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POTENTIAL ROLES FOR ELF3 IN FETAL ALCOHOL SPECTRUM DISORDER AND DEVELOPMENT

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POTENTIAL ROLES FOR ELF3 IN FETAL ALCOHOL SPECTRUM DISORDER AND  
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I would like to dedicate this work to my grandmother, Mary Seaberg. I would not be who or where I am today if not for her.

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## LIST OF ABBREVIATIONS

|                  |  |
|------------------|--|
| FAS              | Fetal Alcohol Syndrome                           |
| FASD             | Fetal Alcohol Spectrum Disorder                  |
| ZGA              | Zygotic Genome Activation                        |
| Hpf              | hours post fertilization                         |
| Sox2             | (Sex determining region Y)-box 2                 |
| FIMO             | Find Individual Motif Occurances                 |
| Elf3             | E-74 like factor 3                               |
| TGF- $\beta$ RII | Transforming Growth Factor Beta Receptor type II |
| IL-1b            | Interleukin-1b                                   |
| TNF- $\alpha$    | Tumor Necrosis Factor alpha                      |
| NF- $\kappa$ B   | Nuclear Factor kappa B                           |
| IL-6             | Interleukin-6                                    |
| WAP              | Whey acidic protein                              |
| SPRR2A           | Small proline-rich protein 2A                    |
| RT-PCR           | Real Time Polymerase Chain Reaction              |
| PCR              | Polymerase Chain Reaction                        |
| MO               | Morpholino                                       |
| BP               | base pair  |

|           |                                      |
|-----------|--------------------------------------|
| Foxm1     | Forkhead box m1                      |
| Her5      | Hairy-related 5                      |
| Rarab     | Retinoic acid receptor alpha b       |
| Slc16a9b  | Solute carrier family 16a, member 9b |
| Rnft1     | Ring finger transmembrane protein 1  |
| Krt23     | Keratin 23                           |
| Zgc123063 | Sex-hormone binding globulin         |
| mRNA      | Messenger Ribonucleic Acid           |
| Ets       | E26 transformation specific          |
| HMG       | High Mobility Group                  |
| cDNA      | Complementary DNA                    |



## ABSTRACT

Farrell, Mark Casey M.S., Purdue University, August 2015. Potential Roles for Elf3 in Fetal Alcohol Spectrum Disorder and Development. Major Professor: James A. Marrs.

Fetal alcohol spectrum disorder is a disease caused by prenatal alcohol exposure. It is characterized by craniofacial abnormalities, growth retardation, central nervous system defects, learning disabilities and a variety of other minor defects. Even though it affects 2-5% of individuals born every year, very little is known about the mechanisms that cause it. The zebrafish (*Danio rerio*) presents as an interesting and efficient model for studying this disease. This study provides some insight into the mechanisms underlying observed FASD phenotypes and, more specifically, the transcription factor *elf3*, which is downregulated in response to ethanol exposure during early embryonic development. Here we show a number of *elf3* target genes that are downregulated during early development in response to ethanol exposure. We also give some insight into the expression pattern of *elf3* in relation to zygotic genome activation. Translation blocking morpholino oligonucleotides were used to implicate Elf3 in epiboly movements during gastrulation and zebrafish tail development. Taken together these results help to strengthen the zebrafish as a model for FASD in addition to giving greater insight into both the expression pattern and role of Elf3 during development.

## CHAPTER 1: INTRODUCTION

### Fetal Alcohol Spectrum Disorder

Fetal Alcohol Syndrome (FAS) is a disease caused by prenatal alcohol exposure, resulting in a variety of birth defects including craniofacial abnormalities, growth retardation, central nervous system defects, and even death (Sampson, Streissguth et al. 1997; Sokol, Delaney-Black et al. 2003). Fetal Alcohol Spectrum Disorder (FASD) is characterized by these same phenotypes in addition to a number of minor defects and learning disabilities. It is estimated anywhere between 2-5% of individuals born each year suffer from FASD with that prevalence being considerably higher among groups of low socioeconomic status (May and Gossage 2001; May, Gossage et al. 2009; May, Baete et al. 2014). In addition, according to a CDC report from 2010, 7.6% of pregnant women between the ages of 18 and 44 admitted to at least some use of alcohol during the last 30 days of their pregnancy, while approximately 1.4% of women of the same age group admitted to binge drinking while pregnant. The average frequency and intensity of women who reported binge drinking was, on average, three times per month with six drinks on each occasion. The first step towards eliminating FASD completely should be education. However, even knowing that alcohol consumption could cause problems

for their children, many women continue to drink while pregnant. Given the prevalence of women drinking while pregnant in the United States and the frequency of individuals born with FASD, it is imperative that the mechanisms underlying this disease be understood to help identify effective preventative and therapeutic measures.

The molecular mechanisms underlying FASD are poorly understood. Studying humans in order to gain a better understanding of this disease is problematic. Most studies must be retrospective, in that they are done on a single individual who has already been born and is displaying symptoms of FASD (which may not be apparent immediately after birth). In addition, ethical considerations rightly limit the ability to study it in humans. As a result, a robust animal model must be developed in order to determine the mechanisms that cause FASD. Rats and mice provide interesting models for study, however these animals present a variety of problems as well. The blood-alcohol content of the mother is hard to control and must be monitored constantly. Such high frequency of blood work stresses her and could potentially skew results. In addition, pups born who are deformed are at risk for destruction before they can be studied (Abel 1981; Driscoll, Streissguth et al. 1990; Bilotta, Barnett et al. 2004). The zebrafish, however, presents as an organism in which FASD can be modeled in a more practical manner.

### Modeling FASD using Zebrafish

Zebrafish eggs are fertilized and develop externally. In addition, ethanol can pass through the chorion with relative ease (Lovely, Nobles et al. 2014). As a result, experimental conditions can be easily and reliably manipulated when using zebrafish in developmental studies. The embryo is also largely transparent throughout development. This provides an advantage as the experiment can be easily monitored without any significant interference with the developing embryo itself. The zebrafish also develops relatively quickly and has a large clutch size, allowing for the study of a large number of animals in a short period of time. Phenotypic defects seen in humans are recapitulated in zebrafish as well. Microphthalmia, decreased body length, craniofacial, and cardiac abnormalities have all been reported in zebrafish developing in the presence of ethanol (Blader and Strahle 1998; Bilotta, Barnett et al. 2004; Muralidharan, Sarmah et al. 2013; Sarmah and Marrs 2013; Muralidharan, Sarmah et al. 2015). If the molecular mechanisms underlying these phenotypic defects can be elucidated in zebrafish, then it could shed light on the causes of FASD in humans.

Identifying critical developmental stages most affected by ethanol exposure in zebrafish will help give insight into sensitive mechanistic events. It was shown that as the zebrafish embryo develops, it becomes less and less sensitive to alcohol (Lovely, Nobles et al. 2014). If this is true for humans as well, it is important to note that some women may become pregnant and are yet unaware. As a result, they

continue on with their normal drinking habits without fear of unknown consequences. The CDC reports that 53.7% of non-pregnant women surveyed had at least one drink during the 30 days prior to taking the survey while 12.1% reported binge drinking. If these drinking patterns are habitual for these women, and some become pregnant but do not yet know it, the alcohol they consume could affect the embryo during its earliest stages of development. The weak barriers against ethanol during the earliest stages of development means that newly fertilized embryos that are undergoing critical early developmental processes such as zygotic genome activation (ZGA) are very susceptible. This event occurs at approximately 3 hours post fertilization (hpf) during the mid blastula transition in zebrafish and at the 4-cell stage in humans. Previous to this event, all mRNA found within the embryo has been maternally deposited. Once the zygotic genome is activated, a number of epigenetic modifications take place, a large number of genes are suddenly expressed, and the embryo begins to produce its own mRNA (Vastenhouw, Zhang et al. 2010; Lindeman, Andersen et al. 2011; Andersen, Reiner et al. 2012; Lee, Bonneau et al. 2013; Potok, Nix et al. 2013). Considering there is evidence that epigenetic changes are, at least, partially responsible for the teratogenic effects of ethanol exposure during development, this important event needs to be studied in greater detail within the context of an FASD model (Haycock 2009).

