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# Investigating a Role for the CCAAT/Enhancer-Binding Protein $\delta$ in the Developing Zebrafish

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Investigating a Role for the CCAAT/Enhancer-Binding Protein  $\delta$  in the Developing  
Zebrafish

by

Alisha Jennifer Beirl

A thesis submitted in partial fulfillment of the  
requirements for the degree of

Master of Science  
in  
Biology

Thesis Committee:  
Bradley A. Buckley, Chair  
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Portland State University  
2014

## Abstract

The CCAAT/enhancer-binding protein delta (C/EBP $\delta$ ) is a highly conserved transcription factor capable of regulating numerous cell fate processes, such as cell growth, differentiation, proliferation and apoptosis. C/EBP $\delta$  is inducible during cellular stress responses, including inflammation and responses to growth factor deprivation or thermal stress. C/EBP $\delta$  is stress-inducible in a diversity of fishes, including the zebrafish *Danio rerio*; however, little is known about its role in fish development. Here I show that overexpression of C/EBP $\delta$  leads to severe developmental defects, including reduced body length, edema, liver malformation and retinal abnormalities. The proportion of individuals that display developmental abnormalities is significantly greater in C/EBP $\delta$ -overexpressing embryos compared to control embryos and overexpression significantly reduces survival of larvae over time. TUNEL analysis suggests C/EBP $\delta$ -overexpressing embryos exhibit a pattern of apoptotic cell death which is spatially distinct from control embryos. These data support a critical role for C/EBP $\delta$  in numerous developmental processes, including promoting programmed cell death during development. Mutations in C/EBP $\delta$  have been implicated in the progression of human tumors, including those of myeloid, hepatocellular and breast cancers. Therefore, the C/EBP $\delta$ -overexpressing zebrafish will serve as a valuable model for examining the role of this gene during development, as a part of the cellular response to stress and in pathological states such as tumor progression.

## Acknowledgments

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## Chapter I: Overexpression of C/EBP $\delta$ in the developing zebrafish

### Introduction

The CCAAT/Enhancer Binding Proteins (C/EBPs) are a highly conserved family of transcription factors, capable of regulating critical cell fate processes, such as cell growth, proliferation, differentiation, cell cycle arrest and apoptosis (Tsukada et al., 2011).

C/EBPs are composed of a highly conserved basic leucine zipper (bZIP) domain, a DNA-binding domain, a regulatory domain and a trans-activation domain (Wedel and Ziegler-Heitbrock, 1995). The C-terminus consists of the basic DNA-binding and leucine zipper regions, and its sequence is over 90% conserved among each isoform. The N-terminus is more variable, having less than 20% sequence homology among each isoform. The N-terminus contains the transactivation domain, which is the region responsible for transcriptional activation or repression of target genes. The basic region is the site of DNA-binding and the leucine zipper domain enables C/EBPs to dimerize, which is necessary for transactivational competence. The regulatory domain is the site at which phosphorylation occurs, in order to facilitate nuclear localization. The transactivation domain is responsible for transcriptional activation or repression. Upon induction, C/EBPs become localized within the nucleus where they bind a ATTGCGCAAT consensus sequence located in the promoter region of their target genes to activate or repress their expression (Lekstrom-Hines and Xanthopoulos, 1998).



Six isoforms ( $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\gamma$ ,  $\zeta$ , and  $\epsilon$ ) of the C/EBP family have been identified in mammals, and each are highly conserved with one another, sharing greater than 90% sequence identity in their DNA-binding domains (Ramji and Foka, 2002). The function of each isoform varies depending on cell and tissue type. In addition to the six isoforms which have been extensively characterized in mammalian models, the zebrafish, *Danio rerio*, expresses a novel isoform, C/EBP1, which has high sequence similarity in the DNA-binding domain compared to other C/EBP members, but lacks sequence homology at the N-terminus to any other known isoform. Expression of C/EBP1 is restricted to myeloid cells in the developing zebrafish (Lyons et al., 2001a).

Thousands of C/EBP target genes with highly diverse cellular functions have been identified to date (Halmos et al., 2004; Zhang et al., 2008; Huber et al., 2012). The sequences which make up the DNA-binding domains of C/EBPs are highly homologous among each isoform. Because these sequences are so similar, it is not possible to predict which target genes each C/EBP isoform will bind and activate based solely on sequence. A study by Zhang et al., (2008) identified hundreds of C/EBP $\delta$  target genes using chromatin immunoprecipitation assays to specifically determine which genomic regions C/EBP $\delta$  can bind. A large proportion of genes that are regulated by C/EBP $\delta$ , function in cell differentiation, proliferation, adhesion, migration, cell growth, cell cycle regulation and apoptosis (Zhang et al., 2008). Upregulation of C/EBP $\delta$ , along with many of these target genes, occurs during the cellular response to stress (Zhou and Dewille, 2007). C/EBP $\delta$  will be the isoform of focus for the remainder of this chapter.

The cellular stress response is the set of molecular mechanisms that cells may carry out in response to a broad range of environmental stressors, such as heat, osmotic imbalance and chemical stimuli (Gasch and Werner-Washburne, 2002; Kultz, 2005). There are numerous molecular programs in place that determine a cell's capacity to deal with stress. Generally, a cell's initial response to environmental insults is to activate survival pathways which will enable them to defend and overcome any macromolecular damage caused by the stress (Fulda et al., 2010). However, in cases of more severe stress, the cell is unable to overcome macromolecular damage and alternatively activates cell death pathways, resulting in elimination of the damaged cell (Fulda et al., 2010).

One cellular stress response mechanism is to alter the expression of genes that are involved in cell fate and the regulation of the cell cycle (Morano and Thiele, 1999). These variations in gene expression in response to environmental insults are conserved among highly diverse groups of organisms (Dutertre et al., 2011). In addition, many of the genes that regulate cell fate processes may also play critical roles during vertebrate embryonic development (Kelsh and Raible, 2002). C/EBP $\delta$  can activate or repress the expression of numerous target genes that are involved in making cell fate decisions.

C/EBP $\delta$  is inducible during cellular stress responses, including inflammation and responses to growth factor deprivation or thermal stress (Yin et al., 2006; Billiard et al., 2001; Buckley et al., 2006). C/EBP $\delta$  is inducible by thermal stress in a broad range of fish species adapted to highly diverse thermal environments. The heat-inducibility of C/EBP $\delta$  has previously been demonstrated in the eurythermal goby, *Gillichthys mirabilis*

(Buckley, 2011), the Pacific bluefin tuna, *Thunnus orientalis* (Buckley, 2009), the cold-adapted Antarctic Notothenoid, *Trematomus bernachii* (Sleadd and Buckley, 2012), and the zebrafish *Danio rerio* (B.A. Buckley pers. comm.). When these fishes are exposed to thermal stress, C/EBP $\delta$  is upregulated in a tissue-specific manner. It is possible that C/EBP $\delta$  upregulation leads to cell cycle arrest and/or apoptosis in response to thermal stress. Although the activation of C/EBP $\delta$  in response to thermal stress is highly conserved among a broad diversity of fish species, little is known about its role in fish development.

The cell cycle consists of a series of phases which result in the growth and proliferation of a given cell. To begin, a cell grows as its chromosomes become prepared for DNA replication during the G<sub>1</sub> phase. This is followed by the S phase, in which a cell synthesizes DNA in order to double its genomic DNA content. As DNA synthesis continues, the G<sub>2</sub> phase occurs, in which the cell continues to grow while ensuring the appropriate mechanisms are in place for mitosis to take place. Finally, once division is completed, the cell enters the G<sub>0</sub> post-mitotic phase, in which the cell has left the cycle and is no longer dividing. This process of cell proliferation is energetically costly, and a cell may exit the cycle at times when energy conservation is essential, such as during the cellular stress response. At given checkpoints, a cell may exit the cycle and undergo cell cycle arrest, a temporary and reversible pause in growth or proliferation. An alternative and irreversible option, which often occurs in more extreme stress states, is to exit the cell cycle permanently and undergo programmed cell death.

Programmed cell death, or apoptosis, is characterized by DNA fragmentation, blebbing of the plasma membrane, mitochondrial swelling, nuclear chromatin condensation and lysis of cellular contents, resulting in the death of the cell (Isaacs, 1993). Apoptosis occurs at various stages of morphogenesis and is essential for the normal development of most organisms (Abud, 2004). For example, in the zebrafish, both the spatial and temporal patterns of apoptosis in the tail region must be highly precise in order for normal development of the embryo to occur (Rojo and Gonzales, 2008). However, apoptosis can also occur in response to numerous environmental stressors and in some pathological disease states. In such cases, increased apoptosis can become detrimental to the organism (Samali and Orrenius, 1998; Arends and Wyllie, 1991).

