebrate eIF2α-kinases *in vitro*, we took the alternative approach of looking at the capacity of cobia eIF2α to be phosphorylated by the kinases *in vitro*. ^{35}S-labeled cobia eIF2α was synthesized in a reticulocyte translation system to which we later added purified mammalian eIF2α-kinases. Phosphorylated and non-phosphorylated forms of eIF2α were separated by vertical slab gel isoelectric focusing (VSIEF) (Figure 3.6). This analysis showed that cobia eIF2α can be phosphorylated by eIF2α-kinases and that PKR and HRI are either not well expressed or not activated by poly I:C or NMPP in CM cells.

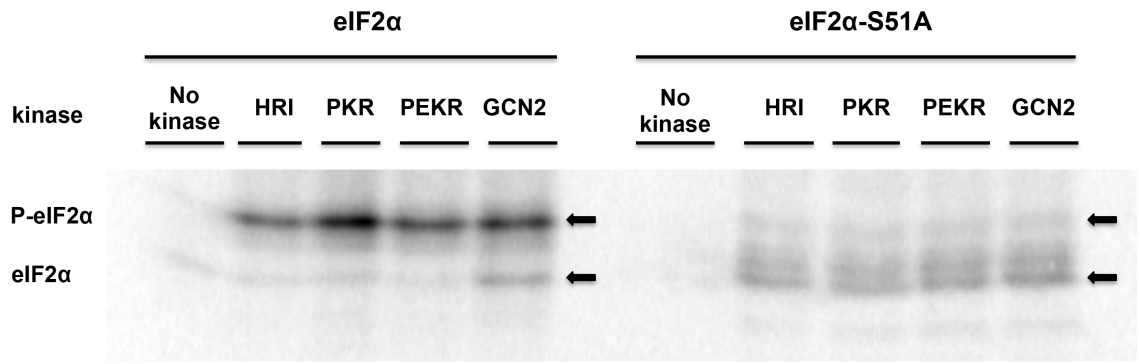

Figure 3.6. ***In vitro*** **phosphorylation of cobia eIF2α by purified recombinant mammalian eIF2α-kinases**

^{35}S-radiolabeled cobia eIF2α, eIF2α-S51A were synthesized in the reticulocyte TnT cell-free transcription/translation system by incubation at 30 ^{o}C for 30 min after which they were supplemented with 8 ng/μl of purified mammalian recombinant HRI (EIF2AK1), PKR (EIF2AK2), PERK (EIF2AK3) or GCN2 (EIF2AK4) followed by incubation at 30 ^{o}C for an additional 10 min. Microcystin (0.5 μM) was included to inhibit phosphatase activity. Each sample was diluted in 40 volumes isoelectric focusing sample buffer prior to analysis. Samples were subjected to vertical slab isoelectric focusing (VSIEF) to separate phosphorylated and non-phosphorylated forms, using a narrow pH range of 4.5–6. Proteins transferred to PVDF and visualized using the Typhoon imager. The red arrow indicates the phosphorylated eIF2αs.

3.4.9 eIF2α phosphorylation in cobia juveniles

3.4.9.1 Comparison of eIF2α phosphorylation in fish fed a fish meal (FM) versus an all plant protein diet (PP)

In order to be useful in aquaculture situations, it was necessary to demonstrate that eIF2α phosphorylation responds to nutritional differences and stress, not just in CM cells, but in fish themselves. During the current investigation, the Place lab was conducting comparisons of different dietary formulations for cobia, focusing on the formulation of plant protein diets. We took advantage of this by sampling fish from a few of the diet trials. Our investigation was not intended to be an exhaustive analysis of eIF2α phosphorylation under all dietary regimes, but samples of fish were taken to ask if changes in eIF2α phosphorylation could be documented. For instance, the performance of cobia juveniles maintained for 12 weeks on an all plant protein diet, PP, was compared

with those on a mixed fish meal/plant protein diet, FM (91). The all plant protein diet was supplemented with menhaden oil to provide essential omega-3 fatty acids, but not with taurine, known to be essential in cobia diets (92). However, the PP diet still contained 0.02 % taurine. The complete formulations are given in Table 3.4.

Table 3.4: Diet formulations and proximate compositions of the experimental diets

Ingredient (g $100g^{-1}$)	Diet1	Diet2
Menhaden meal	45.5	22.9
Poultry meal	7.5	3.8
Wheat Flour	16	15.0
Soy protein concentrate	7.5	3.8
NPFI-3010	--	35.1*
Corn	17	9.4
Menhaden oil	3.9	6.4*
Vitamin pre-mix	1.0	1.0
Trace mineral pre-mix	0.1	0.1
Taurine	1.5	1.5
Lysine HCL	--	0.1*
DL-Methionine	--	0.8*
Proximate Composition (g $100g^{-1}$)[a]		
Moisture (g $100g^{-1}$)	7.2	11.1
Protein (g $100g^{-1}$ dm)	46.3	42.6
Protein on dry matter basis	49.9	47.9
Fat (g $100g^{-1}$ dm)	10.8	11.4
Fiber (g $100g^{-1}$ dm)	1.2	1.0
Ash (g $100g^{-1}$ dm)	9.1	8.8
Carbohydrate[b] (g $100g^{-1}$ dm)	28.72	29.01
Energy (mJ kg^{-1})	18.56	18.97
Performance characteristics		
SGR	3.29+/-0.08	3.45+/-0.08
PER	1.74+/-0.04	1.74+/-0.04
CF	0.637+/-0.04	0.715+/-0.04

[a] New Jersey Feeds Labs analysis, Trenton, NJ.

[b] Calculated by difference (100-Moisture-Protein-Ash-Fat-Fiber).

The specific growth rate (SGR) in cobia juveniles on the FM diet was 4.72+/-0.02, compared with 0.57+/-0.12 for fish on the PP diet. The phosphorylation of eIF2α in livers of these fish at the end of the feeding trial is shown in Figure 3.7.