### Ethanol Exposure and Zygotic Genome Activation

Recent research has provided greater insight into the influence of ethanol on ZGA. Our lab used a GeneChip Microarray to analyze all genes expressed at 4.5 hpf, comparing gene expression in embryos treated with alcohol to untreated control embryos. This time point was chosen to examine how the first set of genes an embryo transcribes are being affected by ethanol exposure. Two hundred ninety three genes were found to be significantly dysregulated in the ethanol treated embryos. Of these genes, 31 encoded transcription factors, including those critical for ZGA such as *sox2*, which is important in maintaining pluripotency as well as neural progenitor identity in addition to a variety of other important biological processes (Graham, Khudyakov et al. 2003). Find Individual Motif Occurrences (FIMO) analysis was run on 11 of these transcription factors to estimate the number and location of binding sites in the zebrafish genome and, therefore, number of potential targets for each transcription factor. These potential targets were then compared with the 293 ethanol dysregulated genes to identify potential transcription targets for these factors. Interestingly, the consensus sequence of a transcription factor named E-74 like factor 3 (Elf3) was found in the largest number of genes dysregulated in response to ethanol at 4.5 hpf (Fig. 1a, b). Many of these targets were also targets of the critical ZGA factor, Sox2 (Fig. 1c). Even though Elf3 has a large number of predicted targets, which include targets shared with Sox2, little is known about its functions, especially within the context of early embryonic development.

### The Structure of Elf3 and other Ets transcription factors

ELF3 (also called ESX, ERT, ESE-1, EPR-1, JEN) is a member of the E26 transformation specific (ETS) family of transcription factors. These transcription factors are so named because they share a highly conserved DNA binding domain named the ETS domain (Fig. 2). These factors can be further divided into sub-families based on the degree of similarity of the amino acid sequence of this domain. All ETS transcription factors share a consensus sequence of “GGA”, which is the core of a 10 BP sequence that is recognized by the ETS domain. The variable sequences that flank this core are what determines which ETS transcription factor(s) will be able to bind (Wasylyk, Hahn et al. 1993). The ETS domain present in ELF3 is unique in amino acid sequence, and doesn't appear to group within any known sub-family of ETS transcription factors. Even though the primary amino acid sequence is different, ELF3 has been shown to function quite similarly to the ETS domains of ETS-1 and ETS-2. In addition to a functionally conserved ETS domain, ELF3 contains two other regions required for its function. The first is the Pointed domain, which is found in other members of the ETS family as well. The role of this domain, however, is not entirely clear. There is some evidence that it plays roles in dimerization, transactivation and transformation in other members of the ETS family. The third catalytic domain present in ELF3 is the A/T Hook domain. Transcription factors that contain this domain, often bind to “AT” rich regions of DNA (Oettgen, Alani et al. 1997; Aravind and Landsman 1998; Kopp, Wilder et al. 2007). Although the Pointed and ETS domains are seen in other members of the ETS family, the A/T Hook

domain is unique, and is usually seen in the High Mobility Group (HMG) family of transcription factors in *Drosophila* (Aravind and Landsman 1998). Taken together, the structure of Elf3 clearly follows that of other ETS transcription factors, while also containing a unique A/T Hook domain, suggesting a unique function for this factor.

### The Role of Elf3 in Cancer and Inflammation

The ETS family of transcription factors has been implicated in a variety of biological processes including proliferation, differentiation and survival of many different cellular lineages during embryonic development. These factors also control oncogenic transformation in adult tissues (Wasylyk, Hahn et al. 1993; Remy and Baltzinger 2000; Kageyama, Liu et al. 2006; Jedlicka and Gutierrez-Hartmann 2008; Haycock 2009). There is a growing body of research that indicates Elf3 is one of the Ets transcription factors that plays a role in the development of cancer. This research has illustrated that ELF3 is involved in the transformation of mammary epithelial cells in breast cancer (Chang, Scott et al. 1997; Kaplan, Wang et al. 2004; Prescott, Koto et al. 2004; Schedin, Eckel-Mahan et al. 2004). There is also evidence that ELF3 positively regulates TGF- $\beta$ RII, inhibiting tumorigenesis (Chang, Lee et al. 2000; Kim, Im et al. 2000; Lee, Chang et al. 2003). In addition to breast cancer, Elf3 has been implicated in colorectal cancer, lung cancers, and synovial sarcomas (Tymms, Ng et al. 1997; Allander, Illei et al. 2002; Lee, Chang et al. 2003; Kohno, Okamoto et al. 2006; Lee, Bahn et al. 2008).



Although the role *Elf3* plays in certain cancers may be important, it plays roles in other biological processes that also must be examined thoroughly.

In addition to its role in oncogenesis, *ELF3* is involved in inflammation. The expression of *ELF3* can be driven by certain proinflammatory cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) via the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) in monocytes, bronchial epithelial cells, chondrocytes and osteoblasts (Rudders, Gaspar et al. 2001; Grall, Gu et al. 2003; Brown, Gaspar et al. 2004; Wang, Fang et al. 2004; Grall, Prall et al. 2005; Peng, Tan et al. 2008; Wu, Duan et al. 2008; Zhan, Yuan et al. 2010; Kushwah, Oliver et al. 2011; Oliver, Kushwah et al. 2012). This is especially interesting considering that under normal conditions, *ELF3* expression is restricted to epithelial tissues (Andreoli, Jang et al. 1997; Oettgen, Alani et al. 1997; Tymms, Ng et al. 1997). Additionally, expression of *Elf3* has been linked directly to increased expression of interleukin-6 (IL-6), leading to the differentiation of certain T-cells during the inflammatory response (Kushwah, Oliver et al. 2011; Oliver, Kushwah et al. 2012). It is clear that *ELF3* is important in a variety of biological processes, but very little is known about it within the context of development, especially early development.

#### The Role of *Elf3* in Development

*Elf3* has been implicated in some developmental processes, but overall, very little is known about its role in early development. There is evidence that *ELF3* is important

during the differentiation of the intestinal epithelium. Approximately 30% of mice with a null mutation of *Elf3* (*Elf3*<sup>-/-</sup>) die in utero. The ones that survive exhibit a “wasting syndrome”. Mice that have this “wasting syndrome” characteristically look malnourished, have watery diarrhea and are generally lethargic. These *Elf3* null mice also have severe morphological defects within their intestine including poor villus and microvillus formation. In addition, many enterocytes and mucus secreting goblet cells fail to fully differentiate. These enterocytes have low levels of TGF- $\beta$ RII which is partially responsible for inducing the differentiation of epithelial cells (Ng, Waring et al. 2002; Oliver, Kushwah et al. 2012). Although these observations are relatively preliminary, *Elf3* appears to play a critical role in intestinal development.

ELF3 appears to be involved in mammary gland development and involution as well. Elevated levels of *Elf3* mRNA have been detected in the mammary gland epithelium during pregnancy and early lactation in mice. In addition, ELF3 positively regulates the whey acidic protein (WAP) gene, which is a major protein found in milk and is important in regulating the proliferation of mammary epithelial cells. Elevated levels of *Elf3* are also found following weaning, during involution of the mammary glands, suggesting it may play a role in the apoptotic pathway as mammary tissues are deconstructed and the epithelial cells are removed (Neve, Chang et al. 1998; Oliver, Kushwah et al. 2012).

ELF3 has also been associated with the development of the epidermal skin epithelia and the epithelia of the cornea. It was shown that *ELF3* expression is induced during human keratinocyte division. It activates expression of small proline-rich protein 2A (*SPRR2A*), *Transglutamase-3*, and *Proflaggrin*, which are all associated with keratinocyte differentiation (Andreoli, Jang et al. 1997; Oettgen, Alani et al. 1997; Oliver, Kushwah et al. 2012). In addition, *ELF3* is upregulated during differentiation of epithelial cells in the cornea of the eye. This is linked to the upregulation of K12 keratin, which is associated with terminal differentiation of corneal epithelia (Yoshida, Yoshida et al. 2000; Oliver, Kushwah et al. 2012).

Additionally, some research has been done on the role of *Elf3* during preimplantation development of mouse embryos. It has been shown that expression of *Elf3* is increased following fertilization. Additionally, siRNA techniques were used to knockdown *Elf3* expression. Less than 60% of treated embryos survived to the blastocyst stage. Triple knockdown of three ETS transcription factors (*Elf3*, *Etsrp71* and *Spic*) resulted in a reduction in expression of genes that contain ETS regulation sites including *Elf3-1a* and *Oct3/4* (Kageyama, Liu et al. 2006). Taken together, it is clear that ELF3 is important in a variety of biological processes. However, very little work has been done on the role it plays in early development. Even less work has been done on the role it plays during zebrafish development. If we are to reveal the

mechanisms underlying FASD phenotypes using zebrafish as a model, we must also gain a greater understanding of the importance Elf3 has during early embryonic development.