Numerous experimental techniques have been developed to assay for apoptosis. One method, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is particularly useful for detecting fragmented DNA *in situ*. In this assay, the enzyme TdT catalyzes the addition of a dUTP onto the exposed 3' hydroxyl terminal end of fragmented DNA. The dUTPs are detected by secondarily labeling them with a dye or a fluorescent antibody. This enables visualization of cells containing fragmented DNA, which is characteristic of apoptosis. Many studies have demonstrated the advantages of the TUNEL assay in efficiently identifying apoptotic cells in culture, as well as in whole mount vertebrate embryos (Loo, 2011).

Gene knockdown has long been recognized as a valuable and widely used method for investigating the role of genes involved in nearly any biological process (Bedell et al.,

2011). However, gene overexpression has additionally been recognized as an alternative but powerful tool for examining gene expression and protein function during development. Researchers can infer the role of nearly any gene of interest based on the phenotype that arises from inducing overexpression of that gene in a wildtype organism (Prelich, 2012). Gene overexpression studies, once limited to yeast and other lower eukaryotes, have become increasingly popular in vertebrate animals due to the recent advancements in technologies that are used to induce overexpression (Langenau et al., 2005). Gene overexpression applications have facilitated the discovery of the role of many genes that have been implicated in numerous human diseases (Prelich, 2012).

The zebrafish, *Danio rerio*, is an excellent model for developmental studies. Its rapid development, external fertilization, and transparent embryos make it an ideal model for examining gene expression in the developing embryo (Westerfield, 2000). The zebrafish genome was recently mapped and as a result, many species-specific antibodies and gene overexpression technologies are commercially available (Dooley and Zon, 2000).

Moreover, sequencing of the zebrafish genome has revealed sequence homology to thousands of human genes that have been implicated in disease (Howe et al., 2013).

A number of experimental techniques have been developed that enable researchers to characterize the genetics of zebrafish development (Sun et al., 2011; Cade et al., 2012). Gene expression can be experimentally altered using microinjection techniques (Xu et al., 2008). Microinjection of genetic material, such as synthetic cDNA, mRNA, or oligonucleotides, which can alter gene expression, is accomplished relatively easily at the

one cell stage of zebrafish development (Hogan et al., 2008). Because of these recent advancements in genetic manipulation techniques, novel roles for many genes have been uncovered and characterized to date in the zebrafish (Sun et al., 2011).

Lyons et al. (2001b) showed that *C/EBP $\delta$*  mRNA localization is tissue-specific in the developing zebrafish from 1 to 3.5d post fertilization (dpf). *C/EBP $\delta$*  mRNA expression in the zebrafish is detected at low levels in the yolk sac beginning at 24hr post fertilization. By 2dpf, expression is increased in the yolk syncytial nuclei and cloaca. *C/EBP $\delta$*  becomes highly expressed at 3 to 3.5dpf, when mRNA expression is observed in the jaw, fin, brain and cloaca. These early developmental expression patterns suggest that *C/EBP $\delta$*  may be required for numerous developmental processes. However, functional studies involving zebrafish *C/EBP $\delta$*  are necessary to determine the specific requirements for this transcription factor during development.

I used the developing zebrafish as a model to investigate a developmental role for *C/EBP $\delta$* . Using microinjection techniques, I induced ectopic overexpression of *C/EBP $\delta$*  at the one cell stage. Here, I demonstrate a critical role for *C/EBP $\delta$*  in the developmental patterning of the zebrafish and in promoting apoptotic cell death.

## Methods

### *Maintenance of research animals*

Adult research animals were housed in an aquatic facility with recirculating water flow, at a temperature range of 28 to 30°C. A 14hr light/10hr dark cycle was maintained to promote spawning. For all experiments, the wildtype AB strain was used. AB adult zebrafish were obtained from Dr. Cynthia Cooper at Washington State University (Vancouver, WA) or the Zebrafish International Resource Center ((ZDB-GENO #960809-7) Eugene, OR). Embryos were collected from either pair-wise or group spawning. Staging of embryos and larvae was completed by observation of previously established morphological characteristics, according to Kimmel et al. (1995). Embryonic and larval zebrafish were raised in embryo media (15mM NaCl, 0.5mM KCl, 1mM MgSO<sub>4</sub>, 0.15mM KH<sub>2</sub>PO<sub>4</sub>, 0.05mM Na<sub>2</sub>HPO<sub>4</sub>, 1.0mM CaCl<sub>2</sub>, 0.7mM NaHCO<sub>3</sub> in Millipore purified water at pH 7.2 ) from 0 to 6d post fertilization. All experimental protocols involving the use of live animals were approved by the Institutional Animal Care and Use Committee at Portland State University.

### *Generation of the zebrafish overexpression construct and microinjections*

For overexpression studies in *Danio rerio*, full length zebrafish C/EBP $\delta$  cDNA was synthesized and cloned into the pCMV-AC-GFP expression vector (Blue Heron Biotech Bothel, WA). Plasmid DNA was purified using a Nucleospin plasmid DNA purification kit (Clontech Mountain View, CA). DNA was quantified using a Thermo scientific Nano Drop 2000 spectrophotometer. Plasmid DNA for microinjections was diluted to 5ng/ $\mu$ L

in 100mM KCl, and 0.25% phenol red was added as a pH indicator and to visualize the solution within the cytoplasm. Injection needles were made from thin-walled capillary glass (World Precision Instruments Sarasota, FL) using a P-80/PC micropipette puller (Sutter Instruments Novato, CA). DNA was loaded into the injections needles using gel loading pipet tips. Each injection needle was calibrated using a Leica 50mm stage micrometer and according to a method previously established by Mullins (2008). Microinjections were completed using an MPPI-3 pressure microinjector (Applied Scientific Instrumentation Eugene, OR). 5 or 10 pg of total plasmid DNA was injected into the cytoplasm of each embryo. Embryos were grown to a range of stages between 24 and 30 hours post fertilization and screened for the appearance of green fluorescent protein, using a Leica DM IRB inverted fluorescence microscope.

#### *Analysis of survival and morphological defects*

Embryos were injected with either *dct*-GFP or C/EBP $\delta$ -GFP encoding plasmid DNA. *dct* is an enzyme that functions in the synthesis of melanin pigmentation and its expression is restricted to the melanocytes (Jackson et al., 1992). Alterations in *dct* expression should have no effect on cell cycle regulation or the promotion of apoptosis; therefore, it was used as a control. *dct*-GFP and C/EBP $\delta$ -GFP injected embryos and larvae, along with uninjected controls, were examined for mortality or developmental deficiencies from 0 to 6 days post fertilization (dpf). Percent survival was quantified beginning at 1 dpf, as a large proportion of both *dct*-GFP and C/EBP $\delta$ -GFP injected embryos died prior to this time point. From 1 to 3 dpf, survival was determined based on the presence or absence of



necrotic tissues. From 3 to 6 dpf, survival was determined by the presence or absence of cardiac activity. Embryos were scored for developmental abnormalities at 24 hours post fertilization. As the abnormalities characteristic of C/EBP- injected embryos cover a broad range of phenotypes, embryos were scored based on the presence or absence of morphological defects such as edema, reduced body length, curved tail and reduced retina. Figure 1C shows an example of a *dct*-injected larvae that was scored as positive for developmental deficiencies. Figures 1D and 1E show C/EBP $\delta$ -injected larvae that were scored positive for developmental deficiencies.

#### *Statistical analyses*

All statistical analyses were completed using GraphPad Prism 5. Statistics for survival data were done using 2-way ANOVA with replication. Statistics for developmental deficiencies were completed using a student's t test. Analyses of cell quantification data were completed using a student's t test.

#### *TUNEL staining and analysis of apoptosis*

To visualize apoptotic cells, chorions were removed and whole embryos or larvae were anesthetized in tricaine (MS-222), placed in 1.5mL microcentrifuge tubes (up to 30 embryos per tube), and fixed in 4% Paraformaldehyde (PFA) in Tris Buffered Saline (TBS) overnight at 4°C. Samples were rinsed 3x5 min in TBS + 0.1% Tween-20 (TBST) and then dehydrated in a graded series of methanol/TBST washes. Samples were stored in 100% methanol and rehydrated in a series of TBST/methanol washes. For embryos older than 24 hours post fertilization (hpf), pigment was bleached by incubating each

sample in a 0.5% KOH/3% H<sub>2</sub>O<sub>2</sub> solution for 10 min at room temp, then each sample was rinsed 2x10 min in TBS. All samples were treated with 10µg/mL proteinase K for approximately 10 min to permeate tissues for the TUNEL assay (treatment time was increased slightly for older larvae). The TUNEL assay was completed using an Apoptag Peroxidase *In situ* cell death detection kit (Millipore Billerica, MA). TUNEL staining was completed according to manufacturer's directions, with a few modifications. Samples were incubated in equilibration buffer for 10 min. For each sample, 5µL of TdT enzyme was mixed with 45µL of reaction buffer. Samples were pre-incubated in TdT enzyme solution for 1-h on ice, followed by 1-h at 37°C. Each sample was then incubated in 50µL stop solution for 1-h at room temp. Samples were blocked in 2% Western blocking reagent in TBS for 1 to 2-h at room temp, or overnight at 4°C. Samples were incubated in Anti-Digoxigenin Alkaline Phosphatase-conjugated antibody (Roche Indianapolis, IN) at 1:1000 in block solution overnight at 4°C. Samples were rinsed 3x30 min in TBST. Staining was completed using Nitro Blue Tetrazolium/Bromo-chloro-indolyl phosphate (NBT/BCIP) as an alternative to peroxidase. Staining time ranged from 1-h at room temp to overnight at 4°C, and varied among embryonic and larval stages. Imaging and cell quantification analyses were completed under bright light using a Zeiss Stemi 2000-CS stereo microscope.