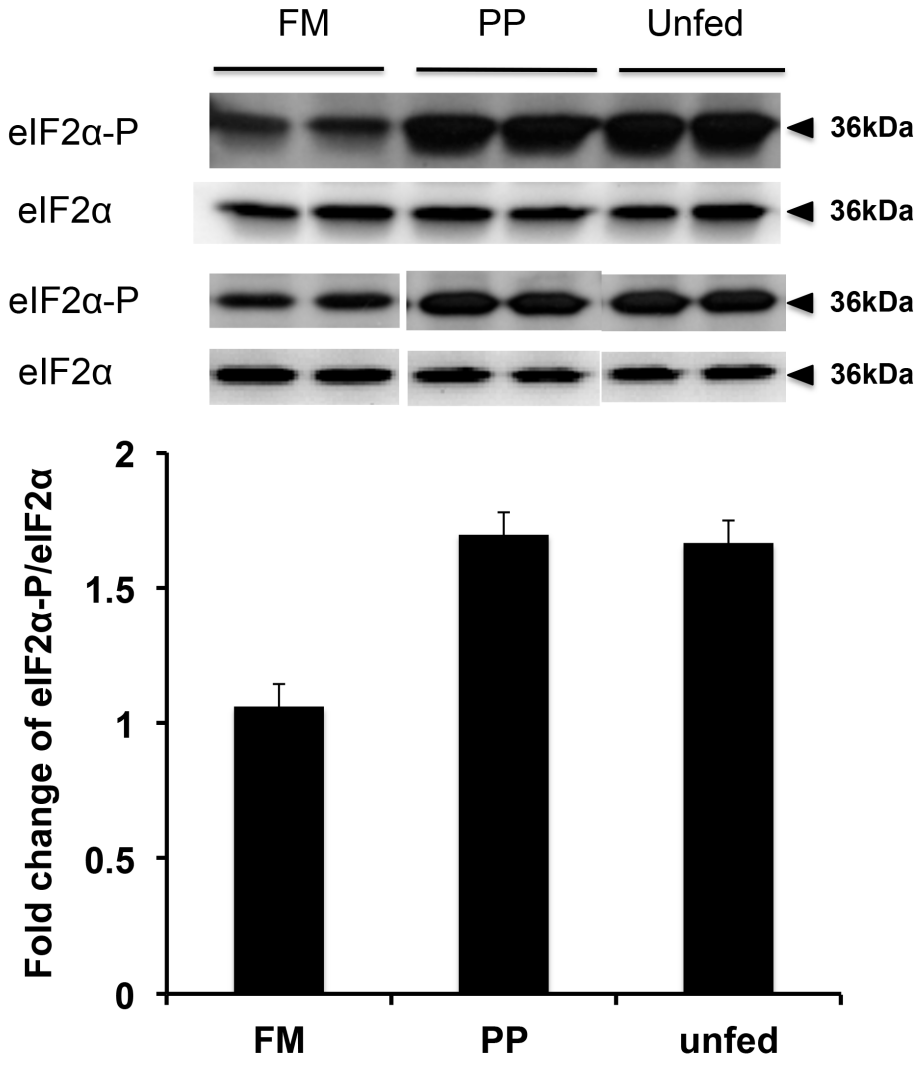

Figure 3.7. Comparison of eIF2α phosphorylation in cobia fed a fish meal versus plant protein diets

Cobia juveniles maintained for 12 weeks on an all plant protein diet, PP, was compared with a mixed fish meal/plant protein diet, FM (91) and unfed fish. The complete formulations are given in Table 3.2. Proteins in tissue extracts were fractionated by SDS-PAGE and transferred to PVDF. Phosphorylated and total eIF2α were visualized by enhanced chemiluminescence, using antibody to phosphorylated eIF2α and (after stripping) antibodies to total eIF2α, as described in legend to Figure 3.5. The bar diagrams in lower panels represent the quantification of the blots corresponding to the respective lanes in the upper panel.

Higher eIF2α phosphorylation levels can be seen in all the fish fed the plant protein diet without taurine supplementation. In fact, the level of eIF2α phosphorylation is as high as that observed in juveniles maintained without feeding for 7 days.

3.4.9.2 Comparison of eIF2α phosphorylation in fish fed a diet with partial replacement of fish meal/poultry by-product/soy protein concentrate with a non-GMO soybean cultivars

Although fish meal can be replaced at least in part by soybean concentrate, most commercially available soybean products are from genetically modified organisms (GMOs), the use of which is not universally accepted. Schillinger Genetics, Inc. has developed multiple cultivars of soybean with potential as fishmeal replacements in diets for aquaculture. Cultivars have been developed with reduced levels of the anti-nutritional factors raffinose, stachyose, and trypsin inhibitors. A diet was formulated, Diet 2, to replace 50 % of protein supplied by fishmeal with a cooked, solvent-extracted soybean meal formulation 3010 from Schillinger Genetics Inc. Poultry meal, wheat flour, soy protein concentrate, corn, and fish oil were all varied to maintain the Diet 2 isonitrogenous, isolipidic, and isocaloric qualities compared with the diet1, FM, and to contain similar amino acid profiles. Diet 2 is also supplemented with 0.15 % taurine (214). The performance characteristics of Diet 2 in a 12-week trial were compared with that of the reference diet, Diet 1, containing 45.5 % fishmeal (214). The complete formulation is given in Table 3.4. Both diets gave very good performance characteristics; diet2 gave slightly better performance characteristics than FM, with an SGR of 3.45 +/-0.08, CF of 0.715+/-0.04

compared to SGR of 3.29+/-0.08, CF of 0.637+/-0.637 (209). This is reflected in the level of eIF2α phosphorylation which is lower in fish fed the diet2 (Figure 3.8). Note that levels of phosphorylated eIF2α are low in each condition, with the ratio of phosphorylated to total eIF2α in the reference diet being equivalent to that observed in the FM diet in Figure 3.7.

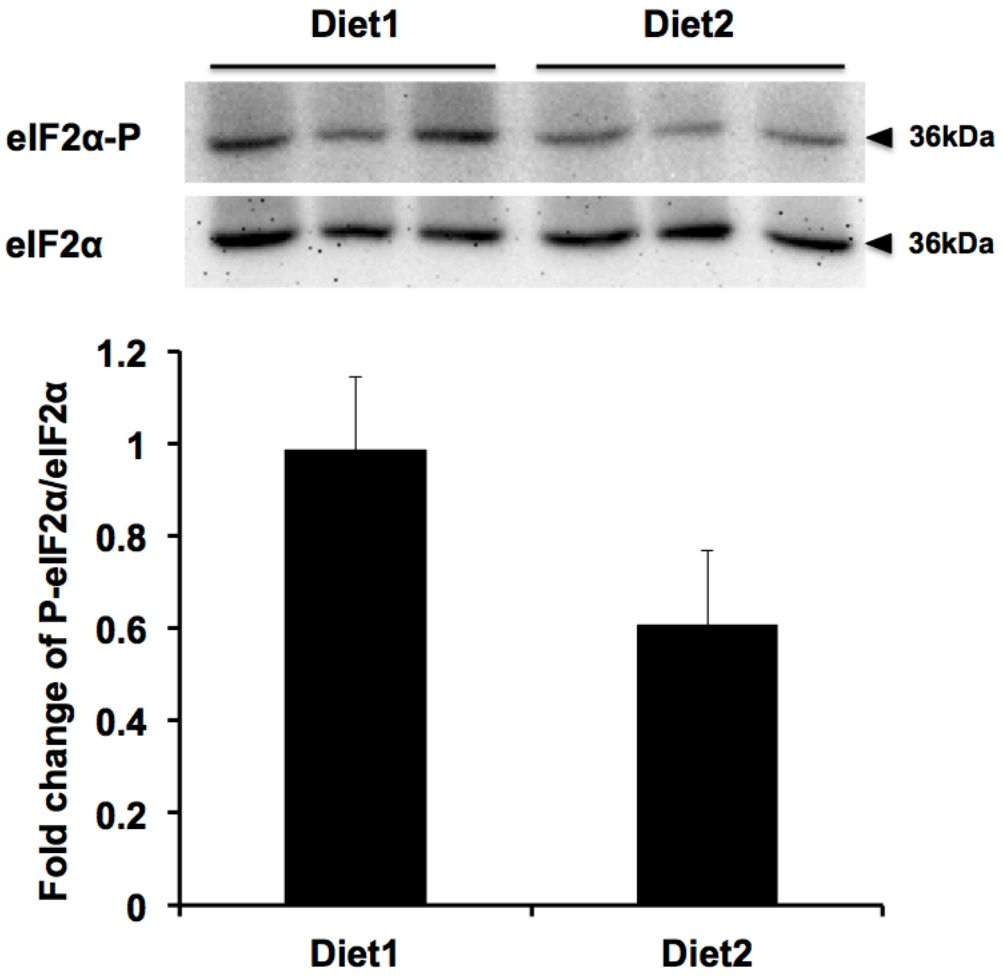

Figure 3.8. Comparison of eIF2α phosphorylation in cobia Juvenile fed diet1 and diet 2 (soy protein concentrate with a non-GMO soybean cultivars)

Juvenile cobia keeps in tank fed diet1; a diet with partial replacement of fish meal, poultry by-product and diet2; soy protein concentrate with a non-GMO soybean cultivars and diet formula given in Table 3.4. Proteins in tissue extracts were fractionated by SDS-PAGE and transferred to PVDF. Phosphorylated and total eIF2α were visualized by enhanced chemiluminescence, using antibody to phosphorylated eIF2α and (after stripping) antibodies to total eIF2α, as described in legend to Figure 3.5. The bar diagrams in lower panels represent the quantification of the blots corresponding to the respective lanes in the upper panel.