### Research Goals

The goal of this research is to strengthen the zebrafish as a model for FASD and give some insight into the role Elf3 plays during early embryonic development. Elf3 appears to be involved in a wide variety of biological processes including inflammation, differentiation of epithelia, and oncogenic transformation. However, there is much more evidence for its involvement in cancer progression than in development. This is especially true for early development. Interestingly, our laboratory's microarray experiment, showed that *elf3* expression was reduced in response to ethanol exposure during early development. Elf3 has a large number of potential target consensus sequences in a large number of genes, many of which are also targets of the important pluripotency transcription factor, Sox2. Other labs have shown that almost one third of *elf3*<sup>-/-</sup> mice die in utero, further implicating its importance in early embryonic development (Ng, Waring et al. 2002). Taken together, it is clear that a greater understanding of the role *elf3* plays during early development and its response to ethanol exposure will both strengthen the zebrafish as a model for studying FASD and give greater insight into the importance of this transcription factor.

Our hypothesis was that downregulation of *elf3* is partially responsible for observed FASD phenotypes in zebrafish and its knockdown will result in similar defects. In order to test this hypothesis, we used Quantitative Polymerase Chain Reaction (qPCR) to measure the dysregulation of Elf3 target genes in response to ethanol exposure at 4.5 hpf. We also used PCR techniques to establish an expression pattern of *elf3* with respect to ZGA. Finally, we examined which developmental processes Elf3 might play a role in by knocking its expression down using a translation blocking morpholino oligonucleotide and testing for phenotypic defects at various stages of early development.

## CHAPTER 2: MATERIALS AND METHODS

### Polymerase Chain Reaction

Embryos were treated with 100 mM ethanol beginning at 2 hpf. Total RNA was extracted from ~30 embryos per group using TRIzol (Sigma). 1 µg of RNA was used to synthesize cDNA using M-MLV Reverse Transcriptase (Promega, Madison, WI). PCR reactions were performed using Taq DNA Polymerase (Roche Diagnostics, Mannheim, Germany), 1 µl of cDNA and 0.8 µl of *elf3* specific primer. Primer sequences - *elf3* situ F1: 5' TCTCAACAACGCCAATCCACC 3', *elf3* situ R1: 5'CCCTATAGTGAGTCGTATTACGGCGTCGAATCTGCTCTCC 3'. A 1.2% agarose gel was cast using ethidium bromide as an intercalating dye and the PCR products were ran through this gel. The gel was visualized using the ChemiDoc XRS+ System (Bio Rad, Hercules, CA).

### Quantitative PCR Analysis

Quantitative PCR analysis was performed as described in Sarmah and Marrs, 2013. Embryos were treated with 100 mM ethanol beginning at 2 hpf. They were raised to 4.5 hpf and total RNA was extracted from ~30 embryos (per group) using TRIzol (Sigma). One µg of RNA was used to synthesize cDNA using M-MLV Reverse Transcriptase (Promega, Madison, WI). 1-2 µl of this cDNA (1:5 dilution) and 1 µl of

each primer were used in each qPCR reaction using Power SYBR Green PCR mix as a detector (Applied Biosystems, Foster City, CA). Two independent experiments were performed (one in duplicate, one in triplicate) and results were confirmed using findings from a previously performed microarray analysis. Thermal cycling was performed by a Lightcycler 480 (Roche). Primer sequences – *her5* F: 5' TGTAATGCTCTGTGACCCC 3', *her5* F: 5' AGCTGGTCTTTTCAGTCTTG 3', *zgc123068* F: 5' GCTGGAATCTCAATGGCAGG 3', *zgc123068* R: 5' TCTCCAGTCACCCAGTACA 3', *slc16a9b* F: 5' GACCCCGTCAAAAAAACC 3', *slc16a9b* R: 5' CCAGCGTCACGTAATTGAG 3', *krt23* F: 5' GGAGAAACTCAAACGACCG 3', *krt23* R: 5' CTCCACTGTTTTGCTCAG 3', *rarab* F3: 5' CCAGTGATTCAGCGACTACAA 3', *rarab* R3: 5'CCGGAAGACACAGTAAAGAG 3', *rnft1* F: 5' CCTCTCACACATCTCCACTG 3', *rnft1* R: 5' GTH=GAATGCACTCATCCCTC 3', *foxm1* F: 5'; GTGGTGATCCCGAAATCAG 3', *foxm1* R: GATGAACTTGTTTCGCCCC 3',

#### Morpholino Oligonucleotide and mRNA Injection

Translation blocking morpholinos (MO) were designed by and purchased from Gene Tools LLC. The *elf3* morpholino (5' TTAGACTAAGTTCGCTTGACGCCAT 3') was designed to target bases 0-25 in relation to the start codon of zebrafish *elf3* mRNA. The standard control morpholino was designed against a splice-generating mutant at position 705 of human beta globin pre-mRNA. It has been shown that this morpholino has no significant biological activity in zebrafish (Gene Tools, Plymouth OR). A BLAST search was used to ensure there were no significant similarities found

between the *elf3* MO binding sequence and other zebrafish mRNA sequences. A significant similarity was defined as a twenty-five BP long sequence having twenty one or more bases in common with the *elf3* MO binding sequence. Approximately 4 nl of 0.98  $\mu\text{g}/\mu\text{l}$  (.1mM) of morpholino dissolved in RNase free water was injected into the yolk of embryos between the one and eight cell stages (approximately 4 ng per embryo).

mMessage mMachine (Ambion, Austin, TX) was used to transcribe mRNA from *elf3* cDNA cloned into the pExpress-1 vector (Dharmacon GE Healthcare Bio-Sciences, Pittsburgh, PA). Approximately 4 nl of 50 ng/ $\mu\text{l}$  mRNA dissolved in RNase free water was coinjected with the *elf3* MO into one cell embryos. In total, approximately 200 picograms of mRNA was injected into each embryo.

#### Epiboly Measurements and Statistical Analysis

Epiboly measurements were performed using ImageJ Software (NIH). A line was drawn horizontally across the front of migrating cells. A line was drawn perpendicular to the horizontal beginning at the horizontal and terminating at the animal pole. The length of this line was measured and then the diameter of the embryo from animal pole to vegetal pole was measured. Percent epiboly was then calculated using these two measurements. Statistical analysis was performed on percent epiboly using unpaired, two-tailed, Student's *t*-test (GraphPad Software, La Jolla, CA USA).



## CHAPTER 3: RESULTS

Potential Elf3 Targets are Dysregulated in Response to Ethanol Exposure at 4.5 hpf

In order to validate our Microarray results, qPCR was performed on a number of potential Elf3 targets that were dysregulated in response to ethanol treatment at 4.5 hpf. Embryos were treated with ethanol at a concentration of 100mM beginning at 2 hpf. Whole RNA was harvested from both control and ethanol treated embryos at 4.5 hpf and was then used to synthesize cDNA. qPCR was then used to examine differences in levels of gene expression between control and ethanol treated embryos of 7 putative Elf3 targets. Each gene was tested in duplicate before being tested in triplicate to further confirm results. *Forkhead box m1 (foxm1)*, *hairy-related 5 (her5)*, *retinoic acid receptor, alpha b (rarab)*, *solute carrier family 16, member 9b (slc16a9b)*, and *ring finger transmembrane protein 1 (rnft1)* were all found to be significantly upregulated following ethanol exposure. *Keratin 23 (krt23)*, and *sex-hormone binding globulin (zgc123063)* were both found to be downregulated in ethanol treated embryos (Figure 3). These changes in gene expression were consistent when tested in duplicate and triplicate via qPCR and further confirmed the results found in the GeneChip Microarray.

### *elf3* mRNA is Not Maternally Deposited

In order to gain a greater insight into the expression patterns of *elf3* within the early embryo, we examined if it was maternally deposited. Whole RNA was extracted from embryos before (1.5 hpf) and after (4.5 hpf) ZGA. Next, cDNA was synthesized from this RNA and PCR was then used to amplify *elf3* in either or both of the samples. Gel electrophoresis was then performed to visualize the results. *elf3* was present at 4.5 hpf, as expected. Interestingly, it was not present at 1.5 hpf (Figure 4). Both samples were then tested in the same manner for the presence of *sox2*, which is a transcript that should be present at both of these time points. Indeed, *sox2* was detected at both 1.5 and 4.5 hpf, confirming that the cDNA that was used was viable, further indicating that *elf3* is likely expressed at 4.5 hpf but not at 1.5 hpf.

