OVERLAPPING FUNCTIONS OF PEA3 ETS TRANSCRIPTION FACTORS IN FGF SIGNALING DURING ZEBRAFISH DEVELOPMENT

by

Wade A. Znosko

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This thesis was presented

by

Wade A. Znosko

It was defended on

June 1, 2010

and approved by

Deborah Chapman, Ph.D., Associate Professor, Department of Biological Sciences

Jeffrey Brodsky, Ph.D., Professor and Avinoff Chair, Department of Biological Sciences

Gerard Campbell, Ph.D., Associate Professor, Department of Biological Sciences

Philip Auron, Ph.D., Professor and Chair, Department of Biological Sciences, Duquesne University

Thesis Advisor: Michael Tsang Ph.D., Assistant Professor, Department of Developmental Biology

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Fibroblast Growth Factors (FGFs) are secreted molecules that activate the RAS/mitogen-activated protein kinase (MAPK) signaling pathway to establish dorsal polarity, maintain the isthmic organizer, and assure proper ventricle formation in the zebrafish. The mechanism of FGF regulation of these processes and the transcription factors involved are still unclear. Expression of the zebrafish PEA3 family of ETS transcription factors, *Etv5*, *Erm,* and *Pea3,* is responsive to FGF signaling, and these factors are likely transcriptional effectors of this pathway. I have determined the role of PEA3 ETS factors in FGF signaling and gene regulation through gain- and loss-of-function studies. Ectopic expression of a constitutively activated form of Etv5 induced FGF target transcripts, *dual specificity phosphatase 6* (*dusp6*) and *similar expression to fgfs (sef).* The simultaneous knock-down of *Etv5*, *Erm*, and *Pea3* produced phenotypes reminiscent of the *fgf8* mutant, including the disruption of the mid-hindbrain boundary, diminished cardiac progenitors, and left/right patterning defects. Furthermore, the expression of FGF target genes was abolished in Etv5/Erm/Pea3 depleted embryos. To understand how FGF signaling and PEA3 ETS factors control gene expression, the transcriptional regulation of *dusp6* was studied in mouse and zebrafish. Conserved Pea3/ETS binding sites were identified within the *dusp6* promoter, and reporter assays show that one of these sites is required for *dusp6* induction by FGFs in both species. In addition, I demonstrated the interaction of PEA3 ETS

factors with the *dusp6* promoter both *in vitro* and *in vivo*. These results revealed the requirement of ETS factors in transducing FGF signals in developmental processes.

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PREFACE

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1.0 INTRODUCTION

The fundamental question in developmental biology involves determining how a single, undifferentiated fertilized egg can undergo changes that require cell proliferation, differentiation, migration, and apoptosis to ultimately form a multi-cellular organism with several types of tissues, organ systems, and functionality. These processes involve a variety of different signaling cascades and transduction pathways to insure proper development. To understand the role of these transduction pathways, we must look at the function of individual components of these cascades. Utilizing zebrafish as a model system, my research focuses on the importance of FGF signaling, and more specifically the activation and function of ETS transcription factors within this pathway to lead to proper zebrafish development.

1.1 THE FIBROBLAST GROWTH FACTOR (FGF) SIGNALING PATHWAY

Fibroblast growth factors (FGFs) are a family of 22 small polypeptide growth factors (in humans) that are critical for proper development. Of these, 18 are true secreted ligands (Furthauer et al., 2004; Itoh, 2007; Ornitz and Itoh, 2001; Sekine et al., 1999; Sun et al., 1999). FGFs have been identified in both invertebrates and vertebrates, ranging from nematodes to humans, but have not yet been identified in unicellular organisms such as *Escherichia coli* or *Saccharomyces cerevisiae* (http://ncbi.nlm.nih.gov/Genbank/idex.html). In invertebrates,

Drosophila have only 3 *Fgf*s, and only 2 have been discovered in *C. elegans* (Itoh and Ornitz, 2004). In contrast, among vertebrates, a large number of *Fgf* genes have been identified. Zebrafish and *Xenopus* have 10 and 6 *Fgf*s respectively, while mice and humans both have 22 genes (Itoh and Ornitz, 2004). This expansion of *Fgf* genes has been hypothesized to occur simultaneously with a phase of global gene duplications that took place during the emergence of vertebrates (Coulier et al., 1997). Across species, most orthologous FGF proteins have a high degree of conservation (>50% amino acid sequence identity), and can be classified into seven groups or subfamilies that share sequence similarity combined with biochemical and developmental properties. All subfamilies share domain similarity and have a high affinity for heparin (Maruoka et al., 1998; Xu et al., 2000).