3.4.9.3 eIF2α phosphorylation during "cold banking" and acclimation

"Cold banking" is a carefully employed technique to slow down fish growth rate. It is especially effective with fingerlings, when an investigator is trying to stagger fish production. A comparison was made of "cold banked" juveniles, maintained at 20 °C and fed a maintenance ration 5 days a week at 1.5 % body weight (bw) daily for 6 weeks prior to acclimation, with cold-banking parameters based on Holt et al. (196). Acclimation to 27 °C and 5 % bw feeding daily was established over the course of two weeks, increasing 1 °C per day and 0.5 % bw per day for one week, followed by maintenance at 27 °C for one week. eIF2α phosphorylation was compared in the cold-banked and acclimated fish in Figure 3.9. The level of eIF2α phosphorylation was higher in the acclimated fish. This surprised us since cold shock activates PERK in mammals (215). However, a similar response is seen in human livers kept at cold temperatures for transportation (216). Cold transportation temperatures activate the IRE-1 pathway component of ER stress early. In contrast, at colder temperatures, ATP levels are lower, so that PERK is not activated (requires autophosphorylation), and the level of eIF2α phosphorylation is low. However, this portion of the ER stress response happens as a second phase once the liver is re-perfused and the temperature increased.

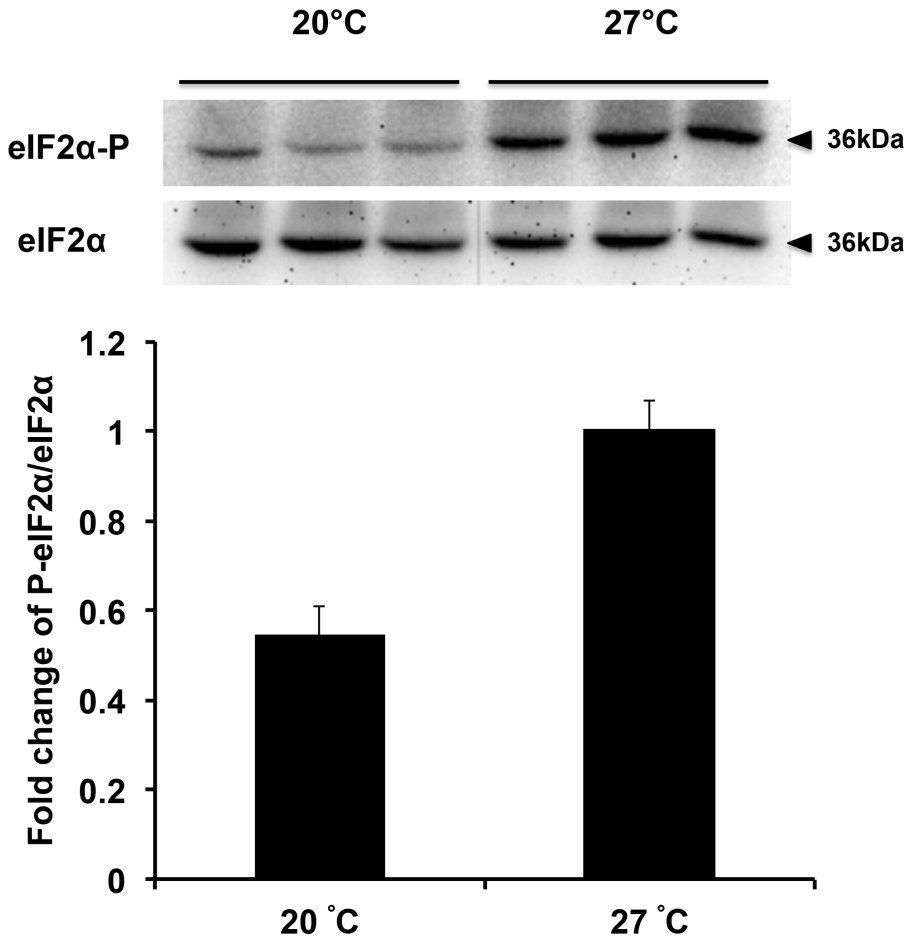

Figure 3.9. Effects of cold banking on eIF2α phosphorylation level

Effects of water temp change on eIF2α phosphorylation level in cobia liver. Juvenile cobia keeps in tank that water temperature holds at 20 ^{o}C and 27 ^{o}C. Proteins in cell extracts were fractionated by SDS-PAGE and transferred to PVDF. Phosphorylated and total eIF2α were visualized by enhanced chemiluminescence, using antibody to phosphorylated eIF2α and (after stripping) antibodies to total eIF2α, as described in legend to Figure 3.5. The bar diagrams in lower panels represent the quantification of the blots corresponding to the respective lanes in the upper panel. Y-axis is the relative ratio of eIF2α phosphorylation compare to total eIF2α.

3.5 DISCUSSION

The present study investigated the response of the marine fish cobia, *Rachycentron canadum*, to stressors as measured by phosphorylation of the α-subunit of the translational initiation factor, eIF2, the target of a family of protein kinases that respond to physiological stressors. The high conservation of eIF2α

throughout vertebrates allowed us to use commercial antibodies to eIF2α and phosphorylated eIF2α for our studies. Also in this study, a new cobia cell line, CM, has been described and demonstrated to be of use in demonstrating the response to nutrient deficiency and ER stress. The responses of CM cells to nutrient deficiency and ER stress mimic those observed in mammalian and ZFL cells (Liu *et al*, m/s in preparation). The lack of response to poly (I:C) and NMPP most likely reflects low level expression of these kinases in the CM cell line. However, investigation of this will depend on uncovering the sequences of cobia eIF2α kinases. The CM cells are likely to be very useful for additional molecular studies to investigate the basis of the dietary requirements of cobia. These cells could be used for investigation of fatty acyl elongase, a key enzyme in the synthesis of polyunsaturated fatty acids. Similarly, the CM cells could be used to investigate the defects in the taurine biosynthetic pathway in cobia in more detail as well as responses to added taurine. Because of the lack of response to poly (I:C), it is unclear whether CM cells will be useful for susceptibility testing for viruses such as iridoviruses and nervous necrosis virus (NVV) important viral pathogens of at the fry and fingerling stages of cobia (217, 218).

Beyond investigations in CM cells, we asked if eIF2α phosphorylation could be used as a rapid indicator of fish physical condition. Our preliminary studies have shown that in cobia juveniles, responses at the level of eIF2α phosphorylation can be observed in response to diet and water temperature. This proof of principle opens the way to a more systematic investigation of responses at the level of eIF2α phosphorylation in cobia and other species of aquaculture

interest. We conclude that eIF2α phosphorylation may be useful as an indicator of fish condition and health in aquaculture situations.

Chapter 4: Adaptation of ZFL cells to long term culture in a serum-free medium

4.1 ABSTRACT

The use of fish cell lines in serum-free conditions is not yet routine. This study reports on the successful adaptation of ZFL cells to the synthetically manufactured serum-free medium UltraMEM^{TM}-ITES and compares the growth rates of adapted ZFLs to cells growing in Leibovitz's L-15 medium with 9 % FBS (L15-FBS). L15-FBS, which has a taurine level of 11.99 ± 1.16 µM, was gradually replaced with UltraMEM^{TM}-ITES over 120 days. Here we show that the ZFL cells adapted to growth in serum-free medium over 250 days, after which the doubling time stabilized at 8.62 ± 0.56 days, approximately three-fold slower than cells growing in L15-FBS. These growth characteristics were stable for over 250 days and the cells could be recovered after storage in liquid nitrogen. The successful adaptation of ZFL cells to UltraMEM^{TM}-ITES allowed us to investigate for the first time the effects of taurine depletion and restoration on expression of taurine biosynthetic pathway and transporter genes. The expression of these genes is upregulated approximately 10-fold in ZFL growing in UltraMEM^{TM}-ITES, but only the taurine transporter, TauT, responds to re-addition of taurine. The development of serum-independent ZFL cells provides a useful tool for a range of toxicological, pharmacological, metabolic and innate immune studies.