### *elf3* Morpholino Injection Results in Epiboly Delay

In order to test the involvement of *elf3* in the overall development of the zebrafish embryo, we injected a translation blocking morpholino that specifically recognizes and binds to *elf3* transcript into the early embryo. This *elf3* morpholino was injected into a group of embryos between the one and eight cell stage (*elf3* MO). An established, standard control morpholino to a human beta-globin intron mutation was injected into a comparable number of embryos between the one and eight cell stages to serve as a negative control (injected control). A third group of embryos were not injected with any morpholino (uninjected control). Embryos were collected at approximately 8.3 hpf and percent epiboly was measured. The *elf3* MO

group of embryos showed a significant delay in epiboly (n=68, 75.9%  $\pm$  6.48% standard deviation) compared to both the injected (n=54, 85.7%  $\pm$  4.19% standard deviation) and uninjected (n=41, 81.5%  $\pm$  4.39% standard deviation) control groups (Fig. 5). In order to examine whether this defect was due exclusively to the downregulation of *elf3* or due to non-specific binding of the morpholino, rat *elf3* message, to which the morpholino should not bind, was coinjected with *elf3* morpholino (*elf3* MO + RNA) in an attempt to rescue the epiboly delay. Percent epiboly (n=57, 75.66%  $\pm$  5.44% standard deviation) in this group of embryos was significantly less (p < .0001) than that of the embryos in both control groups and was nearly identical to that of the *elf3* MO group (Fig. 5).

#### *elf3* Morpholino Injection Results in a Defect in Tail Formation

After we established that injection of the *elf3* MO results in an epiboly delay, we wished to examine if it had any effects on the later stages of development as well. In order to gain a greater insight into this, we injected embryos in a similar manner, again creating an *elf3* MO group in addition to injected and uninjected control groups. We found that a small number of embryos in the *elf3* MO group showed a severe “curled tail” deformity (Figure 6). There were so few embryos that showed this defect, we felt it imperative that we repeat the experiment multiple times to ensure that this was indeed a positive result, and not just an artifact. Repeatedly, we saw the same defect in a small number of embryos injected with the *elf3* morpholino (approximately 4-9%) but not in either of the control groups. In order to confirm

that non-specific binding of the morpholino was not causing this defect, rat *elf3* mRNA was again coinjected with the *elf3* MO in an attempt to rescue the “curled tail” phenotype. As with the epiboly delay, the rat message was unable to rescue this defect as it was produced in a select few embryos in both *elf3* MO and *elf3* MO + mRNA groups but not in either control group.

*elf3* MO injections cause neither brain nor eye defects at 24 hpf

Once we had established that a tail defect was consistently seen in embryos injected with the *elf3* MO, we examined these defective embryos for any other visible defects. We examined especially the developing brain and eyes at 24 hpf. At this stage these two organ systems are easy to visualize with a microscope and any severe phenotypic defects would be obvious. When comparing the developing brain and eyes of the *elf3* MO injected embryos to those of uninjected control embryos, however, there were no obvious phenotypic defects (Figure 7).

## CHAPTER 4: DISCUSSION

These studies have given a greater insight into the importance of Elf3 and the role it plays during development of the zebrafish within the context of FASD. The zebrafish presents as a promising model for FASD, but the affected molecular mechanisms during early development, when the embryo is most susceptible to ethanol exposure, have yet to be identified (Lovely, Nobles et al. 2014). *elf3* expression is affected by ethanol treatment and yet, almost nothing is known about the role it plays during early development. Considering its large number of potential targets and the targets it shares with the important pluripotency factor Sox2, its involvement in developmental processes must be studied.

We showed that a number of putative Elf3 targets are significantly dysregulated in response to ethanol exposure at 4.5 hpf. Our GeneChip Microarray found 56 genes that contained the Elf3 consensus sequence were dysregulated at this time point. When 7 of these genes were tested using qPCR, the results for all of them mirrored those found in the microarray (Fig 3). Some of these genes are of particular interest because of the biological processes in which they are involved. For example, *foxm1* is a gene that was found to be upregulated in response to ethanol exposure. Overexpression of this particular gene has been linked to epigenetic modifications

that can cause oncogenesis (Teh, Gemenetzidis et al. 2012). Another gene of interest that is upregulated in response to ethanol exposure is *her5*. This gene is important in the development of endodermal organs such as the liver and pancreas (Shin, Chung et al. 2008). A third *elf3* target gene tested was *rarab*. This gene has been implicated in the development of the heart, liver, pectoral fin and the midbrain-hindbrain boundary (Linville, Radtke et al. 2009; Garnaas, Cutting et al. 2012; D'Aniello, Rydeen et al. 2013). This is an interesting result as cardiac and central nervous system defects as complications of ethanol exposure have previously been reported in the zebrafish FASD model (Sarmah and Marrs 2013). Although the implications of these dysregulated genes are unknown, here we show that Elf3 has a number of targets that are dysregulated in response to ethanol, some of which play important roles in development and may even play a role in the spectrum of phenotypes observed as a consequence of FASD.

We also found that *elf3* mRNA is likely not maternally deposited. By using *sox2* as a positive control, it is clear that *elf3* is expressed at 4.5 hpf, immediately following ZGA. However, *elf3* message was not detected in the developing zebrafish embryo at 1.5 hpf, immediately before ZGA. Although some *elf3* transcriptional target genes were identified, these studies were largely done in adult tissues (Oliver, Kushwah et al. 2012). Other studies have shown that *elf3* is expressed immediately following fertilization in mice. Although these findings seem to contradict previous results, ZGA in mice occurs at the one cell stage. Therefore, *elf3* is not expressed previous to

fertilization and ZGA in mice but is expressed after. Here, we show that this is also true in zebrafish. Although ZGA occurs at a later time point, the expression pattern around ZGA appears to be similar to that observed in mice. This is an exciting finding as the conservation of this expression pattern across species further strengthens zebrafish as a model for studying embryonic development and FASD.

Using translation blocking morpholinos to knockdown *elf3* expression has given us greater insight into the developmental processes in which this transcription factor functions. Injection of this morpholino repeatedly produced a significant epiboly delay. Epiboly is a process, by which, the three cell layers (enveloping layer, deep cells and yolk syncytial layer) present in the zebrafish embryo 1 hour after the mid blastula transition spread and thin across the yolk, eventually completely enveloping it (Figure 8). At the beginning of epiboly, the yolk appears to bulge towards the animal pole in a process referred to as “doming”. Meanwhile, the deep blastomeres intercalate radially, moving towards the enveloping layer and yolk cell in the deep cell layer. Once doming is completed, the embryo has reached 50% epiboly and the deep cells begin involution (mesendoderm cells move under the ectoderm cells). Meanwhile, all three embryonic layers continue to spread over the yolk and towards the vegetal pole. A marginal band of actin forms in the yolk cell at the vegetal margin of these migrating cells. The purpose of this structure is to contract and assist in the closure of the blastopore (Holloway, Gomez de la Torre Canny et al. 2009). A few factors including Mitogen Activated Protein Kinase

Activated Protein Kinase 2 (*mapkapk2*) and E-Cadherin (*cdh1*) have been implicated in these critical movements. Mutants or knockdown of these genes result in significant epiboly delays. If the epiboly delay is too severe, the actin band begins to squeeze the embryo and it bursts the yolk cell, destroying the developing embryo (Babb and Marrs 2004; Holloway, Gomez de la Torre Canny et al. 2009). These epiboly defects are seen in the *elf3* MO embryos. The actin band begins to contract before the embryo reaches 50% epiboly, resulting in a bulging of the yolk cell and an ovoid embryo shape. Additionally, epiboly is a measure of gastrulation progression. If epiboly is delayed, then it is likely that gastrulation is as well. Gastrulation delays have been shown to cause a variety of defects in both the patterning and development of a number of organs (Cooke 1985). Additionally, it has been shown that ethanol can induce a significant epiboly delay in zebrafish embryos (Sarmah, Muralidharan et al. 2013). We have shown here that Elf3 may play a role in epiboly movements during gastrulation. Therefore, it is possible that the observed epiboly delay in ethanol treated embryos is partially caused by the downregulation of *elf3*. However, more work must be done in order to further examine this complex relationship. Taken together, this work indicates that Elf3 is partially responsible for epiboly and gastrulation movements and may play a role in observed defects in these processes in a zebrafish FASD model.