## Results

### *Overexpression of C/EBP $\delta$ in developing zebrafish*

I examined wildtype control, *dct*-GFP+, and C/EBP $\delta$ -GFP+ embryos for phenotypic changes in developmental patterning, beginning at 24-h post fertilization (hpf). At 24 hpf, C/EBP $\delta$ -GFP+ embryos display severe morphological abnormalities, and many demonstrate delayed development compared to their control counterparts. At 3d post fertilization (dpf) uninjected control larvae have developed normally (Fig. 1A). Most *dct*-GFP+ larvae develop normally, similar to uninjected control larvae (Fig. 1B); however, some show slight abnormalities in development, such as reduced retina (Fig. 1C). C/EBP $\delta$ -GFP+ larvae continue to exhibit severe defects in developmental patterning, such as edema, liver malformation, curved or reduced bodies, retinal abnormalities, jaw defects and swim bladder deficiencies (Fig. 1D and 1E). Expression of green fluorescent protein is distributed randomly throughout C/EBP $\delta$ -GFP+ embryos at 24 hpf (Fig. 1F).

### *Analysis of morphology in C/EBP $\delta$ -overexpressing embryos*

As many C/EBP $\delta$ -overexpressing embryos and larvae exhibit developmental abnormalities, I sought to quantify the percentage of embryos in each clutch that display such defects, in uninjected control, *dct*-injected, and C/EBP $\delta$ -injected fish (Fig. 2A). None of the uninjected control fish displayed developmental abnormalities. A small proportion of *dct*-injected embryos exhibited minor problems with developmental timing or patterning, when compared to uninjected controls, such as reduced body length or

curved tails. It is probable that the abnormalities observed in *dct*-injected embryos are a result of the microinjections, and are not likely attributable to overexpression of this gene.

A significant percentage of C/EBP $\delta$ -injected embryos displayed severe developmental deficiencies compared to either group of control embryos (\* $p < .01$ ).

The C/EBP $\delta$  overexpression phenotype is variable; however, embryos and larvae display a consistent range of phenotypes that are common among most C/EBP $\delta$ -GFP+ fish.

Therefore, it was necessary to determine the number of larvae displaying each phenotype at 3dpf (Fig. 2B). Examples of larvae that displayed each phenotype (or combination of phenotypes) are shown in Figures 2C, 2D, 2E and 2F. A C/EBP $\delta$ -GFP+ embryo with a reduced body phenotype is shown in Fig. 2C. A C/EBP $\delta$ -GFP+ embryo with curved tail and edema is shown in Fig. 2D. A C/EBP $\delta$ -GFP+ embryo with reduced body and edema is shown in Fig. 2E. A C/EBP $\delta$ -GFP+ embryo with reduced body, edema and eye deficiencies is shown in Fig. 2F.

#### *Survival of larvae over time*

I was interested in determining the extent to which the observed developmental abnormalities result in mortality of C/EBP $\delta$ -overexpressing fish over time. Embryos were injected with either 5 or 10pg of plasmid DNA. Injection of 10pg of C/EBP $\delta$ -GFP plasmid DNA was lethal to nearly 100 percent of embryos prior to 1dpf. Therefore, determination of survival was done on embryos injected with 5pg of plasmid DNA. Between zero and 1d post fertilization (dpf) approximately 50% of embryos die after

injection with either *dct*-GFP or C/EBP $\delta$ -GFP plasmid DNA; therefore, I began the percent survival quantifications at 1dpf. Beginning at 1dpf, embryos of uninjected controls, *dct*-injected and C/EBP $\delta$ -injected clutches are all at 100% survival, despite the large proportion of developmental deficiencies observed in C/EBP $\delta$ -overexpressing embryos at this time (Fig. 3A). Between 3 and 4 dpf, the survival of C/EBP $\delta$ -overexpressing larvae begins to decline substantially. From 5 to 6 dpf survival of C/EBP $\delta$ -overexpressing and *dct*-overexpressing larvae is significantly reduced (\* $p < .01$  as determined by 2-way ANOVA). Because cardiac activity is not observable in larvae until 3dpf, embryos were scored for survival based on the presence of necrotic tissues. Figure 3B shows a necrotic embryo which was scored as dead at 2dpf. Figure 3C shows an embryo that was scored as alive at 3dpf, based on the presence of cardiac activity.

*Analysis of apoptosis in C/EBP $\delta$ -overexpressing embryos.*

As C/EBP $\delta$  induction is associated with cell cycle arrest and programmed cell death, I aimed to analyze the degree to which apoptotic cell death is occurring in embryos that overexpress C/EBP $\delta$ . At 26-h post fertilization (hpf) uninjected control embryos display some apoptotic cell death, as expected (Fig. 4A). *dct*-injected control embryos exhibited levels and localization of apoptotic cell death that are consistent with uninjected wildtype controls (Fig. 4B). Apoptosis in C/EBP $\delta$ -overexpressing embryos appears to increase substantially, primarily in the head region, compared to control embryos (Fig. 4C). Areas of increased TUNEL staining are indicated by arrowheads in Fig. 4C. I quantified the number of apoptotic cells in embryos injected with C/EBP $\delta$  compared to *dct*-injected and

uninjected controls. At 24hpf, there was a slight but non-significant increase in apoptotic cell death in *C/EBP $\delta$* -overexpressing embryos compared to control embryos (Fig. 5) ( $p=.06$ ). Although quantification of apoptotic cells does not reveal a difference in cell number, the spatial pattern of cells undergoing apoptosis is distinct in *C/EBP $\delta$* -overexpressing embryos compared to control embryos.

Figure 1 – The *C/EBPδ* overexpression phenotype

A) Wildtype uninjected control larvae display normal development at 3dpf. B) *dct*-GFP+ larvae develop similar to wildtype controls. C) A small proportion of *dct*-GFP+ larvae display some developmental problems. D, E) *C/EBPδ*-GFP+ larvae show severe developmental patterning issues at 3dpf. F) GFP localization is ubiquitous throughout *C/EBPδ*-overexpressing embryos at 24hpf.

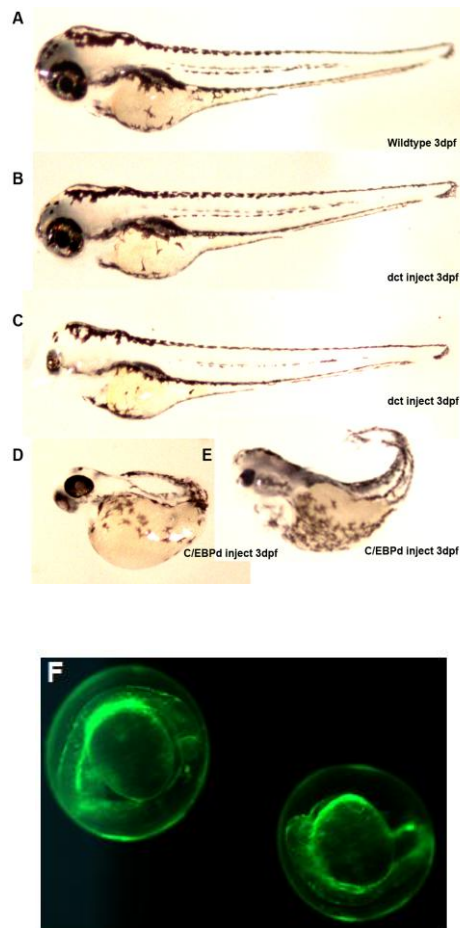
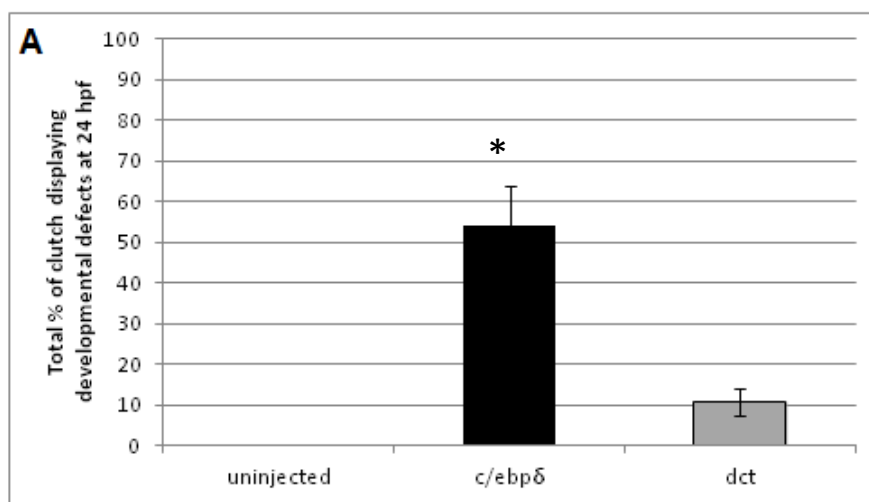


Figure 2 – Summary of the developmental deficiencies associated with C/EBP $\delta$  overexpression.