4.2 INTRODUCTION

Zebrafish (*Danio rerio*) is a widely used model species for human diseases and developmental biology, given its short generation time, well-characterized embryonic development, and sequenced genome (219, 220). However, its small size makes it challenging when isolating tissues for biochemical analysis, making zebrafish cell lines invaluable. Currently, six zebrafish cell lines are commercially available: ZF4, PAC2 and ZEM2 of embryonic origin; AB.9 and SJD.1 fibroblasts obtained from the zebrafish caudal fin; and ZFL obtained from a pool of 10 adult zebrafish livers (ATCC, Manassas, VA, USA: www.atcc.com). The ZFL cell line has characteristics of liver parenchymal cells and exhibits characteristics in culture consistent with differentiated liver cell function (151). ZFL cells have been characterized at the genetic and transcriptomic levels and shown to be suitable as a model cell for zebrafish research (151). They have been used for toxicological (221, 222), pharmacological (223) and innate immune function studies (224). The development of serum-independent ZFL cells will provide a useful tool in a range of such studies. These cells will also be useful in investigating hepatocyte metabolic function such as the example presented here of the regulation of taurine homeostasis. The adaptation strategy and use of UltraMEM^{TM}-ITES should be generally applicable to other fish cell lines.

Synthesis of taurine can occur in two ways (Figure 4.1, reference 1) with both pathways relying on cysteine, a semi-essential amino acid biosynthesized from the essential amino acid methionine. Synthesis occurs mainly in the liver

(225), after which taurine is taken up by other tissues through plasma circulation and a taurine transporter (TauT).

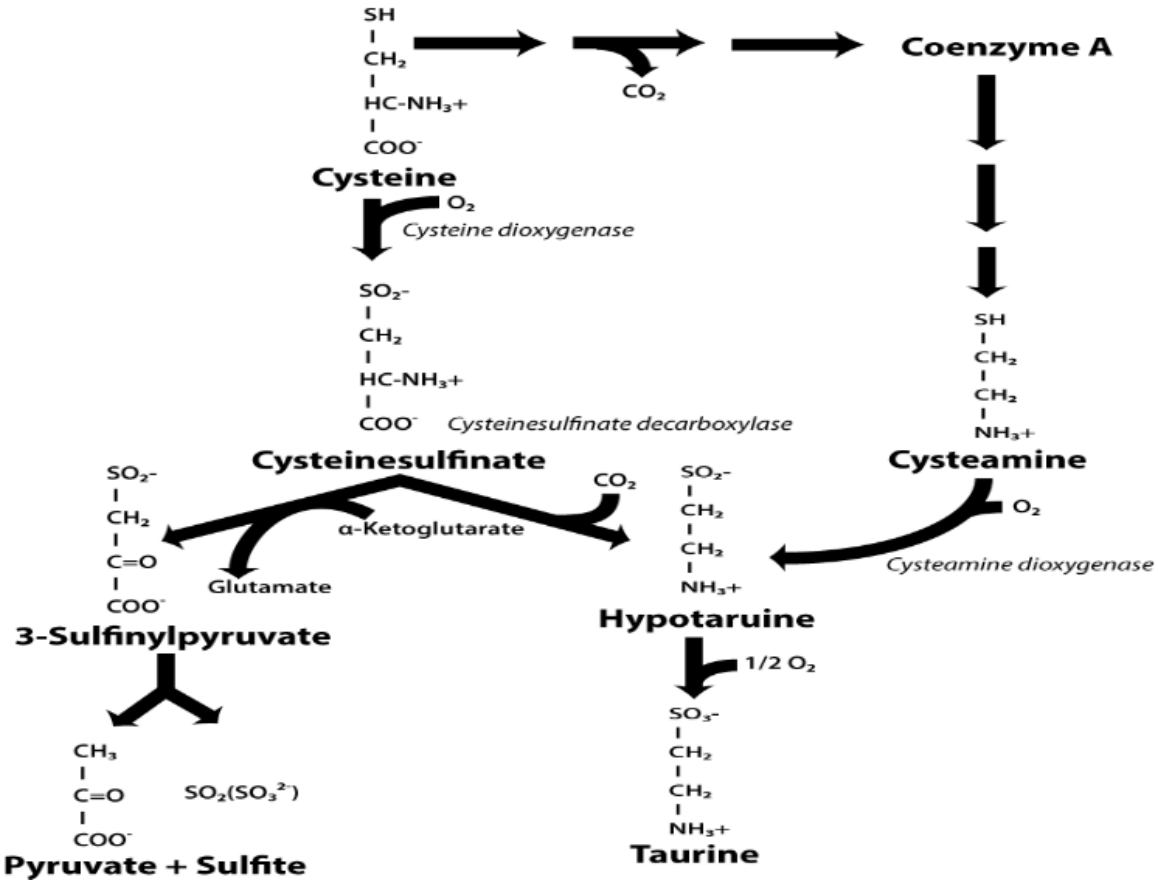

Figure 4.1. Taurine biosynthesis pathway, modified from (1)

4.3 MATERIALS AND METHODS

4.3.1 ZFL cell culture

The zebrafish liver cell line, ZFL, was obtained from ATCC (cat # CRL-2643). Cells were maintained in Leibovitz's L-15 medium (Cellgro, Manassas, VA, USA), 20 mM HEPES-KOH, pH 7, 2 mM sodium pyruvate, 100 units/ml of penicillin and streptomycin, and supplemented with 9 % fetal bovine serum

(Atlanta Biologicals, Lawrenceville, GA, USA) (L15-FBS) in 100 mm tissue culture plates at 28 ^{o}C, lacking sodium bicarbonate and CO_2. L-15 medium is used in preference to MEM because it is designed for supporting cell growth in environments without CO_2 equilibration. L-15 is buffered by phosphates and free base amino acids instead of sodium bicarbonate (220).

4.3.2 Adaptation to UltraMEM^{TM}-ITES culture medium

UltraMEM^{TM}-ITES is a protein-free basal medium supplemented with insulin, transferrin, ethanolamine, selenium (ITES) and L-glutamine. Recombinant human insulin and transferrin are the only protein components of the formulation and are present at a total concentration of 20 µg/ml. ZFL cells maintained in L15-FBS, were initially transferred into medium containing 10 % UltraMEM^{TM}-ITES (Lonza, Walkersville, MD, USA). After two passages, cells were transferred to L15-FBS containing 20 % UltraMEM^{TM}-ITES. Over 123 days in culture, L15-FBS was exchanged with increasing concentrations of UltraMEM^{TM}-ITES in 10 % increments every second passage, going from 0-100 % UltraMEM^{TM}-ITES. Over this period, FBS concentrations decreased concomitantly from 9-0 %. Medium changes were done gently so as not to stress the cells. Cell health was monitored by regular microscopic observation.

4.3.3 Passage number and determination of doubling time

Cells were counted every 5-8 days by trypsinization in 0.05 % trypsin/0.03 % EDTA in PBS, for 10 min at room temperature and counted using a hemocytometer. Average cell counts were calculated from the number of cells in 4 quadrants. Doubling times were calculated using the formula DT=T

ln2/ln(Xe/Xb), in which DT is the cell doubling time, T is the incubation time, Xb is the cell number at the beginning and Xe is the cell number at the end of the period in culture.

4.3.4 Primer design for RT-qPCR analysis of zebrafish taurine transporter protein and enzymes of the taurine biosynthetic pathway

Primers used to amplify cDNAs for zebrafish cysteamine dioxygenase (ADO), cysteine dioxygenase (CDO), cysteinesulfinate decarboxylase (CSD), taurine transporter (TauT) were designed based on published zebrafish sequences in GenBank (http://www.ncbi.nlm.nih.gov) by Primer 3 software (version 0.4.0) (143) (Table 4.1).