Injection of the *elf3* MO also produces a severe tail deformity in a significant number of injected embryos. Although this phenotype does not appear in all of *elf3* MO



embryos, it does happen consistently. It is also important to note that this defect never appears in either the uninjected or injected control groups. The relatively low frequency of individuals with this defective phenotype could be due, in part, to complementation. Indeed, it has been previously hypothesized that the high conservation seen within the ETS domain could allow for other ETS transcription factors to act redundantly in most individuals injected with the *elf3* MO, producing the wild-type phenotype (Ng, Waring et al. 2002; Kageyama, Liu et al. 2006). This finding is especially interesting because *elf3* was not implicated in tail development in any other animals, making this a novel observation. Interestingly, a similar defect has been seen separately in both embryos overexpressing inhibitors of Wnt signaling as well as Bmp7 mutants. (Leyns, Bouwmeester et al. 1997; Wang, Krinks et al. 1997; Schmid, Furthauer et al. 2000; Imai and Talbot 2001; Agathon, Thisse et al. 2003; Thorpe, Weidinger et al. 2005). It has been established that both Wnt and Bmp are critical in establishing the zebrafish tail organizer (Agathon, Thisse et al. 2003). More specifically, Wnt8 seems to act through a transcription factor, Sp5l, to promote proper tail development (Thorpe, Weidinger et al. 2005). Interestingly, *sp5l* was one gene identified in our microarray that contains the Elf3 consensus sequence upstream of the transcription start site. Considering the *elf3* MO injected embryos display a similar phenotype to embryos in which Wnt signaling is inhibited, and both of these factors appear to act on *sp5l* (an important factor for tail development), it is possible that Elf3 is associated with Wnt signaling during tail

development. Further work must be done to examine whether there is any functional connection between Wnt signaling and Elf3 during this process.

Additionally, tail defects have been observed in the context of a zebrafish FASD model. It has been shown that ethanol exposure can induce a slight “kink” in the tail at 28 hpf; the severity of which appears to be dose-dependent (Sarmah and Marrs 2013). Our results suggest Elf3 is important in regulating tail development. Therefore, it is possible that the downregulation of Elf3 in response to ethanol is partially responsible for this observed tail defect. Further analysis must be done, however, to examine whether or not this is likely.

Injection of *elf3* MO appeared to cause defects in both epiboly and tail development. However, neither of these defects were rescued by injection of rat *elf3* mRNA. At first glance, this may mean that the defects produced by *elf3* MO injections are an artifact, and may not be a true result of *elf3* translation inhibition. It is also possible that the rat protein is not structurally similar to zebrafish Elf3, and the phenotypes observed were not necessarily due to unintended, off target effects. Although the DNA binding ETS domains of the rat and zebrafish Elf3 protein are highly similar (91.76% identity), the A/T Hook (52.17% identity) and Pointed (40% identity) domains are not as well conserved (fig. 8). Consequently, it is plausible that the amount of rat mRNA injected was unable to rescue the observed defects because the

transcribed protein was too dissimilar to that of the zebrafish and could not, therefore, participate in the necessary protein-protein and/or DNA binding interactions.

It is also likely that we did not inject enough rat *elf3* mRNA in order to rescue the observed defect. The amount of injected mRNA usually ranges from 50pg-1ng. We injected approximately 200pg of mRNA. Therefore, we injected a relatively low amount of what is considered acceptable with regards to amount of mRNA with the potential to rescue a given phenotype (Bill, Petzold et al. 2009). Additionally, we injected 4ng of *elf3* MO. Therefore the amount of mRNA and MO injected may not have been balanced enough to rescue the epiboly and tail defects. Further work must be done injecting more mRNA and/or less morpholino in an attempt to rescue the observed phenotype.

There is also some evidence that the MO we used could not produce off target effects by binding to other mRNA. The *elf3* MO binding sequence is twenty-five base pairs long. Any mRNA that contains a sequence similar enough (at least 21/25 bases in common) would, theoretically, be able to bind to the morpholino. After performing a Reference Sequence (RefSeq) RNA BLAST search, the transcript identified as containing a twenty five bp sequence that is most similar to the *elf3* MO binding sequence was *isl lim homeobox (isl1)* mRNA. This sequence shared seventeen out of twenty five base pairs with the *elf3* MO binding sequence. As this sequence does not

have the minimum of twenty-one bases in common with the first twenty-five bases of the *elf3* transcript, it is unlikely to bind the morpholino. Considering this sequence is the most similar to the *elf3* MO binding sequence out of all the zebrafish mRNA sequences in the database, it is unlikely that any endogenous zebrafish mRNA would be able to bind to the morpholino other than *elf3* mRNA. Although *elf3* MO injection produced an observable epiboly and tail formation defects, more research will be needed to validate that knockdown of *elf3* is indeed responsible for these phenotypes.

As the tail is not the only tissue beginning to form at the 24 hpf stage when this defect was seen, we thought it important to look for phenotypic defects in the other developing tissues at this time point. After thorough examination of the forming brain and eyes, we found no observable defect. Interestingly, the transcription factor *rarab*, which was identified as a potential *elf3* target in our microarray and was found to be significantly upregulated according to qPCR, is involved in midbrain-hindbrain boundary formation (Linville, Radtke et al. 2009). Even considering this relationship, no defect was observed in the brain. The eyes were also examined for potential defects as they are also developing at this time point, but no obvious defect was observed.

Taken together, these results implicate Elf3 in a variety of developmental processes. Multiple Elf3 target genes are dysregulated in response to ethanol, some of which

have been implicated in important developmental processes. Additionally, we established that *elf3* transcript is not maternally deposited, and is likely only expressed following ZGA. We also showed that injection of an *elf3* morpholino produces both epiboly and tail defects. These results are both novel and exciting. More work needs to be done, however, to confirm whether *elf3* is involved in these developmental processes and how it functions. Additionally, whether or not *elf3* is partially responsible for similar phenotypes in the zebrafish FASD model is a question that warrants further examination. Overall, however, this work provides insight into a transcription factor whose function is not well known, but is potentially a major player in multiple developmental processes and disease, specifically Fetal Alcohol Spectrum Disorder.

## LIST OF REFERENCES

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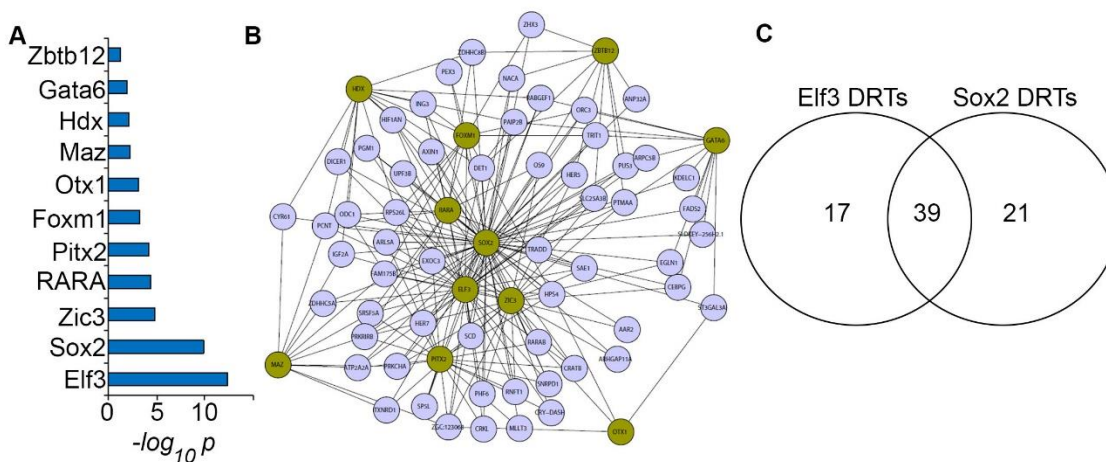


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## FIGURES



**Figure 1. Elf3 shows the most interactions out of 11 transcription factors at 4.5 hpf.** (A) 11 transcription factors showed significant with other genes in our ethanol dysregulated dataset, which is represented using hypergeometric probability (B; gold circles, transcription factors; blue circles, other factors). (C) Elf3 and Sox2 share 39 targets but exclusively bind to 17 and 21 targets respectively in the ethanol dysregulated dataset.