FGF ligands bind to a family of transmembrane protein tyrosine kinase receptors (FGFRs), all of which contain a heparin-binding sequence and extracellular immunoglobulin-like (Ig) domains. The Ig domains are connected by a single pass transmembrane region to a cytoplasmic tyrosine kinase domain that serves as the intracellular signal transducer (Johnson et al., 1990; Lee et al., 1989; Ullrich and Schlessinger, 1990). Four mammalian FGFR genes give rise to a large number of receptor isoforms due to alternative splicing of pre-messenger RNAs, or by the expression of different FGFR genes. This process regulates the number of Ig domains (two or three) and the specific sequence of Ig domain III (IIIa, IIIb, and IIIc isoforms) (Ornitz, 2000; Powers et al., 2000). Ornitz *et al.* (1996) determined the specificity of different FGFs for receptor isoforms by overexpressing these isoforms in Baf3 cells, a murine bone marrow derived cell line which does not normally express FGFRs. The results indicated that diversity in FGF signaling is obtained by different FGFs binding to different receptor splice variants and different *fgfr* gene products. All FGFRs exist as inactivated monomers, only activated when two

molecules of FGF bind to the Ig domains of the receptor, leading to homodimerization. This dimerization allows the intracellular domains to come together, causing transautophosphorylation of critical tyrosine residues (Schlessinger et al., 2000) (**Figure 1A**).

FGFRs, as all receptor tyrosine kinases, transfer extracellular signals to various cytoplasmic signal transduction pathways via tyrosine phosphorylation. After ligand binding and dimerization at the cell membrane, the receptor is capable of phosphorylating specific tyrosine residues on their own and each other's cytoplasmic tails (Lemmon and Schlessinger, 1994). Phosphorylated tyrosine residues, in turn, recruit other signaling molecules to the activated receptors to propagate the signal through a variety of possible transduction pathways (Pawson, 1995) (**Figure 1 A**). The activated tyrosine kinase receptor recruits target proteins of signaling cascades to its cytoplasmic tail and modifies them by phosphorylation. Several signaling cascades are activated in this way, including the phospholipase C gamma (PLC-γ), phosphatidylinositol-3 kinase (PI3K) which activates Akt/protein kinase B, and the rat sarcoma homologue (RAS)/mitogen-activated protein kinase (MAPK) cascade. Within this signaling arm, phosphorylation of a MAPK protein, *extracellular signal-regulated protein kinase* (ERK), results in the ability of ERK to enter the nucleus, and modify transcription factors, thus leading to gene expression (**Figure 1 A**) (Dailey et al., 2005; Powers et al., 2000; Tsang and Dawid, 2004).