Table 4.1. Sequences of the primer pairs used for real-time quantitative PCR determination of the transcript levels of zebrafish cysteamine dioxygenase (ADO), cysteine dioxygenase (CDO), cysteinesulfinate decarboxylase (CSD), taurine transporter (TauT) and ribosomal protein L13A (L13A)

Gene	5'-3' forward primer	5'-3' reverse primer	GenBank accession no.
***D. rerio* ADO**	5'-TTACAGACTGCTGGGAAAAA-3'	5'-GGCTTGAAACAAGCAAATAA-3'	NM_001008634.1
***D. rerio* CDO**	5'-GAACCTGATGGAGTCCTACC-3'	5'-AACTTTCCGTTTCCTTCATC-3'	NM_200741.1
***D. rerio* CSD**	5'-AGCTGAGATCTCTCCTGGAC-3'	5'-TGGTATTGAGGGTTTCAGTG-3'	NM_001007348
***D. rerio* TauT**	5'-ATCACCTGTTGGGAGAAACT-3'	5'-CAGGTAGTACAAGCCACAGG-3'	NM_001037661.1
***D. rerio* L13A**	5'-TCTGGACTGTAAGAGGTATGC-3'	5'-AGACGCACAATCTTGAGAGCAG-3'	NM_212784.1

The primers were designed to span exon-exon junctions. The suitability of the primers was verified by end-point RT-PCR using RNA from ZFL cells. Primers for ribosomal protein, L13A, used as the reference gene, were same as published primer sequences (226).

4.3.5 Preparation of RNA from ZFL cells, cDNA synthesis and RT-qPCR analysis

RNA was prepared from ZFL cells using the Ambion PureLink total RNA extraction mini kit (Life Technologies Corporation, Carlsbad, CA, USA) following the manufacturer's protocol. The RNA was spectrally analyzed for concentration at 260 nm, and for purity using both 260/280 and 260/230 ratios by Nanodrop ND-1000 spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA) and by automated electrophoresis using the Agilent 2100 BioAnalyzer (Agilent, Santa Clara, CA, USA). Values of >2 for 260/280 and 260/230 ratios were considered to be of sufficient purity. Generation of cDNA amplicons of the predicted size was confirmed by end-point RT-PCR. cDNA was synthesized using Revertaid™ M-MULV reverse transcriptase (Fermentas GmbH, St. Leon-Roth, Germany) and random hexamer primers (Qiagen, Valencia, CA, USA). The cDNA produced was diluted to represent that produced from 10 ng/µl RNA for qPCR analysis and stored at -80 °C. The quality of all cDNA preparations was assessed by end point PCR amplification using the primers described in Table 4.1. Amplification of the cDNA for large ribosomal protein L13A was used as a reference for both endpoint and qPCR analysis. Real time PCR was performed on a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR green fluorescent label. Reactions included Taqman™ Universal master mix (Bio-rad, Hercules, CA, USA), 1:100 SYBR green (100 U stock), 5 µM of each primer. Each 20-µl sample was run in optically clear 96-well plates. Cycling parameters were: 50 °C × 10 min, 95 °C × 2 min, followed by 40 cycles of 95 °C× 15 sec/60 °C × 30 sec. The dissociation step was performed at the end

of the amplification phase to verify a single, specific melting temperature for each primer set, reflecting a single product. Data generated by real-time PCR were compiled using 7500/7600 Sequence Detection Software (Life Technologies, Foster City, CA, USA). Transcript levels of transcripts for TauT, ADO, CDO and CSD were expressed relative to ribosomal protein L13A.

4.3.6 Measurement of taurine levels

Taurine content was determined via LC-MS as described previously by (92). Full amino acid composition of UltraMeM^{TM}-ITS was analyzed using the AccQ Tag method (Waters Corporation, Milford, MA USA) on an Agilent 1200 Infiinity Series HPLC (Santa Clara, CA. USA).

4.4 RESULTS AND DISCUSSION

4.4.1 Adaptation of ZFL cells to serum-free medium

The adaptation of ZFL cells to growth in 100 % UltraMEM^{TM}-ITES is shown in Figure 4.2. The exchange from L15-FBS to 90 % L15-FBS/10 % UtraMEM^{TM}-ITES had no effect on ZFL cell doubling time. With each successive exchange from 20-90 % UltraMEM^{TM}-ITES, cell doubling time increased less than two-fold, with the cells adapting to the new medium formulation over 7-10 days and returning to the initial doubling time of 2.54 ± 0.28 days.

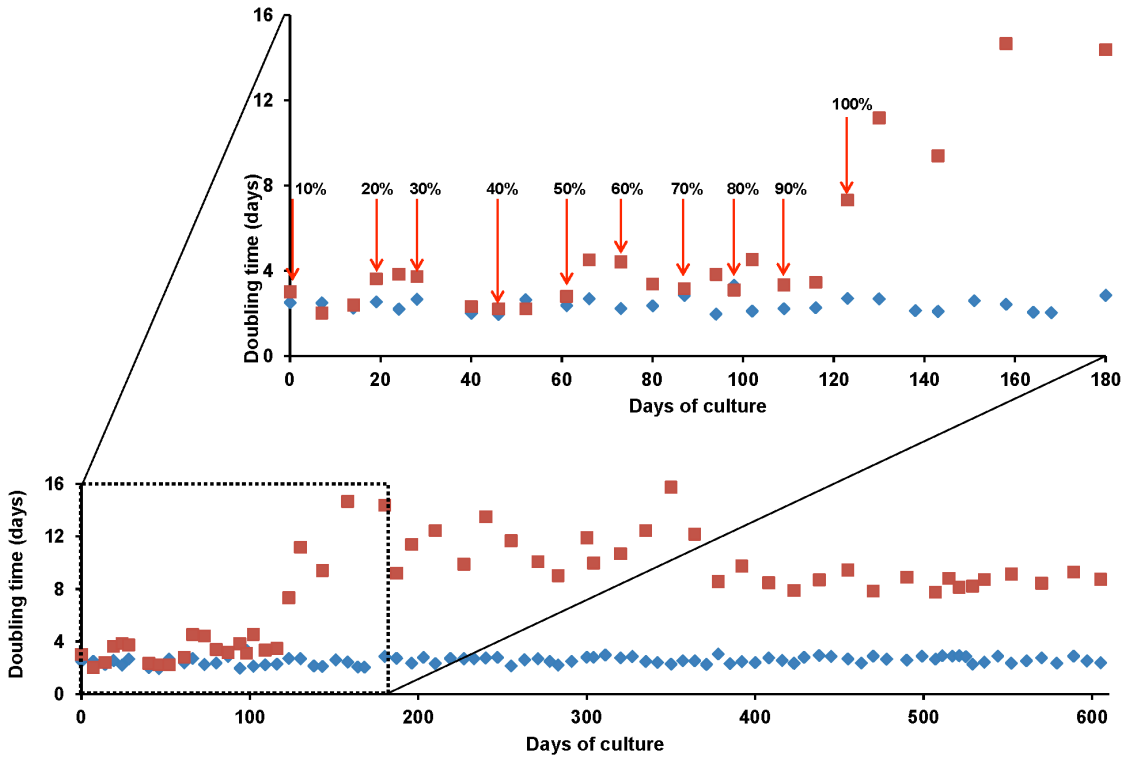

Figure 4.2. Doubling times of ZFL cells during adaptation to growth in UltraMEM™-ITES

ZFL cells were exchanged into decreasing percentages of L15-FBS and increasing percentages of UltraMEM™-ITES. Medium substitutions were made every second passage. Cell counts were taken every 5-8 days. Doubling times were calculated for ZFL growing in L15-FBS alone (blue diamonds) or in L15-FBS supplemented with a range of UltraMEM™-ITES concentrations (red squares) over 600 days.