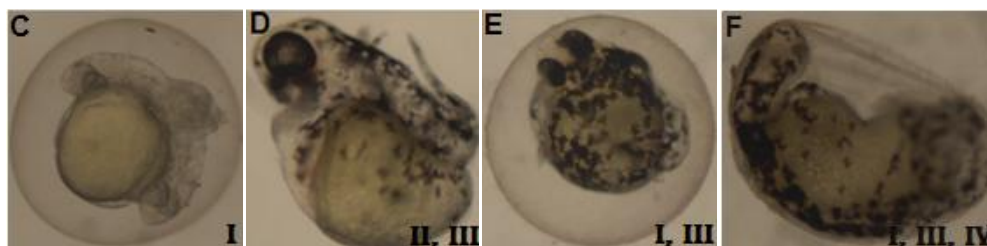
A) The percentage of embryos per clutch that display phenotypic delay or deficiencies in developmental patterning at 24hpf is significantly increased in C/EBP $\delta$ -GFP+ fish (\* $p < .01$ ). B) The range of developmental phenotypes which are observed for each genotype at 3dpf . C) A C/EBP $\delta$ -GFP+ embryo with the reduced body phenotype. D) A C/EBP $\delta$ -GFP+ embryo with curved tail and edema. E) A C/EBP $\delta$ -GFP+ embryo with reduced body and edema. F) A C/EBP $\delta$ -GFP+ embryo with reduced body, edema and eye deficiencies.





**B**

Phenotype	# of uninjected Control Individuals (n=72)	# of <i>C/EBPδ</i> -GFP+ Individuals (n=33)	# of <i>dct</i> -GFP+ Individuals (n=20)
# surviving / # injected	72 / 0	33 / 56	20 / 38
Reduced body	0	20	0
Curved tail	2	5	2
Edema	0	18	0
Eye deficiencies	0	10	2



- I) Reduced body
- II) Curved tail
- III) Edema
- IV) Eye deficiencies

Figure 3 – Analysis of embryonic and larval survival over time.

The percentage of each clutch surviving was quantified over time. From 1 to 2dpf all wildtype control, *dct*-injected and C/EBP $\delta$ -injected embryos have survived. At 2 and 3dpf, some *dct* and C/EBP $\delta$ -injected larvae have begun to die. Between 4 and 6dpf the percentage of *dct*-injected and C/EBP $\delta$ -injected larvae that can survive was significantly decreased in comparison to wildtype control larvae (\* $p < .01$ ). B) A necrotic embryo that was scored as dead at 1dpf (necrotic tissue denoted by arrow). C) Active heart of a larvae that was scored as alive at 3dpf (arrow indicates location of the larval heart).

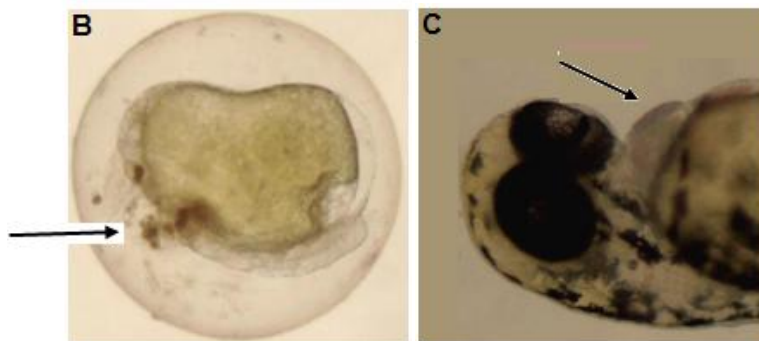
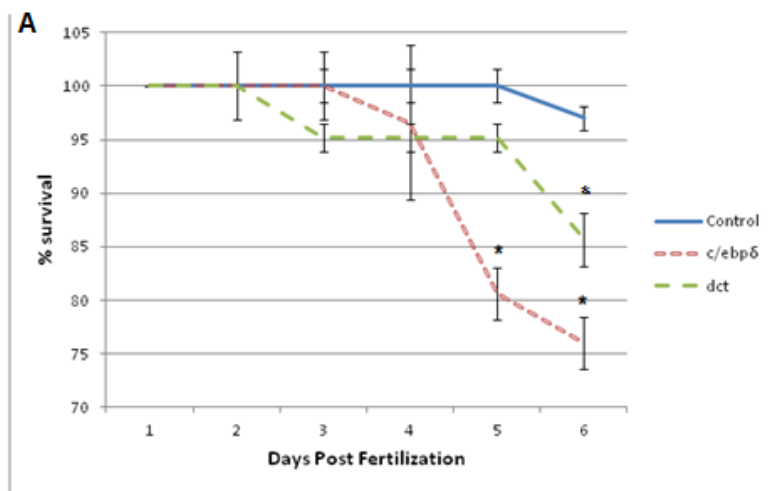


Figure 4 – Analysis of apoptosis during development.

A) At 26hpf, uninjected wildtype control embryos display apoptotic cells, primarily in the tail and head region, as anticipated. B) *dct*-GFP<sup>+</sup> embryos show similar localization of apoptotic cells at 26hpf. C) *C/EBPδ*-GFP<sup>+</sup> embryos display spatial patterns of apoptotic cells which are distinct from either of the control fish (increased TUNEL staining is denoted by arrow).

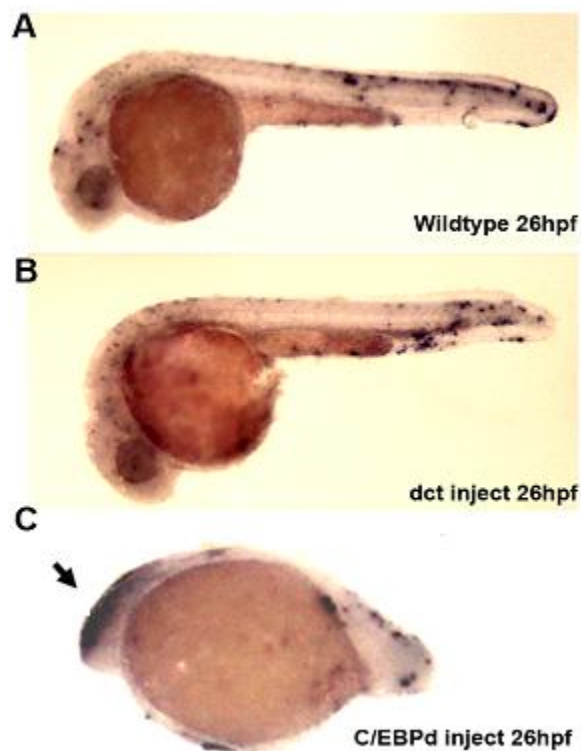
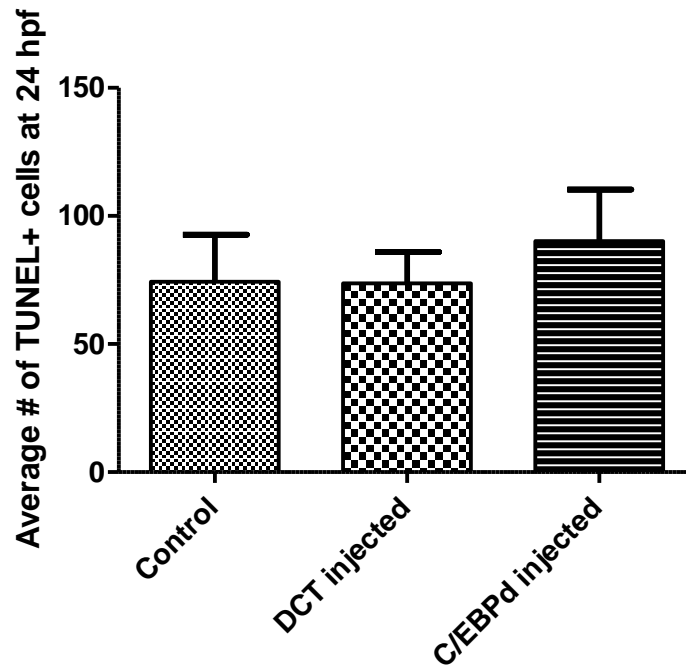


Figure 5 – Quantification of apoptotic cells

Quantification of TUNEL positive cells at 24hpf reveals a similar number of apoptotic cells among uninjected, *dct*-GFP and C/EBP $\delta$ -GFP embryos. There is a slight but non-significant increase in the number of TUNEL-positive cells in C/EBP $\delta$ -GFP embryos (\*p=0.06).