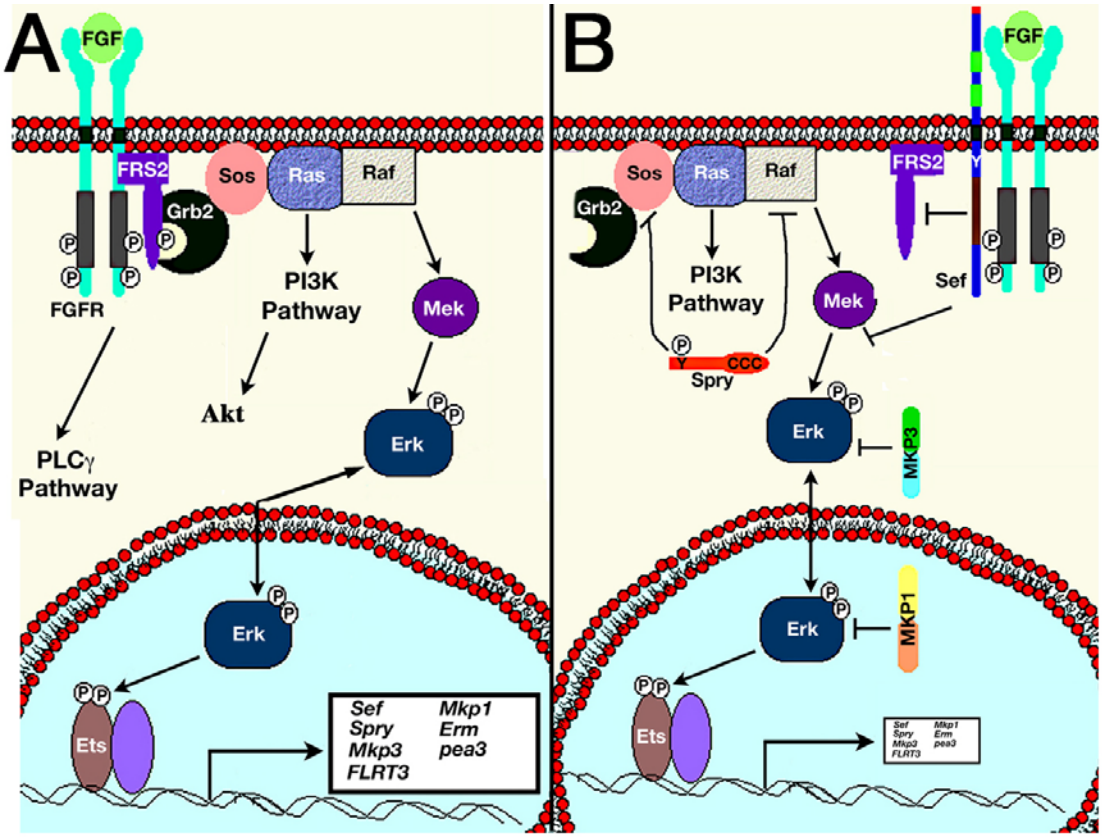

Figure 1: FGF Signal Transduction Pathway.

(A) Schematic representation of the major signal transduction pathways activated upon FGF ligand binding to the transmembrane receptor. **(B)** Feedback attenuators of the FGF pathway, including Spry, Sef, Mkp1 (also known as Dusp1), and Mkp3 (also known as Dusp6), function to limit FGF target gene expression in the FGF/RAS/MAPK pathway.

1.2 THE REGULATION OF FGF SIGNALING

Due to FGF signaling influencing multiple transduction cascades, tight control of the signal is essential to regulate the many FGF-mediated developmental processes (Thisse and Thisse, 2005; Tsang and Dawid, 2004). To limit FGF signaling, several feedback attenuators have been identified within this pathway. Negative feedback regulators, such as the *Sprouty* (*Spry*) family

of genes, *Dual Specificity Phosphatase 6* (*Dusp6*), and *Similar Expression to FGFs* (*Sef*) function to regulate the RAS/MAPK signaling arm of the FGF pathway at multiple points (Furthauer et al., 2002; Furthauer et al., 2001; Hacohen et al., 1998; Tsang et al., 2002; Tsang et al., 2004) (**Figure 1 B**). Spry proteins are evolutionarily conserved and contain four members (Spry1-4) in mammals (Dikic and Giordano, 2003). Regulated by tyrosine phosphorylation on their invariant tyrosine phosphorylation site (Tyr55), Spry proteins have been shown to be both induced by and antagonize FGF signaling (Furthauer et al., 2001; Furthauer et al., 2004; Mailleux et al., 2001; Minowada et al., 1999; Nutt et al., 2001). In studies using both mouse (Spry2) and *Xenopus* (Xspry1) protein, association was identified between the Spry protein and the SH2 domain of Grb2 after FGFR-induced phosphorylation of Spry at Tyr55 (Hanafusa et al., 2002). Due to this, Grb2 can no longer interact with FRS2 (Fibroblast Growth Factor Receptor Substrate 2) and the transducing FGF signal is repressed (**Figure 1 B**). In addition, Spry2 and Spry4 have been shown to interact with Raf through a motif in the C-terminal domain, thus indicating multiple levels of functional inhibition by Spry proteins within the FGF pathway (**Figure 1B**).