After 123 days of adaptation, ZFL cells were switched from 90 to 100 % UltraMEM™-ITES. This exchange resulted in an increased doubling time of up to 16 days. However, over the next 250 days, the cells adjusted and adopted a stable doubling time of 8.62 ± 0.56 days, approximately three times that of the initial doubling time in L15-FBS. The ZFL cells adapted to the UltraMEM™-ITES adhered strongly to the culture plate and displayed some reductions in cell size (Figure 4.3). The doubling time was monitored for a further 250 days, over which

time it remained stable. These cells could be recovered after storage in liquid nitrogen.

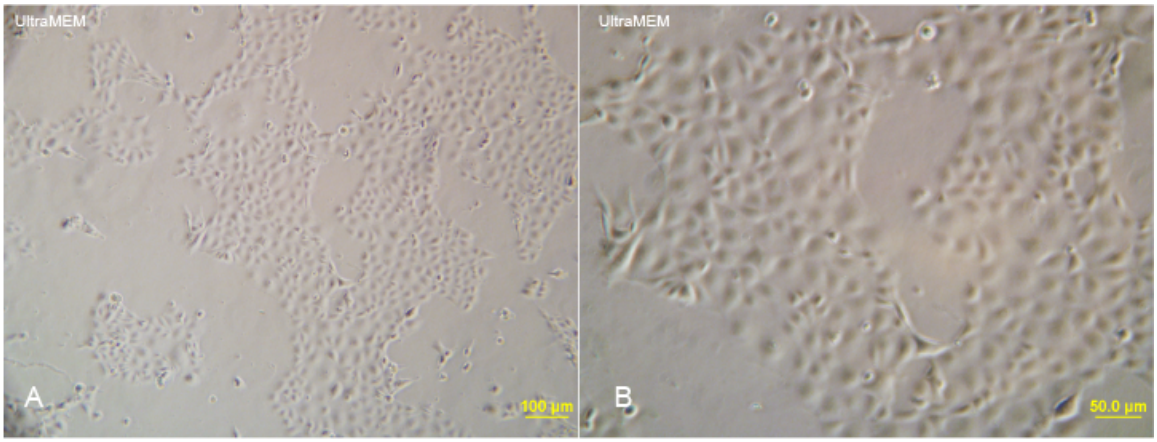

Figure 4.3. Phase-contrast photomicrograph of ZFL cell line monolayers in 100 % UltraMEM™-ITES. ZFL cell line at passage 28 in 100 % UltraMEM™-ITES.

4.4.2 Effect of taurine treatment on expression of taurine biosynthesis and taurine transporter genes

Unlike many carnivorous fish, the omnivorous zebrafish has a complete taurine biosynthetic pathway and is not dependent on taurine in the diet, being able to convert methionine into taurine (227). The usefulness of ZFL cells adapted to serum-free medium was demonstrated by investigating the effect of taurine on expression of the genes involved in taurine biosynthesis and taurine transporter genes in ZFL cells supplemented or not with taurine. ZFL cells grown in L15-FBS could not be used for such an investigation since taurine levels are 11.2 ± 0.8 µM and 20 ± 4.8 µM in L-15 and FBS, respectively, giving an overall 11.99 ± 1.16 µM in L15-FBS (see Table 4.2). In contrast, taurine concentration in UltraMEM™-ITES is undetectable, allowing the effects of taurine to be tested directly.

Table 4.2. Taurine concentrations in L-15, FBS and UltraMEM™-ITES

Source	Taurine concentration, μM
L-15	11.2 ± 0.8
FBS	20 ± 4.8
L-15 + FBS	11.99 ± 1.16
UltraMEM™-ITES	0
Taurine added	160
ZFL cells	145 nmol per cell

Taurine concentration was measured by LC-MS as described (92). The taurine content of ZFL cells was that measured in ZFL cells adapted in L-15-FBS.

Using ZFL cells fully adapted to growth in 100 % UltraMEM™-ITES, cells were treated without or with 160 μM taurine for 24 h. After 24 h, the cells were collected and RNA extracted for RT-qPCR analysis of TauT, ADO, CDO and CSD. The transcript levels were expressed relative to those of ribosomal protein L13A (Figure 4.4). Surprisingly little effect was seen on expression of the biosynthetic pathway genes, ADO, CDO and CSD. However, a 30 % reduction was seen in transcript levels of the taurine transporter, TauT, presumably to decrease the uptake of taurine into cells. The expression of all these genes was approximately ten-fold higher than in ZFL cells growing in L15-FBS (Figure 4.4, inset). Although not directly comparable, these responses are in contrast to what is observed in zebrafish grown for eight weeks on diets with low (0.02 % ± 0.001) or high (4.08 % ± 0.21) levels of taurine (91). Whole body taurine levels, expressed as a percentage of body weight of 1.37 % ± 0.03 were found in fish fed the low taurine diet versus 2.04 % ± 0.28 in fish fed the high taurine diet. In the liver of fish fed the low taurine diet, transcript levels of ADO and CSD were significantly higher than in fish fed the high taurine diet, although no differences were seen in the transcript levels of CDO and TauT (91). This may reflect the observations of Eide *et al* who demonstrated a reduced capacity of ZFL cells to

upregulate transcription compared to primary hepatocytes (152). Alternatively, it could reflect the differences in the period of exposure or the response of cells versus whole organisms.

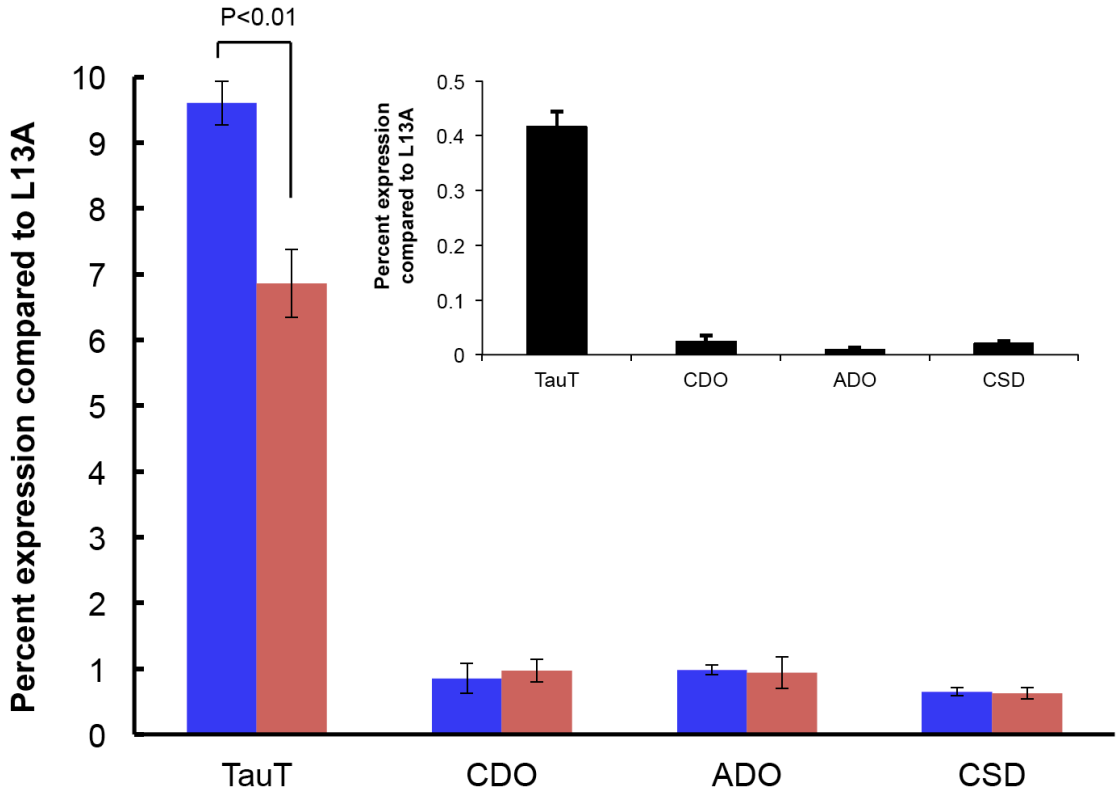

Figure 4.4. Effect of taurine on transcript levels of taurine pathway and taurine transporter genes in ZFL cells growing in UltraMEM™-ITES

Quantitative RT-PCR was performed using cDNA from 10 ng RNA and primers given in Table 1. Expression levels of zebrafish cysteamine dioxygenase (ADO), cysteine dioxygenase (CDO), cysteine sulfinate decarboxylase (CSD), taurine transporter protein (TauT) are expressed relative to 60S ribosomal protein L13A transcript levels. Data are presented as the mean ± S.D. (n = 3 replicates).