## Discussion

The function of C/EBP $\delta$  in controlling cell fate in cultured mammalian cells has been well characterized for many years (Lekstrom-Himes and Xanthopoulos, 1998; Ramji and Foki, 2002). The induction of this gene in response to thermal stress has also been described in several marine and freshwater fishes (Buckley et al., 2006; Buckley, 2009; Buckley, 2011; Sleadd and Buckley, 2012). However, a developmental role for this gene has remained largely elusive, particularly in non-mammalian vertebrates. I have presented the first evidence that C/EBP $\delta$  holds a critical role in fish development.

I have shown that overexpression of C/EBP $\delta$  leads to a series of abnormalities in developmental patterning, as well as delayed development in the zebrafish (Fig 1D & 1E). Because C/EBP $\delta$  normally functions in the arrest of the cell cycle and in promoting programmed cell death, it is likely that apoptosis occurs at inappropriate times and in populations of cells which would not normally undergo programmed cell death at these stages. In any circumstance in which C/EBP $\delta$  is induced, its expression patterns are typically transient in nature (Balamurugan and Sterneck, 2013). This implies that continuous and elevated expression of C/EBP $\delta$  may have adverse consequences to organismal development, which is consistent with the overexpression results presented here.

The use of the CMV promoter in my overexpression studies leads to ectopic expression of C/EBP $\delta$ -GFP that is ubiquitous throughout the embryo. The CMV promoter can drive overexpression of C/EBP $\delta$  in cell lineages which do not normally express this

transcription factor. In addition, the localization of green fluorescent protein expression at 24-h post fertilization shows the spatial pattern of C/EBP $\delta$  overexpression is randomly distributed throughout the entire embryo (Fig 1F & 1G). Therefore, I predict that ubiquitous overexpression of C/EBP $\delta$  may account for some of the morphological defects and the reduced survivability of these larvae.

C/EBP $\delta$  can regulate the expression of hundreds of downstream target genes (Zhang et al., 2008). Therefore, overexpression of C/EBP $\delta$  may alter the transcription of many of these genes which are activated or repressed in response to C/EBP $\delta$  induction.

Examination of protein expression, using markers for known C/EBP $\delta$  targets, would be useful to determine specifically which targets may be contributing to the C/EBP $\delta$  overexpression phenotype that I observe. In particular, it would be interesting to examine the expression of target genes which function in the promotion of apoptosis and cell cycle arrest.

C/EBP $\delta$  has been shown to promote apoptosis in a tissue-specific manner in multiple organisms (Tsukada et al., 2011). However, the extent to which overexpression of C/EBP $\delta$  will induce apoptosis in developing embryos has remained unclear. The data presented here suggest that C/EBP $\delta$  overexpression induces increased apoptosis, primarily in tissues where C/EBP $\delta$  is normally expressed. For example, C/EBP $\delta$  is expressed in the zebrafish brain during development (Lyons et al., 2001). I detected increased levels of TUNEL staining in the brain region of C/EBP $\delta$ -overexpressing embryos, beginning at 1 day post fertilization (Fig. 4C arrowhead). In addition, cells of

the yolk sac, which normally express C/EBP $\delta$ , display increased TUNEL staining at this stage (Fig. 4C).

I quantified the number of apoptotic cells in whole embryos at 26-h post fertilization. I saw no significant increase in TUNEL positive cells in C/EBP $\delta$ -overexpressing embryos compared to control embryos, which I did not anticipate (Fig. 5). With the significant reduction in body size and the delay in developmental timing, it is difficult to ascertain if this result is due to an overall reduction of cells that make up the C/EBP $\delta$ -overexpressing embryos. In addition, many C/EBP $\delta$ -overexpressing embryos displayed regions of increased staining, which were non-quantifiable, due to the inability to distinguish individual cells in these regions (see arrowhead in Fig. 4C). As a result, these individuals were not included in the TUNEL cell quantification data.

In summary, overexpression of C/EBP $\delta$  is harmful to the development of embryonic and larval zebrafish and reduces their long term ability to survive. Transient expression of C/EBP $\delta$  is crucial for normal embryonic development (Lyons et al., 2001b). However, the data presented here show that induced overexpression has numerous adverse consequences to the developmental program. The data also reveal a distinct role for C/EBP $\delta$  in promoting apoptosis in the developing zebrafish, in regions of the embryo which are spatially distinct from wildtype embryos.

## Chapter II: Knockdown of C/EBPs and analysis of protein expression and cell proliferation in the zebrafish

### Introduction

#### *Knockdown of zebrafish C/EBPs using TALEN-mediated mutagenesis*

Gene knockdown is a valuable tool for understanding the role of genes during development. The emergence of the zebrafish as a model system has facilitated the discovery of highly effective technologies which can induce gene knockdown (Sun et al., 2011). While overexpression studies have vastly improved our understanding of the role of many genes, gene knockdown studies are of great importance as well for further elucidating the genetics of zebrafish development (Skromne and Prince, 2008).

Transcription activator-like effector nucleases (TALENs) have recently been shown to elicit rapid and highly efficient targeted gene knockdown in the zebrafish (Cade et al., 2012). TALENs consist of a specific Transcription activator-like effector DNA-binding domain and a non-specific Type IIS FokI endonuclease cleavage domain (Cade et al., 2012). These synthetic nucleases bind to specific sites of DNA and induce a double stranded break in nearly any desired target region of the genome (Dahlem, et al., 2012). The double stranded break is repaired via error prone non-homologous end joining, which often introduces a single base insertion or deletion at the site of cleavage (Cade et al., 2012). These insertions or deletions can result in reading frame shifts, which often cause premature termination of translation, rendering a protein non-functional.



*Analysis of protein expression in C/EBP $\delta$ -overexpressing zebrafish larvae*

Growth arrest DNA damage 45 $\alpha$  (Gadd45 $\alpha$ ) and proliferating cell nuclear antigen (pcna) are two proteins which are critical for the initiation of apoptosis and the regulation of the S phase of the cell cycle, respectively (Sanchez et al., 2010). pcna expression is linked to increases in cell proliferation in numerous tissues across the developing embryo (Leung et al., 2005). Increased Gadd45 $\alpha$  expression is detected in apoptotic cells within the developing zebrafish (Rai et al., 2008). Because C/EBP $\delta$  can regulate expression of pro-apoptotic genes as well as those involved in cell cycle regulation, it is likely that alterations in C/EBP $\delta$  expression will translate to changes in Gadd45 $\alpha$  and/or pcna expression.

*Analysis of cell proliferation in zebrafish embryos*

Variations in C/EBP $\delta$  expression are linked to changes in cell proliferation (Balamurugan and Sterneck, 2013). Therefore, it is of particular interest to understand changes in cell proliferation that result from overexpression of C/EBP $\delta$  in zebrafish.

Bromodeoxyuridine (BrdU) labeling is one method which can be used to assay for cell proliferation in whole mount embryos.

BrdU is a Thymidine analogue that becomes incorporated into newly synthesized DNA during the S phase of the cell cycle. BrdU replaces Thymine during DNA replication. A common method to assay for cell proliferation in live whole mount embryos is to treat live embryos with BrdU and then process them for immunohistochemistry. Primary antibodies that target incorporated BrdU within the nucleus are commercially available.

Embryonic cells that were undergoing proliferation at the time of embryonic fixation are then secondarily labeled with a fluorescent antibody and can be detected using fluorescence microscopy.