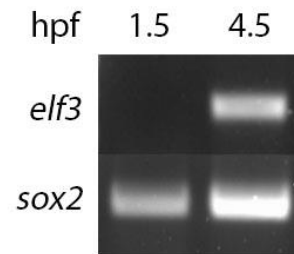
|      |  |   |                  |
|------|--|---|------------------|
| -119 |  | TTAGAGCCGGGTAGGGGAGC  | -100             |
|      |  | ***   |                  |
| -99  | GCAGCGGGCCAGATACCTCAGCGCTACCTGGCGGAACCTGGATTTCTCCCGCCTGCCGGCTGCCTGCCACAGCCGGACTCCGCCACTCCGGTAGSCCTC    | -1  |                  |
| 1    | ATG GCT GCA ACC TGT GAG ATT AGC AAC ATT TTT AGC AAC TAC TTC AGT GCG ATG TAC AGC TCG GAG GAC TCC ACC    | 75  |                  |
| 1    | M A A T C E I S N I F S N Y F S A M Y S S E D S T  | 25  |                  |
| 76   | CTG GCC TCT GTT CCC CCT GCT GCC ACC TTT GGG GCC GAT GAC TTG GTA CTG ACC CTG AGC AAC CCC CAG ATG TCA    | 150   |                  |
| 26   | L A S V P P A A T F G A D D L V L T L S N P Q M S  | 50  |                  |
| 151  | TTG GAG GGT ACA GAG AAG GCC AGC TGG  | 225   |                  |
| 51   | L E G T E K A S W  | 75  |                  |
|      |  | TTG GGG GAA CAG CCC CAG TTC TGG TCG AAG ACG CAG GTT CTG GAC TGG                         | 225              |
|      |  | L G E Q P Q F W S K T Q V L D W   | 75               |
| 226  | ATC AGC TAC CAA GTG GAG AAG AAC AAG TAC GAC GCA AGC GCC ATT GAC TTC TCA CGA TGT GAC ATG GAT GGC GCC    | 300   |                  |
| 76   | I S Y Q V E K N K Y D A S A I D F S R C D M D G A  | 100   | <b>A</b>         |
|      |  |   | <b>(POINTED)</b> |
| 301  | ACC CTC TGC AAT TGT GCC CTT GAG GAG CTG CGT CTG GTC TTT GGG CCT CTG GGG GAC CAA CTC CAT GCC CAG CTG    | 375   |                  |
| 101  | T L C N C A L E E L R L V F G P L G D Q L H A Q L  | 125   |                  |
| 376  | CGA GAC CTC  | 450   |                  |
| 126  | R D L  | 150   |                  |
|      |  | ACT TCC AGC TCT TCT GAT GAG CTC AGT TGG ATC ATT GAG CTG CTG GAG AAG GAT GGC ATG GCC TTC | 450              |
|      |  | T S S S S D E L S W I I E L L E K D G M A F   | 150              |
| 451  | CAG GAG GCC CTA GAC CCA GGG CCC TTT GAC CAG GGC AGC CCC TTT GCC CAG GAG CTG CTG GAC GAC                | 525   |                  |
| 151  | Q E A L D P G P F D Q G S P F A Q E L L D D  | 175   |                  |
|      |  | GGT CAG CAA   | 525              |
|      |  | G Q Q   | 175              |
| 526  | GCC AGC CCC TAC CAC CCC GGC AGC TGT GGC GCA GGA GCC CCC TCC CCT GGC AGC TCT GAC                        | 600   |                  |
| 176  | A S P Y H P G S C G A G A P S P G S S D  | 200   | <b>ESE-1b</b>    |
|      |  | gTC ACC GCA GGG   | 600              |
|      |  | gTC ACC GCA GGG   | 200              |
| 601  | ACT GGT GCT TCT CGG AGC TCC CAC TCC TCA GAC TCC GGT GGA AGT GAC GTG GAC CTG GAT CCC ACT GAT GGC AAG    | 675   |                  |
| 201  | T G A S R S S H S S D G S D V D L D P T D G K  | 225   |                  |
| 676  | CTC TTC CCC AGC GAT GGT TTT CGT GAC TGC  | 750   |                  |
| 226  | L F P S D G F R D C  | 250   |                  |
|      |  | AAG AAG GGG GAT CCC AAG CAC GGG AAG CGG AAA CGA GGC CGG CCC                             | 750              |
|      |  | K K G D P K H G K R K R G R P   | 250              |
| 751  | CGA AAG CTG AGC AAA GAG TAC TGG GAC  | 825   |                  |
| 251  | R K L S K E Y W D  | 275   |                  |
|      |  | TGT CTC GAG GGC AAG AAG AGC AAG CAC GCG CCC AGA   | 825              |
|      |  | C L E G K K S K H A P R   | 275              |
|      |  | GGC ACC CAC CTG   | 825              |
|      |  | G T H L   | 275              |
| 826  | TGG GAG TTC ATC CGG GAC ATC CTC ATC CAC CCG GAG CTC AAC GAG GGC CTC ATG AAG TGG GAG AAT CGG CAT GAA    | 900   |                  |
| 276  | W E F I R D I L I H P E L N E G L M K W E N R H E  | 300   |                  |
| 901  | GGC GTC TTC AAG TTC CTG CGC TCC GAG GCT GTG GCC CAA CTA TGG GGC CAA AAG AAA AAG AAC AGC AAC ATG ACC    | 975   |                  |
| 301  | G V F K F L R S E A V A Q L W G Q K K K N S N M T  | 325   |                  |
| 976  | TAC GAG AAG CTG AGC CGG GCC ATG AGG TAC TAC TAC AAA CGG GAG ATC CTG GAA CGG GTG GAT GGC CGG CGA CTC    | 1050  |                  |
| 326  | Y E K L S R A M R Y Y Y K R E I L E R V D G R R L  | 350   |                  |
| 1051 | GTC TAC AAG TTT  | 1128  |                  |
| 351  | V Y K F  | 371   |                  |
|      |  | GGC AAA AAC TCA AGC GGC TGG AAG GAG GAA GAG GTT CTC CAG AGT CGG AAC TGA GGGTTGGAATCA    | 1128             |
|      |  | G K N S S G W K E E E V L Q S R N   | 371              |
|      |  | ***   |                  |
| 1129 | TACCCTGGACCAAACTCAGGACCCTCGAGGCCCTCGAAACCTTCCTGGGAGGACAGGCAGGCCAGATGCCCTCCACTGGGAAATGCTCCAGCTGTG       | 1228  |                  |
| 1229 | CTGTGGAGAGAAGCTGATGTTTGGTGTATTGTCCAGCCATCGTCTCGGACTCGGAGACTATGGCCCTCGCCTCCCCACCCCTCCTCTTGGAAATTACAAGCC | 1328  |                  |
| 1329 | CTGGGGTTTGAAGCTGACTTTATAGCTGCAAGTGTATCTCCTTTATCTGGTGCCTCCTCAAACCCAGTCTCAGACACTAAATGCAGACAAACACCTTCCT   | 1428  |                  |
| 1429 | CCTGCAGACACCTGGACTGAGCCAAAGGAGCCCTGGGGAGGCCCTAGGGGAGCACCCTGATGGAGAGGACAGAGCAGGGCTCCAGCACCTCTTCTTCTGGA  | 1528  |                  |
| 1529 | CTGGCGTTCCACCTCCCTGCTCAGTCTGGGCTCCACGGGAGGGGTCAGAGCACTCCCTAATTTATGTGCTATATAAATATGTCAGATGTACATAGAGA     | 1628  |                  |
| 1629 | TCTATTTTTTCTAAAACATTCCTCCCTCCCACTCCTCTCCACAGAGTGTGGACTGTCCAGGCCCTCCAGTGGGCTGATGCTGGGACCTTAGGATGGG      | 1728  |                  |
| 1729 | GCTCCAGCTTCCTTCTCCTGTAATGGAGGCAGAGACCTCCAAATAATGTCCTTCTGGGCTTTTCT                                      | 1796  |                  |

**Figure 2. Complete nucleotide sequence and predicted amino acid sequence of ESE-1.** The nucleotide sequences of human ESE-1a and ESE-1b, with the deduced amino acid sequence (one-letter code) of the major open reading frame, are shown. Nucleotides are numbered at the right; amino acids are numbered at the left. The alternative (alt.) 69-bp exon of ESE-1b inserted into the central portion of ESE-1a is boxed by a dashed line and shaded. The Pointed domain, the ETS domain, and the A/T hook domain are boxed, shaded, and marked on the right. The termination codons in frame with the reading frame upstream and downstream are indicated by asterisks. The putative polyadenylation sequence, AATAAA, close to the polyadenylated 3' end of the mRNA is double underlined. The ATTTA motif involved in mRNA turnover is underlined.

Oettgen, P., R. M. Alani, et al. (1997). "Isolation and characterization of a novel epithelium-specific transcription factor, ESE-1, a member of the ets family." *Mol Cell Biol* 17(8): 4419-4433.