Dusp6 is a member of a family of phosphatases that specifically inactivates phosphorylated forms of ERK (Farooq et al., 2001; Muda et al., 1996). Since *Dusp6* itself is regulated via RAS/MAPK signaling, studies in mouse and zebrafish have indicated that they represent feedback modulators of FGF signaling (Eblaghie et al., 2003; Kawakami et al., 2003; Tsang et al., 2004). In chick, ectopic expression of *Dusp6* in the limb bud results in limb outgrowth disruption, a characteristic phenotype of blocking FGF signaling (Eblaghie et al., 2003; Kawakami et al., 2003). In addition, *Dusp6* limits FGF activity in the zebrafish embryo, resulting in a disruption of dorsal-ventral polarity (Tsang et al., 2004).

Similarly, *Sef* protein, conserved among zebrafish, mouse, and human, functions as an antagonist of FGF signaling by interfering with FGF signal transduction at the level of MEK (Furthauer et al., 2002), and/or FGFR1/2 (Kovalenko et al., 2003; Tsang et al., 2002; Xiong et al., 2003). Ectopically expressing *sef* leads to a ventralized phenotype in zebrafish, consisting of a reduced tail and cyclopia, opposite of phenotypes caused by ectopic expression of *fgf8*, indicating the role of *Sef* as an antagonist to FGF signaling (Furthauer et al., 2002; Tsang et al., 2002).

In addition, several positive regulators of the FGF pathway have been studied. XFLRT3 is a transmembrane protein induced after activation of FGF signaling and down-regulated after inhibition of this pathway. In gain- and loss-of-function studies in *Xenopus*, FLRT3 was shown to mimic FGF signaling functions, thus indicating a positive regulatory role for this protein (Bottcher et al., 2004). PEA3 ETS (E26 transformation-specific) factors are thought to function as transcriptional regulators of the FGF pathway that allow proper signaling levels to be reached and maintained during development (Munchberg et al., 1999; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). Due to the importance of the correct regulation of FGF signaling during embryogenesis, the proper balance of both positive and negative feedback attenuators are necessary to achieve proper signaling levels in time and space during development.

1.3 THE ROLE OF FGF SIGNALING IN DEVELOPMENT AND DISEASE

1.3.1 FGF Signaling in Human Genetic Disorders

The roles of FGF signaling in human development and disease have been intensely studied. Specific mutations among the FGFRs have been involved in multiple genetic disorders. In humans, point mutations within the critical Ig domains of FGFR1, FGFR2, and FGFR3 are associated with the development of Apert, Crouzon, and Pfeiffer syndromes, resulting in premature closure of the joints in the skull, which inhibits proper brain formation and growth (Chen and Deng, 2005; Coumoul and Deng, 2003). Unique to Apert syndrome, patients also display syndactyly of the limbs. This dominantly acting mutation involves specific mutations on two adjacent residues on FGFR2, S252 and P253, an area that lies in the linker region between IgII and IgIII (Bellus et al., 1996; Park et al., 1995; Wilkie et al., 1995). Although the ligands bind to the receptor under the correct stoichiometry (2:2), there is a decrease in the dissociation kinetics of FGFR2, leading to activation of FGF signaling under conditions where availability of ligand binding is limited (Anderson et al., 1998). Interestingly, a subset of patients (10%) diagnosed with Apert syndrome also developed cardiovascular abnormalities, including a narrowing of portions of the aorta, resulting in death (Cohen and Kreiborg, 1993; Skidmore et al., 2003). Furthermore, Cardio-facio-cutaneous (CFC) syndrome is due to mutations downstream in the FGF pathway. Again, this defect is associated with craniofacial and atrial malformations as a result of missense mutations in KRAS, MEK1, and MEK2 (Niihori et al., 2006; Rodriguez-Viciana et al., 2006). Further investigations into these mutations revealed a hyperactivation within the FGF signaling pathway due to a hyper-phosphorylation of ERK (Niihori et al., 2006; Rodriguez-Viciana et al., 2006).