## Methods

### *Genomic DNA isolation, PCR and sequencing of TALEN zebrafish*

TALENs encoding zebrafish C/EBP $\delta$ ,  $\alpha$  and  $\beta$  were designed in collaboration with the Zebrafish International Resource Center (Eugene, OR), using ZiFiT Targeter software (Sander et al., 2010). TALENs designed to target each C/EBP isoform were cloned into FokI plasmids by Addgene (Cambridge, MA). Plasmid DNA encoding TALENs were linearized using the restriction endonuclease SgfI, and were purified using Nucleospin Plasmid DNA purification kit (Clontech). Plasmid DNA was transcribed into mRNA using a T7 in vitro transcription kit (New England Biolabs) and mRNA was purified by phenol/chloroform extraction. Embryos were injected with mRNA encoding C/EBP $\delta$ ,  $\alpha$  or  $\beta$  TALENs at the one cell stage. A group of control embryos was injected with a mammalian mRNA that is not designed to induce knockdown of C/EBPs.

Microinjections were carried out according to a previously established protocol (Xu et al., 2009). Embryos were screened for developmental phenotypes that were specific to C/EBP $\delta$ ,  $\alpha$  or  $\beta$  knockdown embryos and were not observed in the control embryos.

To confirm successful knockdown of C/EBP $\delta$ ,  $\alpha$  or  $\beta$ , embryos injected with either C/EBP $\delta$ ,  $\alpha$  or  $\beta$  knockdown or control mRNAs were grown to 3 days post fertilization. C/EBP $\delta$ ,  $\alpha$  or  $\beta$  -TALEN injected and control larvae were collected at 3dpf and genomic DNA was isolated according to a previously established method by Sharma et al. (2003). PCR was completed using the following primers designed to amplify the specific region of C/EBP $\delta$ ,  $\alpha$  or  $\beta$  in which the mutation was predicted to lie within:

*C/EBP $\delta$* :

F: AAAGACACACACTTTCCTTGGGA

R: TGTAGGCGCTGAAGTCGATG

*C/EBP $\alpha$* :

F: GGCCACTAATGACCAGCCTT

R: GCTCTTGTTTGATGGCCACG

*C/EBP $\beta$* :

F: TGCACTTGTAGTCGGTGAAACTTGC

R: GTTCCCCAGGCTGCCGCTC

PCR fragments from individual *C/EBP $\delta$* ,  $\alpha$  or  $\beta$  TALEN-injected or control larvae were purified then sequenced at Oregon Health and Science University's core DNA sequencing facility (Portland, OR). Sequences were analyzed using Geneious 4.0 (Auckland, NZ). DNA sequences from *C/EBP* knockdown and control larvae were compared to determine the presence or absence of a mutation.

*Protein quantification and Western blot analysis*

Western blots were completed on whole-larval lysates obtained from pools of (n=50) *C/EBP $\delta$* -overexpressing and control (uninjected and *dct* injected) individuals (see chapter I). Pooled larvae were collected, anesthetized in MS-222 and were stored in 200 $\mu$ L lysis

buffer (2% SDS in 32mM Tris pH 6.8) at -80°C. Larvae were briefly homogenized and total protein was isolated then quantified using a Bradford protein assay (Bio Rad). Samples were processed for SDS-Polyacrylamide Gel Electrophoresis and Western blotting (15 to 50 µg total protein weight range) using a method previously described by Buckley (2011). Blots were incubated in primary antibody (Gadd45 $\alpha$ , pcna or C/EBP $\delta$ ) at various concentrations ranging from 1:250 to 1:1000 in 5% Western blocking reagent (Bio Rad) for either 1.5-h at room temperature or overnight at 4°C. After several rinses in PBST, blots were incubated in secondary Anti-Horseradish peroxidase antibody at a concentration of 1:5000 or 1:10,000 in 5% Western blocking reagent for 1-h at room temp.

#### *Bromodeoxyuridine labeling and immunohistochemistry*

I used BrdU labeling to examine cell proliferation in whole mount embryos at 24hpf. Live embryos were treated with 10mM BrdU + 15% Dimethyl sulfoxide (DMSO) in embryo media (or 15% DMSO in embryo media for control treatments) for 30 min at 4°C, then for 1-h at room temperature. Embryos were dechorionated, anesthetized in MS-222, and fixed in 4% PFA in PBS overnight at 4°C. Samples were rinsed 3x5 min in PBST, dehydrated in a graded series of MeOH/PBST, then rehydrated. Samples were processed for immunohistochemistry using a method previously established by Ma et al. (2008).

### Figure 6 - C/EBP $\alpha$ TALEN injection phenotype

At 24hpf most C/EBP $\alpha$  TALEN injected embryos develop similar to uninjected wildtype controls. However, a small number of embryos display developmental abnormalities, including curved tail or delayed development (denoted by asterisks).

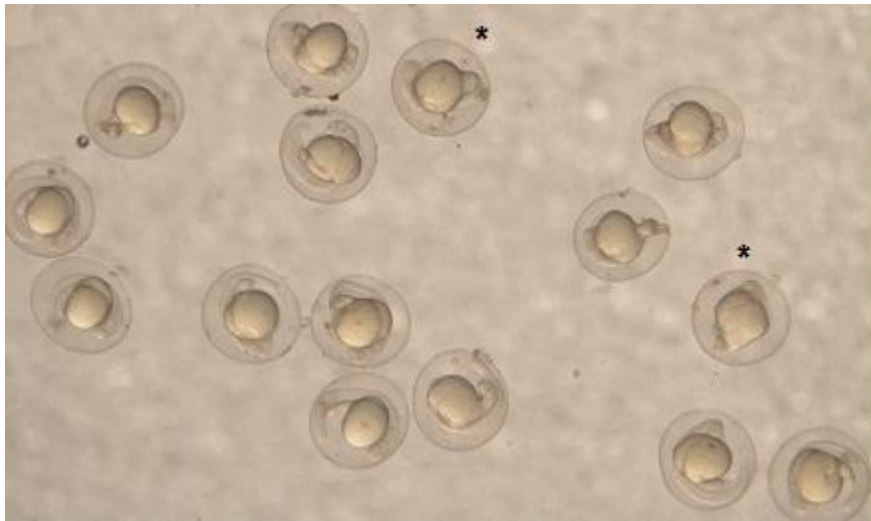
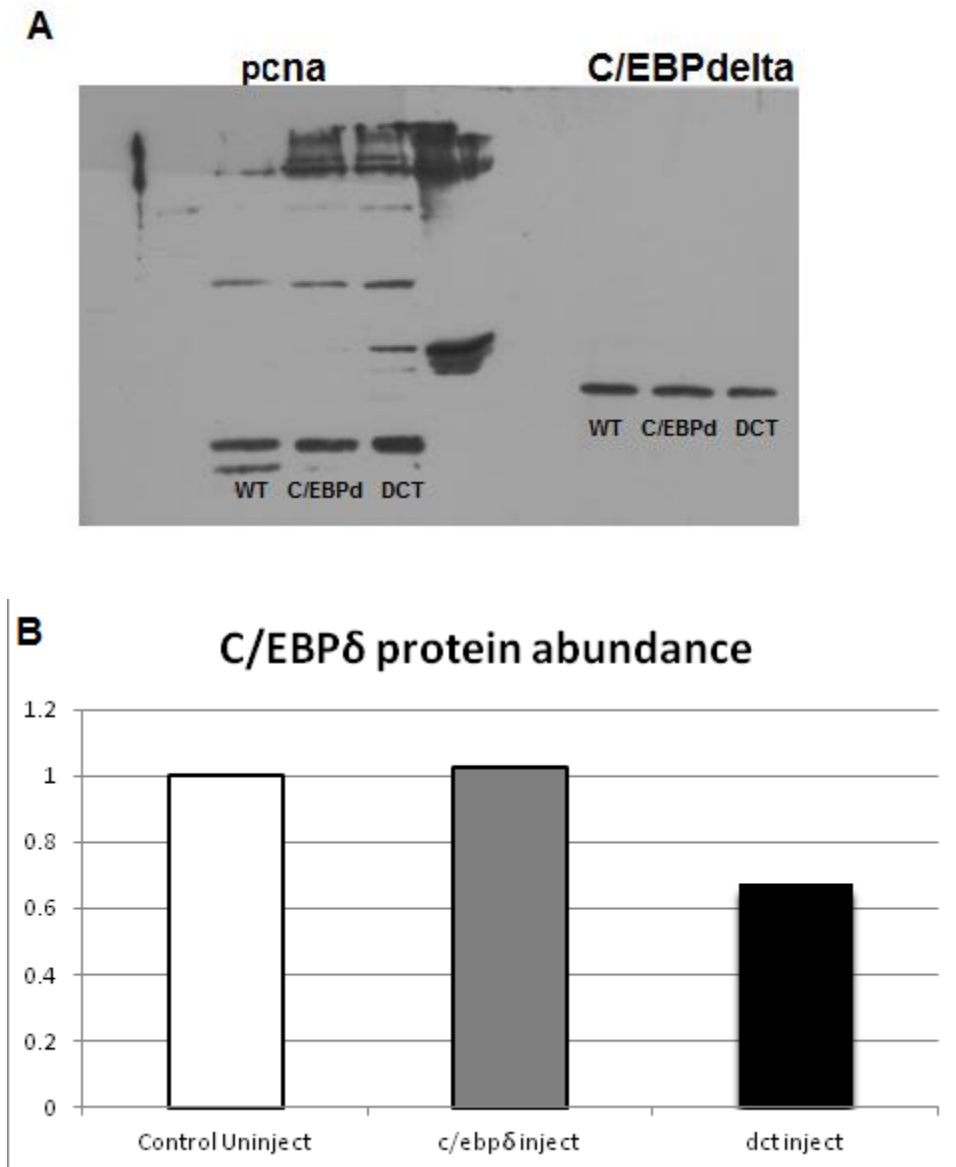


Figure 7 – Analysis of *C/EBP $\delta$*  and *pcna* protein expression in *C/EBP $\delta$* -overexpressing larvae.