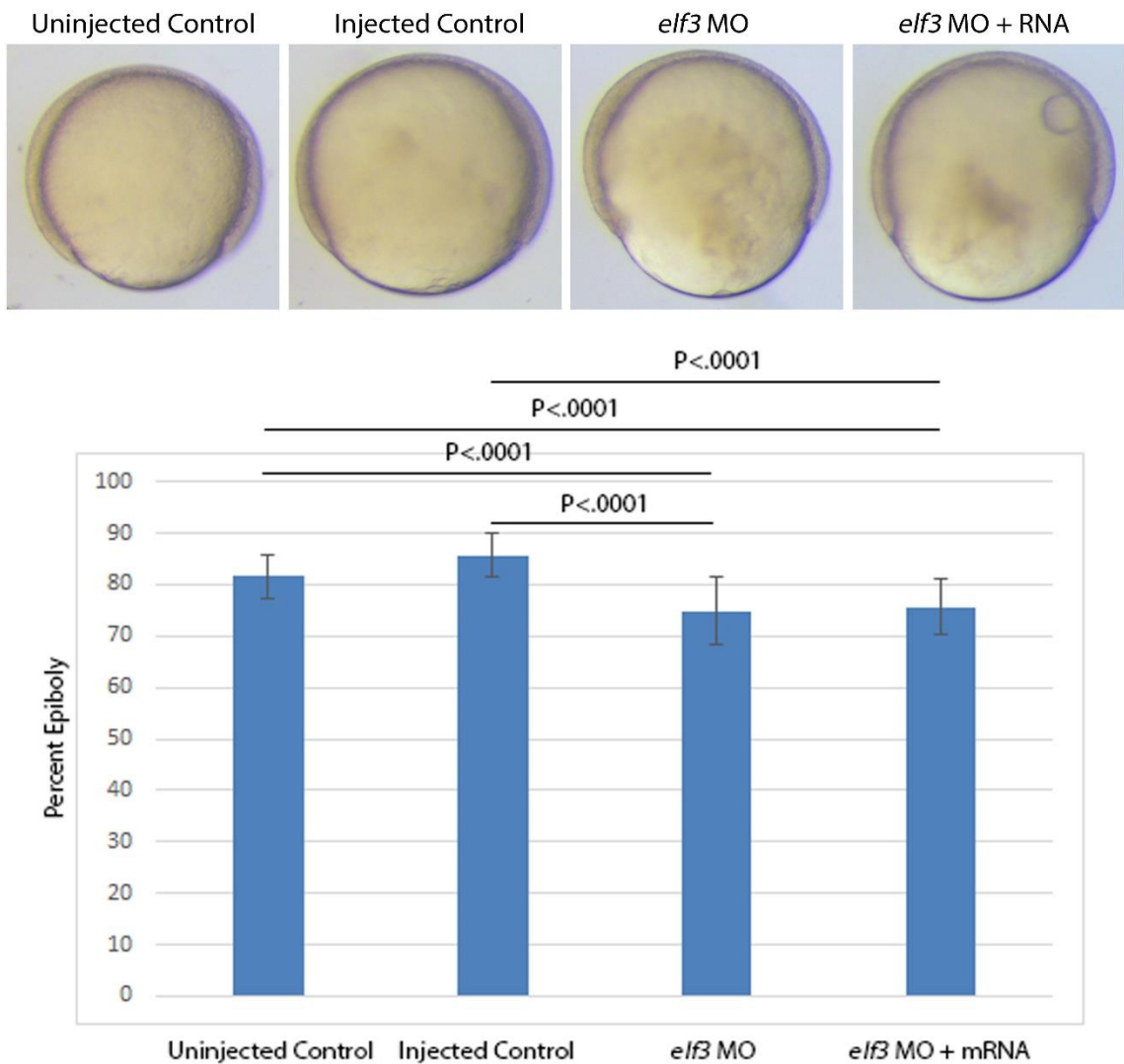
| <u>Gene Name</u> | <u>Up or Down</u> | <u>Fold change in<br/>Microarray</u> | <u>Fold change in<br/>RT-PCR</u> |
|------------------|-------------------|--------------------------------------|----------------------------------|
| <i>foxm1</i>     | Up                | 1.25                                 | 1.42                             |
| <i>her5</i>      | Up                | 1.21                                 | 1.43                             |
| <i>krt23</i>     | Down              | -1.55                                | -3.33                            |
| <i>zgc123063</i> | Down              | -1.25                                | -2.23                            |
| <i>rarab</i>     | Up                | 1.46                                 | 1.53                             |
| <i>slc16a9b</i>  | Up                | 5.9                                  | 1.27                             |
| <i>rnft1</i>     | Up                | 1.49                                 | 1.24                             |

**Fig 3. Potential Elf3 Targets are Dysregulated in Response to Ethanol Exposure at 4.5 hpf.** Out of the 56 putative Elf3 targets that were dysregulated in response to ethanol exposure, 7 were tested for dysregulation by using RT-PCR. Five of these genes (*foxm1*, *her5*, *rarab*, *slc16a9b*, and *rnft1*) were found to be upregulated while the remaining two (*krt23*, and *zgc123063*) were found to be downregulated in response to ethanol exposure.

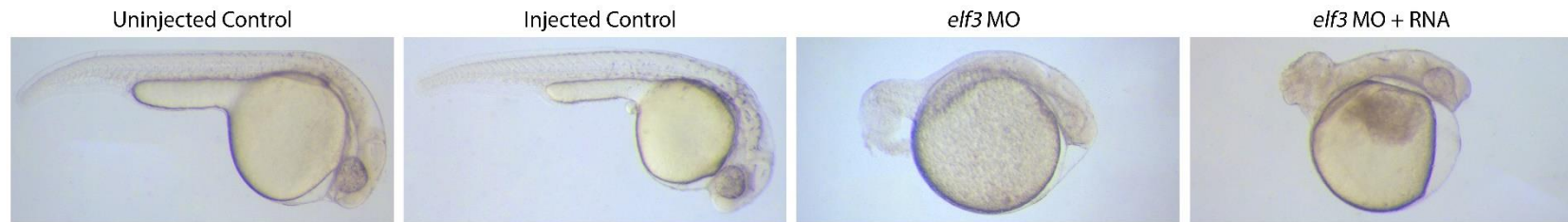


**Fig 4. Elf3 mRNA is not maternally deposited.** *elf3* mRNA was detected at 4.5 hpf but not at 1.5 hpf while *sox2* mRNA was detected at both of these time points.