4.4.3 Usefulness of ZFL cells adapted to growth in serum-free medium

The adaptation of a cell line to an FBS-free growth medium can be a labor-intensive and time-consuming procedure. Moreover, there is no guarantee that a cell line will survive indefinitely in the FBS-free medium. This successful

adaptation of ZFL cells to growth in UltraMEM^{TM}-ITES has allowed the first investigation of the effects of taurine on expression of taurine biosynthetic pathway and taurine transporter genes in a defined cell type. ZFL cells adapted to growth in serum-free medium will expand the use of this cell line in a range of toxicological, metabolic and innate immune studies. The adaptation strategy described and the synthetic medium used should be applicable to other fish cell lines such as the cobia CM cells (Liu *et al*, m/s in prep) since, unlike zebrafish, cobia have an absolute requirement for taurine in their diets.

Chapter 5: Conclusions and future directions

5.1. Summary/Conclusions

In this chapter, I reaffirm the research objectives and review the contributions and significance of my work in meeting my objective of assessing eIF2α phosphorylation as a rapid indicator of fish health in aquaculture situations. I also discuss several questions arising from my data and approaches that can be used to answer these questions.

There is now an accumulation of reports of the eIF2α kinases in fish. The investigations have focused on the dsRNA activated eIF2α kinase, PKR, rather than nutrition because of its role in defense against virus infections. PKR has been identified in zebrafish (181, 228), three-spined stickleback, *G. aculeatus* (181, 228), fugu, *Takifugu rubripes*, (181, 184, 228), puffer fish (*Tetraodon nigroviridis*) (228), common carp, *Cyprinus carpio* (228), crucian carp, *Carassius auratus* (228, 229), grass carp, *Ctenopharyngodon idellus (230)*, fathead minnow, *Pimephales promelas*, (228), medaka, *Oryzias latipes*, Atlantic salmon, *Salmo salar* (228), and Japanese flounder, *Paralichthys olivaceus* (182). All are interferon-stimulated genes and play roles in interferon–mediated antiviral responses. It has been proposed that PKR may also form part of the adaptive response to prevent further accumulation of energy in the overfed state (45). PKR is normally activated by double-stranded RNA, and interestingly the RNA-binding domain of PKR has been shown to be required for its activation in

response to lipids. Consequently, endogenous RNA species produced during metabolic stress could be required for the activation of this pathway.

PERK has been identified in Chinese rare minnow in which it is involved in viral pathogenesis (187), in medaka, *Oryzias latipes* in which it is involved in ER stress (186), and in zebrafish in which it is involved in the response to tributyl tin and other metal poisoning (189, 231). In chinook salmon CHSE cells, PERK activation arising from ER stress from production of aquatic birnavirus proteins ininfected cells leads to down-regulation of Bcl-2 family proteins and ultimately cell death (188). PERK is the other eIF2α kinase involved in the response to nutritional changes. One of the problems that can arise with unbalanced fish diets is the development of fatty liver disease (FLD) and PERK has been reported to be involved in FLD in zebrafish larvae (232).

HRI has only been reported so far in Japanese flounder where it responds to heat shock (185).

So far, the results reported here in ZFL and cobia cells represent the first report of GCN2 activation in fish. Since GCN2 is the primary responder to nutritional deprivation, this opens up the whole area of fish diet and eIF2α phosphorylation. GCN2 is also involved in a variety of organismal functions in vertebrates such as feeding behavior (53, 233). To insure an adequate supply of nutrients, omnivores choose among available food sources. This process is exemplified by the well-characterized innate aversion of omnivores to otherwise nutritious foods of imbalanced amino acid content. Brain-specific inactivation of GCN2 impairs this aversive response (53). In mice, GCN2 is rapidly activated in

the mediobasal hypothalamus (MBH) after consumption of a leucine-deficient diet (233). Knockdown of GCN2 in this particular region shows that MBH GCN2 activity controls the onset of the aversive response. Investigation of cobia juveniles gave changes in eIF2α phosphorylation consistent with the changes seen in the ZFL and CM cell lines. Figure 5.1 shows the component of the cascade of eIF2α phosphorylation in my work, all four protein kinases have been identified that specifically phosphorylate the α subunit of eIF2 on the residue Ser51. My studies reported here highlight the usefulness of monitoring eIF2α phosphorylation in the establishment of optimum diets and provides a means to investigate reasons underlying poorly accepted diets.

This successful adaptation of ZFL cells to growth in the serum-free medium, UltraMEM^{TM}-ITES has allowed the first (preliminary) investigation of the effects of taurine on expression of the taurine biosynthetic pathway and taurine transporter genes in ZFL cells. Taurine is thought to play a role in reduction of stress and in the improvement of metabolism in a cell or organism, although the mechanisms involved are unknown. Taurine treatment can decrease oxidative stress and tissue damage in rat liver (234) and in Chinese hamster ovary (CHO cells) (235). Taurine protects against ER stress in *Caenorhabditis elegans* (236) and in mouse PC12 cells and a primary neuronal cell culture (237). Taurine may also stimulate cell proliferation by improving mitochondrial functioning (238). The ZFL cells adapted to growth in serum-free medium will expand the use of this cell line in a range of toxicological, metabolic and innate immune studies. The adaptation strategy described and the synthetic medium used should be

applicable to other fish cell lines.