## Results and Discussion

Based on previous studies which have described knockdown of both *C/EBPδ* and *C/EBPβ* in mouse models, I predicted that *C/EBPδ* and *C/EBPβ* knockdown zebrafish embryos and larvae would display normal developmental phenotypes similar to those of control embryos (Yan et al., 2013; Tanaka et al., 1997). *C/EBPδ* mouse mutants display reductions in adipocyte differentiation, but develop normally and are capable of surviving to adult stages (Tanaka et al., 1997). *C/EBPβ* knockdown mice display similar phenotypes to those of *C/EBPδ* mutants and have also been shown to exhibit reduced osteoblast differentiation and delayed bone formation; however, these phenotypes are not detrimental to the long term survival of the animal (Tominaga et al., 2008). Mutant models of *C/EBPα* have also been described previously in both cell culture and embryonic models (Porse et al., 2005; Zhang et al., 1997). Mutations in *C/EBPα* severely impede cell cycle progression and inhibit adipocyte differentiation in the mouse. In contrast to *C/EBPδ* and *C/EBPβ* mutants, *C/EBPα* mutant phenotypes are detrimental to the survival of the animal. I predicted that *C/EBPα* knockdown would cause abnormal development, leading to increased mortality in developing zebrafish, which would be consistent with these previously reported mouse mutant phenotypes.

Screening of *C/EBPδ* and *C/EBPβ*-TALEN injected embryos showed normal developmental patterning at 24hpf. Screening of *C/EBPα*-TALEN injected embryos at 24hpf revealed a small number of embryos with slight developmental problems, such as curved tail (These individuals are denoted with an asterisk in Fig. 6).



Sequencing analysis of TALEN injected C/EBP $\alpha$ ,  $\beta$  and  $\delta$  individuals did not uncover any single base insertion or deletions when compared to wildtype uninjected control larval sequences. C/EBP $\alpha$  TALEN-injected larvae that showed some abnormalities in development were sequenced, as these individuals were anticipated to be potential mutants due to their phenotype, which was distinct from wildtype controls. However, these larvae showed no mutations in C/EBP $\alpha$  when compared to wildtype sequences.

*Analysis of protein expression in C/EBP $\delta$ -overexpressing zebrafish larvae*

I completed Western blot analysis using mammalian antibodies for Gadd45 $\alpha$  and Pcn $\alpha$  to determine if C/EBP $\delta$  overexpression can induce apoptosis or inhibit cell proliferation. In addition, I completed Western blots using a custom antibody designed to target fish (*Gillichthys mirabilis*) C/EBP $\delta$  to establish if overexpression of the C/EBP $\delta$  gene will result in protein-level increases in its expression.

I predicted that C/EBP $\delta$  overexpression would result in increased abundance of the C/EBP $\delta$  protein. Increases in C/EBP $\delta$  transcripts do not necessarily translate to increases in the C/EBP $\delta$  protein. Therefore, protein-level analyses are necessary in order to determine if C/EBP $\delta$  overexpression leads to upregulation of the C/EBP $\delta$  protein. Because expression of Gadd45 $\alpha$  is associated with cell cycle arrest and apoptosis, I expected to see an increase in expression of this protein in C/EBP $\delta$ -overexpressing embryos compared to control embryos. pcna upregulation is associated with increased cell proliferation. Given that C/EBP $\delta$  induction often leads to arrest of cell proliferation,

I expected to observe a reduction in *pcna* expression in C/EBP $\delta$ -overexpressing embryos compared to control embryos.

I observed similar levels of C/EBP $\delta$  protein production in larvae overexpressing C/EBP $\delta$  compared to uninjected control larvae (Fig. 7A). Unexpectedly, densitometric analysis suggests a slight but non-significant decrease in the abundance of C/EBP $\delta$  protein in larvae injected with *dct*-GFP plasmid DNA (Fig. 7B). Because overexpression of C/EBP $\delta$  is not driven in every cell or tissue across each individual, it may be difficult to detect changes in protein expression with the use of whole larval lysates. In addition, the use of mammalian antibodies that are not specific to zebrafish Gadd45 $\alpha$  or *pcna* may hinder the reliability of these experiments or interfere with the ability to detect these proteins altogether. Future experiments involving zebrafish-specific antibodies would be useful to further elucidate protein-level changes in expression of C/EBP $\delta$ , Gadd45 $\alpha$  and *pcna* that arise from C/EBP $\delta$  overexpression.

#### *Investigation of cell proliferation in C/EBP $\delta$ -overexpressing embryos*

C/EBP $\delta$  induction is associated with cell cycle arrest and reductions in cell growth and proliferation. Therefore, I wanted to determine the effects of C/EBP $\delta$  overexpression on cell proliferation in the developing embryo. I expected to observe a reduction in the number of proliferating cells in C/EBP $\delta$ -overexpressing embryos compared to their control counterparts. I observed variations in fluorescence and non-specific antibody staining throughout multiple trials of BrdU labeling. It was difficult to discern staining in individual cells and therefore detect and changes in cell proliferation in C/EBP $\delta$ -overexpressing embryos compared to control embryos.

## Chapter III: Effects of embryonic and larval survivability in response to acute and chronic exposure to cold and thermal stress

### Introduction

Exposure to both acute and chronic fluctuations in environmental temperatures can have negative consequences to organismal development. Rapid changes in temperature can activate signaling pathways associated with an animal's cellular stress response. One result of the cellular stress response is variation in the expression of genes which are crucial for normal development and survival of the embryo. Previous developmental and transcriptomic profiling studies have examined alterations in gene expression that arise in response to temperature stress in the zebrafish (Long et al., 2013; Scott and Johnston, 2012). Understanding the range of temperatures which can affect an organism's survivability is important in order to carry out future experiments that could uncover genes that vary expression in response to changing temperature. I characterized the range of temperatures which inhibit the survival of embryonic and larval zebrafish under acute exposure, as well as over time.

## Methods

### *Thermal and cold stress exposures in embryonic and larval zebrafish*

For acute exposures, embryos were staged to 1 or 3dpf (24 or 72hpf) according to Kimmel et al. (1995). Embryos and larvae were of the AB strain and were obtained from pair-wise crosses. 1dpf embryos were exposed in their chorions, while 3dpf larvae hatched prior to treatments, and were therefore exposed without chorions. Embryos and larvae were incubated at each designated temperature for 3-h, in glass beakers containing embryo media that were placed in water baths or a hybridization oven. Immediately following each exposure, fish were examined for survival. The percentage of fish surviving per temperature was calculated for each age ( $n \geq 20$  for all treatment temperatures).

For chronic exposures, embryos were staged to 1dpf (24hpf) and moved to water baths set to 25° (cold), 28.5°C (control) or 31°C (hot) for a period of five days. Larvae were examined each day for survival, and the number of fish surviving per day was quantified through 6dpf ( $n > 40$  for each treatment). From 1 to 3dpf, survival was determined by the presence or absence of necrotic tissues. From 3 to 6dpf, survival was determined by the presence or absence of cardiac activity.

Figure 8 – Acute exposure to thermal and cold stress in larval and embryonic zebrafish.

Embryos and larvae (1dpf and 3dpf, respectively) were exposed to cold (4°C, 12°C and 16°C), control (28.5°C) and heat (34°C, 37°C and 42°C) for 3 hours. None of the 3dpf larvae are capable of surviving acute exposure to 4°C, while all 1dpf embryos can survive this exposure. None of the 1dpf or 3dpf fish survived acute exposure to 42°C.