1.3.2 FGF Signaling in Axis Formation

In addition, the importance of FGF signaling in multiple developmental processes including axis formation and limb morphogenesis is highly conserved across other species. Early during development, evidence suggests maternal β-catenin initially established the dorsal-ventral (D/V) axis among vertebrates. In concert with the Wnt signaling pathway, β-catenin is prevented from being sequestered to the cytoplasm and can accumulate in the nucleus within the presumptive dorsal region of the embryo. This asymmetric nuclear localization of β-catenin is the earliest marker of the D/V axis (Dougan et al., 2003; Schneider et al., 1996). Soon after the mid-blastula transition, β-catenin activates the expression of a number of zygotic genes, including *bozozok*, *chordin*, *squint*, and FGF signals within dorsal blastomeres (Dougan et al., 2003; Fekany et al., 1999; Feldman et al., 1998; Furthauer et al., 2004; Kelly et al., 2000; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). This induction and patterning of the mesoderm was one of the earliest events in which FGF signaling was found to be essential. In *Xenopus*, FGF was shown to induce mesoderm formation, and when inhibiting components of the FGF pathway, mesoderm formation was blocked, inducing gastrulation and posterior defects (LaBonne et al., 1995; MacNicol et al., 1993; Umbhauer et al., 1995; Whitman and Melton, 1992). Furthermore, zebrafish have been extensively studied to address the involvement of FGFs in establishing the D/V axis. BMP acts as a morphogen secreted ventrally within the developing zebrafish embryo to specify ventral cell fates. This restricted ventral expression coincides with FGF activity from the dorsal side of the embryo, suggesting FGF signaling is implicated in the dorsal down-regulation of *BMP* gene expression (Schmid et al., 2000). Consistent with this, general activation of FGF signaling within the entire embryo inhibits *BMP* gene expression in the whole

blastula, and inhibition of FGF signaling causes *BMP* gene expression to expand dorsally (Furthauer et al., 2004). Therefore, it can be concluded that FGFs act upstream of ventral morphogens and are one of the initial signals for establishing D/V patterning.

1.3.3 FGF Signaling in Limb Development

Evidence has also been shown that FGF signaling plays a direct role in limb initiation and morphogenesis. Initial limb development is described as the formation of a bud, containing lateral plate mesoderm (LPM) cells and the overlying surface ectoderm. The cells within this bud proliferate and eventually give rise to the skeletal framework and connective tissue of the mature limb, whereas the muscle within this limb is derived from migrating cells from the somites (Chevallier et al., 1977; Christ et al., 1977). Limb induction is initially triggered by a combination of FGF8 and FGF10, where FGF8 induces the formation of the apical ectodermal ridge (AER). This region of cells undergoes cell proliferation and outgrowth as a result of FGF2/4/8 signaling in concert with *sonic hedgehog* (Crossley et al., 1996; Fallon et al., 1994; Laufer et al., 1994). Studies using mice further address the importance of FGFs during limb development. In the absence of FGF8 in mice, the proximal elements of the limb are reduced or completely absent. However, distal elements form normally; an indication that other FGFs are involved in this process (Lewandoski et al., 2000; Moon and Capecchi, 2000). Furthermore, in *FGF8* mutant mice, the total size of the limb bud is smaller. Since this phenotype is detected immediately following the onset of FGF8 expression, the possibility that FGF8 alters cell proliferation or cell death can be excluded, indicating instead that FGF8 affects morphogenetic movements and cell adhesion (Sun et al., 2002). Initial studies looking at another FGF ligand expressed within the limb bud, FGF4 indicated that knocking out this protein had no effect on

fore- or hindlimb formation (Moon et al., 2000; Sun et al., 2000). However, in additional studies where *fgf4* was expressed in place of *fgf8,* all of the skeletal defects caused by inactivation of *fgf8* are rescued, conclusively demonstrating that FGF4 can functionally replace FGF8 in limb skeletal development (Lu et al., 2006). This result reiterates the importance of FGF signaling in limb formation, and also the importance of the temporal/spatial specificity of FGFRs due to the multiple functionalities of FGF ligands.