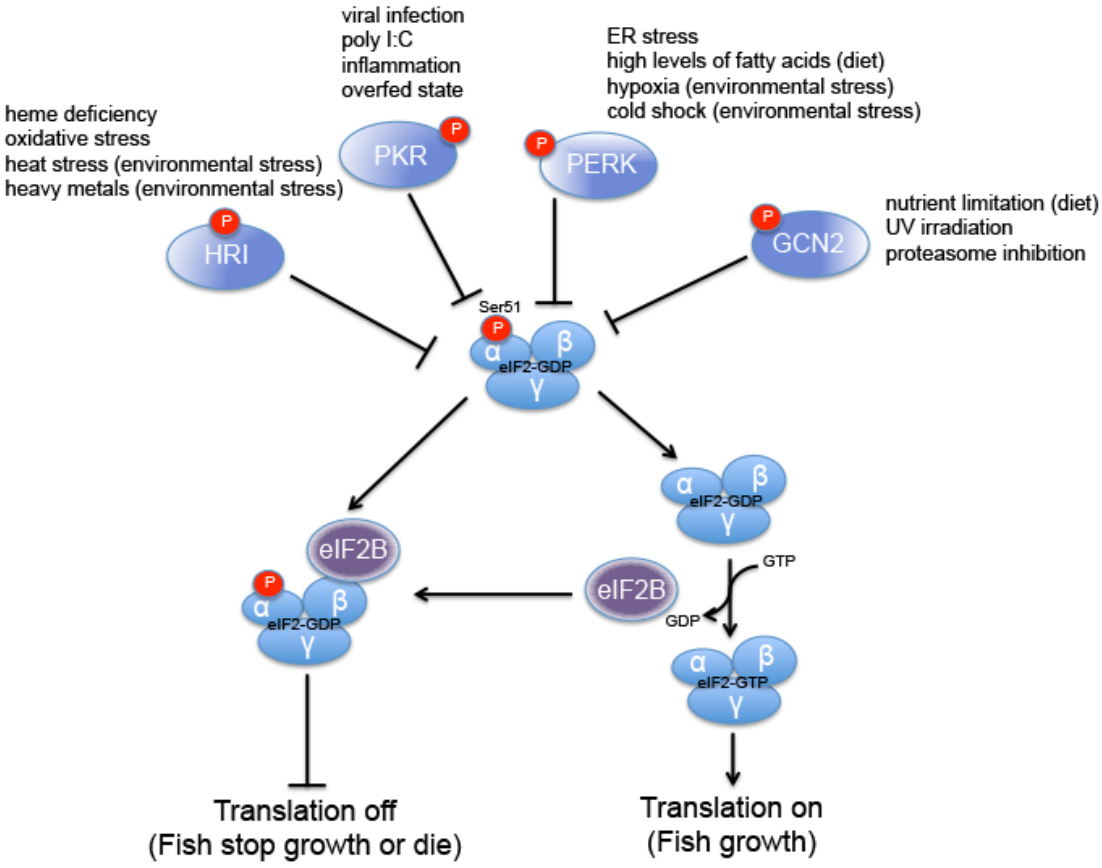

Figure 5.1. Regulation of translation initiation via phosphorylation of Ser51 in eIF2α in fish. Four protein kinases have been identified that specifically phosphorylate the α subunit of eIF2 on the residue Ser51. These kinases are activated under various cellular stress conditions including: plus NMPP (heme deficiency activated HRI), poly I:C (dsRNA-dependent PKR), thapsigargin and cold shock (ER stress activated PERK), and nutrient limitation, leucinol and poor cobia diet (amino acid starvation activated GCN2).

5.2. Future Directions

The research I have summarized raised a number of questions that future research needs to address.

1. Is the determination of the level of eIF2α phosphorylation likely to become a routine assay in aquaculture situations? I have been successful in showing changes in the level of eIF2α phosphorylation in responses to nutritional deficiencies and other stressors in the ZFL and CM cell lines, as well as in cobia maintained under different conditions. Nevertheless, the development of eIF2α phosphorylation as a routine assay for fish health is unlikely because of level of technical skill needed to use the method usefully. However, monitoring eIF2α phosphorylation provides a very useful tool for analysis in dissecting out where dietary deficiencies may lie and what molecular processes underlie nutritional pathologies.

2. Is GCN2 the primary sensor of nutrient deficiency in fish? Despite the importance of GCN2 as the primary sensor of nutritional deficiency, no investigations of the role of GCN2 in any fish species have yet been reported. This should be undertaken for both zebrafish, as a model organism as well as cobia.

In cobia, the sequence of GCN2 (as well as those of PERK, PKR and HRI) should be determined and used to develop antibodies and antibodies to the activated (phosphorylated form) as has been done for the mammalian eIF2α kinases. These could be used for monitoring specific kinase activation in liver and muscle under a range of aquaculture conditions and diets, as well as in CM cells.

Much more can be done in zebrafish. The sequences of the four eIF2α kinases are known and can be used to develop antibodies and antibodies to the activated (phosphorylated form). Notwithstanding that GCN2 is our primary focus, the promoters for each of the eIF2α kinases can be determined from the genome and used in expression constructs behind a reporter gene such as GFP. After microinjection into one-cell stage embryos, these could be used to monitor tissue distribution in cell embryos and whether different stressors lead to their upregulation. PERK has already been looked at in this way in medaka at 1 day post hatching and shown to be ubiquitously expressed (186). The expectation is that GCN2 will be the same. Expression of the different eIF2α kinases can also be determined by whole-mount *in situ* hybridization (WISH) using DIG-labeled riboprobes.

It is of course possible to generate knockout zebrafish for each of the four eIF2α kinases using the new CRISR/Cas technology and determine their responses to nutritional deficiencies and a range of stressors. Again my emphasis would be on GCN2. In fact, development of eIF2α(S51A) knock-in zebrafish, which affect the outcome of activation of all the eIF2α kinases, may be the place to start. However, it may be most useful to begin my investigations using the transgenic zebrafish developed by the Tsai laboratory harboring a construct in which the uORF sequence of human CCAAT/enhancer-binding protein homologous protein gene (hu*chop*) (239). This uORF is a mRNA element in the 5'-untranslated region (5'-UTR), that increases mRNA translational efficiency under conditions of increased eIF2α phosphorylation (see Chapter 1.2)

which means that GFP is only seen in the embryo in response to stressor that increase eIF2α phosphorylation. The Tsai laboratory has used this transgenic line to show that thapsigargin, heatshock at 40 ^{o}C and 1.5 % alcohol all increase the protein level of CHOP and that in response to these stressors (which probably activated both PERK and HRI). Tissues such as brain and spinal cord were particularly sensitive. In embryos 72 hpf, PERK is sensitive to ER-associated stresses, both at transcriptional and translational levels in zebrafish embryos with neural tissue responding with particular sensitivity (240). In addition, they have shown that this transgenic line is an effective bioindicator for detecting environmental toxicants such as Cu^{2+}, Cd^{2+} and chlorpyrifos (chromium) (241). Such a transgenic line would be of great value in investigating the effects of nutritional defects.

3. Will the ZFL cells adapted to serum-free medium be useful for future studies? The ZFL cells adapted to serum-free medium need more characterization. Although the growth characteristics of the cells are stable, the doubling time is three times longer than in the normal medium. The easiest next step, will be to add the taurine back to the serum free medium to see if that restores the growth. Moreover, because of the ability of taurine to function as a powerful anti-oxidant and anti-stress agent, we expected taurine to affect eIF2α phosphorylation. My preliminary analysis of this (data not shown in the thesis) is that there is no obvious effect at 24 h after addition of taurine back to the culture medium. However, the effects of taurine on eIF2α phosphorylation may be short-lived. Like the effects of thapsigargin, the effect of taurine could be acute and my

preliminary examination was only after 24 h. In the future, I would like to look at the effects of taurine on the ZFL cells growing in the serum-free medium over a time course and also to look at a wider range of concentrations. It should be remembered that there are three branches to the unfolded protein response. Three families of signal transducers, ATF6, PERK, and IRE1 all sense the protein-folding conditions in the ER lumen and transmit that information, resulting in production of bZIP transcription regulators that enter the nucleus to drive transcription of UPR target genes (reviewed, 70). Each pathway uses a different mechanism of signal transduction: ATF6 by regulated proteolysis, PERK by translational control, and IRE1 by nonconventional mRNA splicing. In addition to the transcriptional responses that largely serve to increase the protein-folding capacity in the ER, both PERK and IRE1 reduce the ER folding load by down-tuning translation and degrading ER-bound mRNAs, respectively. This means I should look at some of the alternate markers for ER stress, protein levels of ATF4, BiP/Grp78 and CHOP, as well as the UPR-induced splicing of XBP1.

Related questions on the ZFL cells adapted to growth in serum-free medium will be to determine whether their use can be expanded for a range of toxicological, metabolic and innate immune studies. A further question would be whether adaption to serum-free medium is applicable to other fish cell lines such as the cobia CM cells since, unlike zebrafish, cobia have an absolute requirement for taurine in their diets.

Overall, my work has demonstrated that the monitoring of eIF2α phosphorylation will be a useful investigational assay in evaluating nutritional

deficiencies and other stressors in fish and that it is likely to be a useful analytical tool in determining the basis of nutrient pathologies. The work underscores the lamentable lack of information on the eIF2α kinase, GCN2, the kinase most likely to be the main responder to nutritional deficiencies. The work has also underscored the value of established cell lines for the investigation of marine fish of high aquaculture value, as well as the benefits of optimizing the growth of cell lines in ways that enable them to be more useful tools.

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