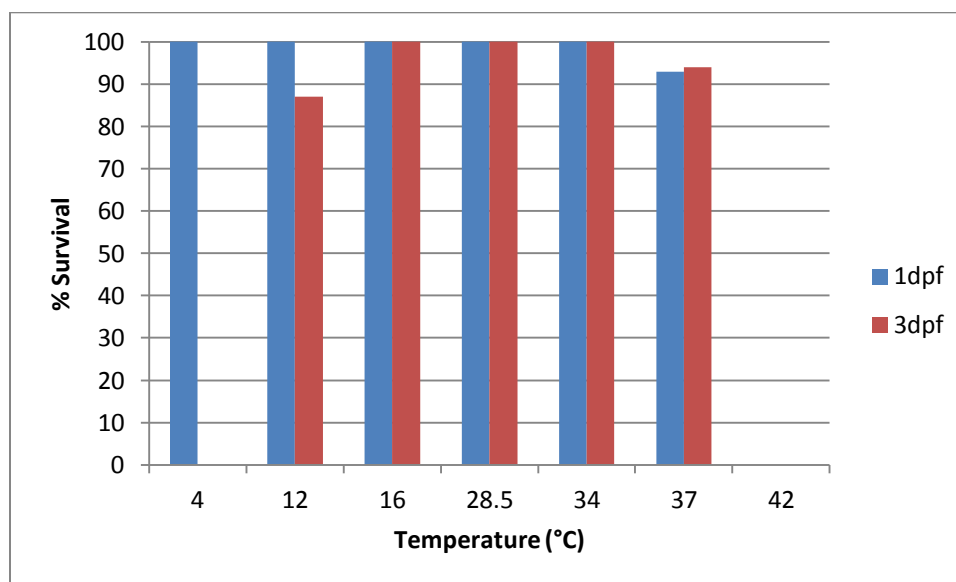
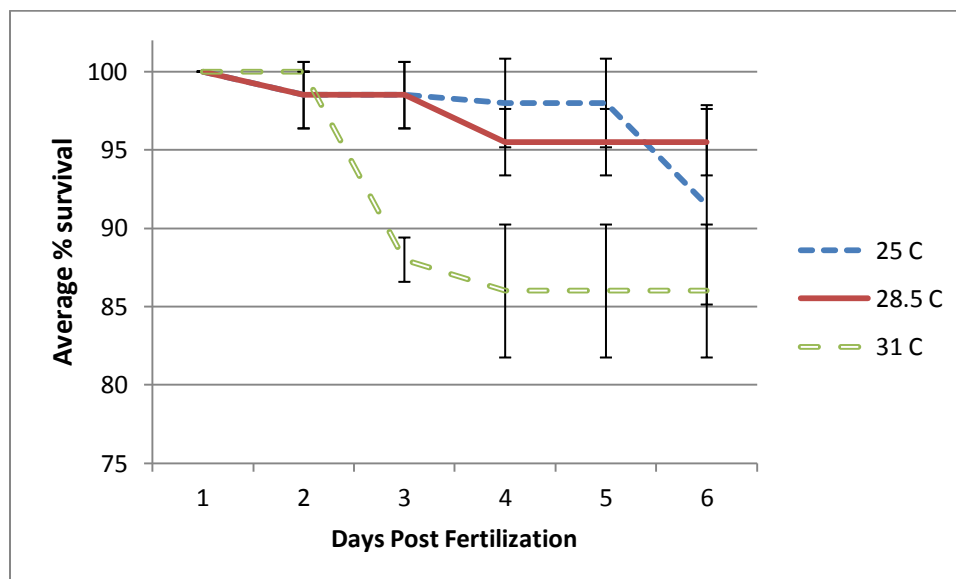


Figure 9 – Chronic exposure to thermal and cold stress in developing zebrafish over time.

Embryos were exposed to chronic cold (25°C), control (28.5°C) and heat (31°C) from 1dpf to 6dpf. This graph suggests a general trend to increased tolerance to cold exposure compared to heat over time (p values are >0.05 for all data points).



## Results/Discussion

### *Acute exposure to temperature stress*

Figure 8 shows the percentage of embryos and larvae which survived each acute exposure. All embryos and larvae incubated at the control temperature (28.5°C) survived acute exposure, as expected. Interestingly, all 1dpf embryos survived acute exposure to 4°C while none of the 3dpf larvae survived at this temperature. None of the embryonic or larval fish survived acute exposure to 42°C. Embryonic zebrafish have a more robust tolerance to rapid changes in temperature compared to larval zebrafish. Perhaps protection against temperature stress is provided at least partially by the chorion in 1dpf embryos. The results of this experiment provide a framework for future studies involving gene profiling in response to acute changes in temperature.

### *Chronic exposure to temperature stress*

Figure 9 shows the percentage of embryos and larvae which survived chronic exposure to 25° (cold), 28.5°C (control) or 31°C (heat) from 1 to 6dpf. Greater than 95% of control embryos survive through 6dpf. Exposure to 31°C results in reduced survival beginning between 2 and 3dpf. In contrast, exposure to 25°C results does not lead to significantly reduced survival until 5dpf. These data reveal distinct temporal requirements for survival in response to cold versus heat exposure in developing larvae. These results will be useful for future experiments to uncover changes in gene expression in response to temperature variations over time in developing zebrafish.

## Significance

Cancer is the leading cause of death worldwide (World Health Organization, 2012).

Many genes that are critical for embryonic development in vertebrates are also implicated in the progression of human diseases, such as cancer. Loss of *C/EBP $\delta$*  function or reductions in expression has been implicated in the progression of human tumors, including those of myeloid, hepatocellular and breast cancers (Agrawal et al., 2007; Balamurugan and Sterneck, 2013; Yin et al., 1996; Zahnow, 2002). Ectopic expression of *C/EBP $\delta$*  reduces the rate of growth in mammary epithelial cells, indicating a potential use for overexpression as a gene therapy target for breast cancer (Pawar et al., 2010). In addition, upregulation of *C/EBP $\delta$*  leads to cessation of the cell cycle, blocking uncontrolled cell proliferation (Johnson, 2005). Therefore, our *C/EBP $\delta$* -overexpressing zebrafish will serve as a valuable model for examining the function of this gene during development, as a part of the cellular response to stress and in pathological states such as tumor progression. The results of this work will provide us with an opportunity to examine novel cancer treatments that may target the expression of *C/EBP $\delta$* .



## Future directions

Because of their significant sequence similarity, all C/EBPs have the capacity to heterodimerize with one another and interact to activate or repress target gene transcription. For example, C/EBP $\delta$  and C/EBP $\beta$  heterodimerize in order to activate transcription of C/EBP $\alpha$  (Ramji and Foki, 2002). Numerous additional studies have identified interactions among C/EBP $\delta$  and other C/EBP family members (Zao et al., 1991, Tanaka et al., 1997, Tang and Dewille, 2003). Therefore, it would be interesting to examine combinatorial overexpression of C/EBP  $\delta$ ,  $\beta$ , and  $\alpha$  during development to determine the co-requirements for these transcription factors at various developmental stages.

I have shown that CMV promoter-driven ubiquitous overexpression of C/EBP $\delta$  is deleterious to the developing embryo. Because the CMV promoter can induce overexpression in any tissue or cell type, it remains unknown whether or not I would obtain similar results if C/EBP $\delta$  overexpression was restricted to a single tissue or cell type. Examination of overexpression using a tissue-specific promoter would be of interest, particularly in liver or brain tissue, where C/EBP $\delta$  is normally expressed.

Gene knockdown remains a useful approach for further identifying the role of genes during development. To date, no mutant models of C/EBP $\delta$  have been characterized in the zebrafish. Mutations in C/EBP $\delta$  have previously been characterized in Murine models (Tanaka et al., 1997; Gigliotti et al., 2003), and these mutants are homozygous viable, making them an ideal model system for studying the effects of C/EBP $\delta$

knockdown from early development to adult stages. Because C/EBP $\delta$  structure and function is highly conserved among vertebrates, it is likely that C/EBP $\delta$  zebrafish mutants would show similar viability to these previously identified mouse mutants. Therefore, C/EBP $\delta$  knockdown zebrafish would serve as valuable models for further elucidating the role of this gene during development as well as examining temporal requirements through adult stages. Furthermore, some interesting future studies would involve exposing C/EBP $\delta$  mutant zebrafish to thermal stress and examining the effects of knockdown on developmental patterning, in the absence of this critical cellular stress response gene.

Reductions in C/EBP $\delta$  expression are prevalent in cells of human breast, myeloid and hepatocellular carcinomas (Zahnow, 2002; Agrawal et al., 2007; Yin et al., 1996). Future studies aimed at overexpression of C/EBP $\delta$  in human cancer cell lines may provide insight into the mechanisms leading to the progression of cancer and tumorigenesis in humans. Additionally, these studies may uncover potential treatments for these debilitating diseases which could employ C/EBP $\delta$  as a gene therapy target. As C/EBP $\delta$  overexpression promotes apoptotic cell death in the embryo, perhaps overexpression of C/EBP $\delta$  can induce increased apoptosis in cultured cells, halting the proliferation of cancer cells.

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