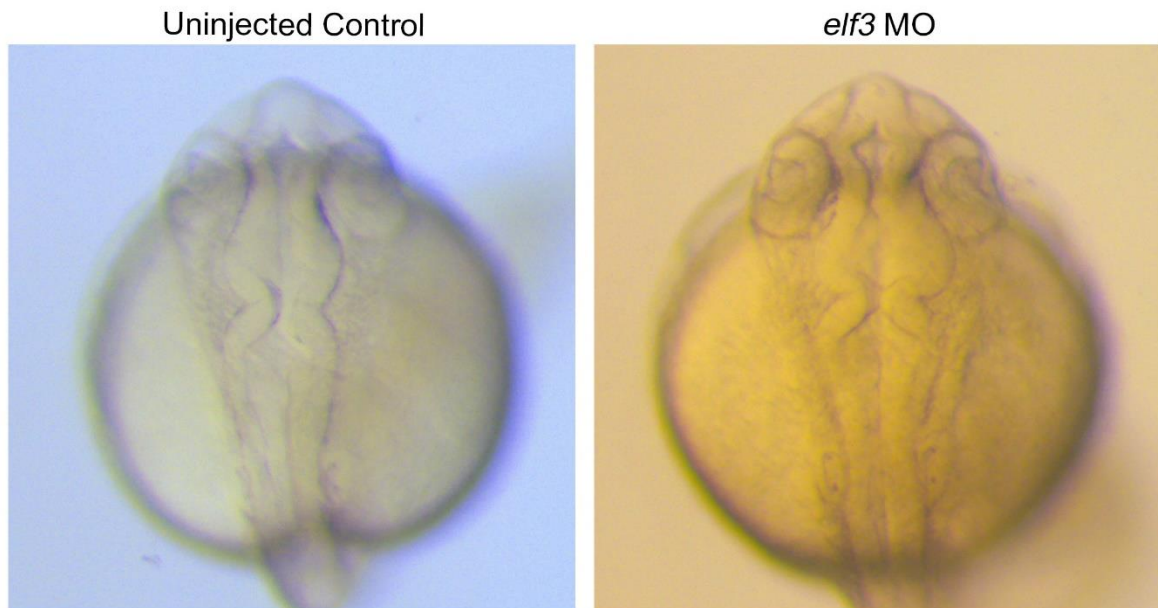




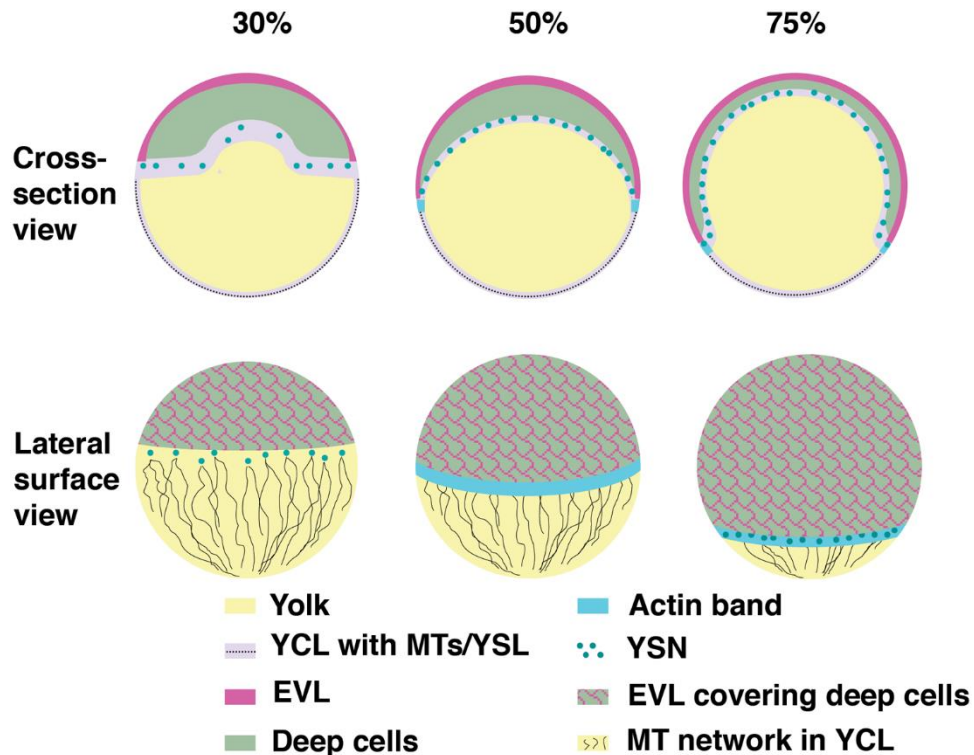
**Fig 5. *elf3* morpholino injection results in epiboly delay.** Percent epiboly of each embryo was measured at 8.3 hpf and averaged for each group. The average percent epiboly was significantly less in both the *elf3* MO group and *elf3* MO + mRNA groups when compared to both the uninjected and injected control groups.



**Fig. 6 *elf3* Morpholino Injection Results in a Defect in Tail Formation** A small number of embryos injected with *elf3* MO displayed tail development defects at 24 hpf. This defect was not seen in either the uninjected or injected control groups. This defect was not rescued by injection of rat *elf3* mRNA.



**Figure 7.** *Elf3* MO injections cause neither brain nor eye defects at 24 hpf. The eyes and brains of both uninjected and *elf3* MO injected embryos were examined and compared. Both developing organs appeared to be developing normally in both groups of embryos.



**Figure 8. Schematic of epiboly progression.** Epiboly begins when the yolk (yellow) domes toward the animal pole (top), concomitant with radial intercalation of the deep cells of the blastoderm (green) to cover 30% of the embryo surface. At 30% epiboly, the yolk syncytial nuclei (YSN, dark-green circles) are maintained within the yolk syncytial layer (YSL) beneath the blastoderm and are associated with microtubules (black lines, surface view) that are oriented toward the vegetal pole within the very thin cortical, yolk cytoplasmic layer (YCL, light blue in cross-section). The enveloping layer (EVL, pink) forms a thin epithelial-like sheath on the surface of the deep cells and is connected to the YSL at the cell margin. The YCL cannot be distinguished from the yolk when viewed from the lateral surface. At 50% epiboly, the EVL and deep cells cover 50% of the embryo and lie more vegetally than the YSN, masking the YSN in the lateral surface view. An actin band (turquoise) forms in the YSL just vegetal to the margin at 50% epiboly and likely functions as a contractile mechanism to close the blastopore throughout the remainder of the epiboly process. By 75% epiboly, the YSN are located beneath the blastoderm, as well as vegetal to it (surface view). The blastopore is the exposed vegetal yolk, which continually decreases in circumference as epiboly progresses.

Holloway, B. A., S. Gomez de la Torre Canny, et al. (2009). "A novel role for MAPKAPK2 in morphogenesis during zebrafish development." *PLoS Genet* 5(3): e1000413.

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Whole protein: 47.19% identity

Zebrafish  -MASSELSLILNN-----ANANLYPEPQASLLSMCRPPHLSEITFNS 41
Rat        MAATCEISNVFSNYFNAMYSSDPTLAPAPLTTFGTEDFVLTLLNQHMSP 50
          *:.*:* :.*      :.* * * * : :      *.: :..

Zebrafish  NSTLG-----SWDQVNPQMWSRQNVLEWIG 66
Rat        EGPVGCVPQTRSQRDRTEPPAVLHLAEKASWTGERPQFWSKTQVLDWIS 100
          :..:*          **  .**:**: **:**.

Zebrafish  FHVEESRFDAGLLNLYCTMDGLTLCATSKEALMSMFGPELGNLLHHSLE 116
Rat        YQVEKNKYDASSIDFSRCDMDGATLCNCALEELRLVFGP-LGDQLHAQLR 149
          :**.:**.* :.:. * ** * * * : * * :** * * : * .*.

Zebrafish  SLKARYVNDLSENTVLSESEFELLDNQLNSFSPMHGPGGLPLETVVVQ 166
Rat        DLTSSSDDELWIIELLEKDGMTFQEGLDGSGPFDQGS---PPAQELDD 196
          .*.: :*** * *.: : : : * * * * .*. * : : : :

Zebrafish  STIPTDNSDAKYSYFDEYTSQLTPESDHGYDSLTFQSSHAGSFLNPSS 216
Rat        GRQASPYYGSSYG-----PGAPSPGSSDFSTSGTDTTPQSSHS----- 233
          . .: :.:. * . . : * *.. * * : : ** * :

Zebrafish  PESNSSDSDPEYSETPYSSISS--KNFSKQPEKTKRGRGRPPKLRDSDGF 264
Rat        SDSGGSVDLDLTDKVFPRDGFDPYKKEPKHGKRKRGRPKL--SKEY 281
          .:*..** * : : : . : :.* ** : * * * * * * * . * :

Zebrafish  DHFIQSKKSKHAPRGTHLWEIFIRDILIHPEQNQGLMKWEDRRDGVFKFLK 314
Rat        WDCLEGGKSKHAPRGTHLWEIFIRDILIHPELNEGLMKWENRHEGVFKFLR 331
          . : : .***** * :*****.:*****:

Zebrafish  SEAVAQLWGQKKKNSMTEYKLSRAMRYYYKREILERVDRRLVYKFGKN 364
Rat        SEAVAQLWGQKKKNSMTEYKLSRAMRYYYKREILERVDRRLVYKFGKN 381
          *****

Zebrafish  STGWRIETGY--- 375
Rat        SSCWKEEVGESQN 395
          *:*: **.*

Pointed Domain: 40% identity

Zebrafish  -----SWDQVNPQMWSRQNVLEWIGFHVEESRFDAGLLNLYCTMDGLTLCATSKEAL 53
Rat        LHLAEKASWTGERPQFWSKTQVLDWISYQVEKNKYDASSIDFSRCDMDGATLCNCALEEL 60
          **  .**:**: **:**.*****: :.:. * ** * * * : * *

Zebrafish  MSMFGPELGNLLHHSLESLSKA- 74
Rat        RLVFGP-LGDQLHAQLRDLTSS 81
          :** * * : * .*.:.

AT Hook Domain: 52.17% identity

Zebrafish  KQEPKTKRGRGRPPKLRDSDGFD- 24
Rat        KGEKHKGRKRGRPKLS-KEYWDC 24
          * ** : * * * * * * . : : *

Ets Domain: 91.76% Identity

Zebrafish  APRGTHLWEIFIRDILIHPEQNQGLMKWEDRRDGVFKFLKSEAVAQLWGQK 50
Rat        APRGTHLWEIFIRDILIHPELNEGLMKWENRHEGVFKFLRSEAVAQLWGQK 50
          ***** * :*****.:*****:*****

Zebrafish  KKNSMTEYKLSRAMRYYYKREILERVDRRLVYK 85
Rat        KKNSMTEYKLSRAMRYYYKREILERVDRRLVYK 85
          *****

elf3 MO Binding Site

Zebrafish  ATGGCGTCAAGCGAACTTAGTCTAA
Rat        ATGGCTGCCACCTGTGAGATCAGCA
          ***** * * * * * *

```

**Figure 9. The zebrafish and rat *Elf3* protein are dissimilar.** ClustalW2 software was used to align the rat and Zebrafish *elf3* proteins. The A/T Hook, Pointed and ETS domains were also aligned in a similar manner. “\*” represent a shared amino acid while “:” represents two different, yet highly similar amino acids. The *elf3* MO binding site was also aligned to ensure that the morpholino would not bind to the rat *elf3* mRNA.