1.3.4 FGF signaling in mid-hindbrain (MHB) formation in Zebrafish

Creation of a large number of different cell types within the nervous system requires both cell intrinsic programs and coordination between neighboring cells. Generating cell diversity with the vertebrate central nervous system begins as early in development as gastrulation. Specification of the individual parts of the zebrafish brain, the forebrain, midbrain, and hindbrain, occurs at this time in response to several signaling pathways including FGFs, retinoic acid, Nodals, and Wnt proteins (Wilson et al., 2002; Wilson and Rubenstein, 2000). Here, designated populations of cells exist in the neural plate that influence cell fate in surrounding neural plate cells. Following the establishment of the initial primordia, each brain region is thought to develop largely independently under the influence of local organizing centers. One of these organizing centers, the isthmic organizer (IsO), is located between the midbrain and rhombomere 1 (r1) of the hindbrain, a region commonly referred to as the mid-hindbrain boundary. Within this region, three of the four FGFRs, *Fgfr1*, *Fgfr2*, and *Fgfr3*, are expressed, with *Fgfr1* being the most diffuse (Blak et al., 2005; Trokovic et al., 2005; Walshe and Mason, 2000). Thus, inactivation of *Fgfr1* in mouse mutants causes the most dramatic effects on midbrain-r1 development (Blak et al., 2007). Furthermore, *Fgf8* is highly expressed in the most anterior of the hindbrain (Crossley

and Martin, 1995; Heikinheimo et al., 1994). Ectopic FGF8 can mimic an IsO tissue transplant, and can transform cell identity into isthmic, r1, or midbrain fate in the posterior hindbrain region (Crossley et al., 1996; Martinez et al., 1999). In chick, when two differentially spliced isoforms of FGF8, FGF8a and FGF8b, are misexpressed, expansion of the midbrain and transformation of the midbrain into cerebellum occurs, respectively (Sato et al., 2001).

In addition to defining the cell fates of the midbrain-r1 region, FGF signaling is also important for induction and patterning of adjacent brain units. When MHB tissue is transplanted in a more caudal region of the forebrain primordium, MHB markers are not only expressed in the in transplanted tissue, but also in the surrounding forebrain tissue (Bally-Cuif et al., 1992; Gardner and Barald, 1991; Martinez et al., 1991). Similarly, transplantation of the MHB cells into an area far removed from the brain region, such as the dorsal spinal chord, leads to an induction of a second cerebellum (Martinez et al., 1995). In *Fgfr1* mouse mutants, a coherent border between the cells of the midbrain and r1 is lost and the two populations appear to mix with one another. This phenotype is similar to embryos defective in heparin sulphate in the neuroectoderm, in which heparin in critical in allowing FGFR dimerization and phosphorylation (Inatani et al., 2003). The zebrafish *fgf8* mutant *acerebellar* (*ace*) can first be recognized at the 5-somite stage, where a thicker neural keel in the developing midbrain is formed, followed by a lack of MHB constriction in the pharyngula stage (Brand et al., 1996). Due to this, *ace* embryos have a tectum that appears to be larger than their wildtype siblings. This further provides evidence of the role of FGF8 in compartmentalization of brain units in vertebrates.

1.3.5 FGF Signaling in Heart Development in Zebrafish

Organogenesis is a highly complex developmental process that involves specification and differentiation of multiple cell lineages. Furthermore, assembly of these different cell types requires detailed regulation of cell movements and cell interactions. Coordinating patterning and morphogenesis is critical for proper organ formation. The vertebrate heart is the first organ to both form and function within an embryo, and thus has become a highly studied organ. Even a simple structure, such as the embryonic heart tube, contains multiple cell types including myocardiocytes and endocardiocytes. Further diversification creates subpopulations such as ventricular and atrial myocardiocytes, that have distinct physiological characteristics (Franco et al., 1998; Marques and Yelon, 2009; Satin et al., 1988). Heart development within the zebrafish can be categorized into early and late heart development. The role of FGFs has been shown to be critical in both of these phases of heart development.

1.3.5.1 FGF Signaling during Early Heart Development

Through fate mapping studies and utilization of laser-mediated activation of caged fluorescein, both atrial and ventricular progenitor populations have been identified as early as 40% epiboly, just prior to gastrulation within the zebrafish embryo. Myocardial progenitors are located within the first four tiers of blastomeres on both sides of the embryo, 60-140^{o} from the dorsal midline, in an area termed the lateral marginal zone (LMZ) (Keegan et al., 2004; Warga and Nusslein-Volhard, 1999) (**Figure 2A-B**). These cardiac progenitor cells are intermingled with progenitors of other lineages found within the LMZ, including endoderm, endothelium, pectoral fin mesenchyme, blood, head muscle, and pharyngeal tissue (Keegan et al., 2004; Kimmel et al., 1990; Warga and Nusslein-Volhard, 1999). But, despite this mingling of different cell types,

ventricular and atrial myocardial progenitors remain relatively organized and compact during and following gastrulation (Keegan et al., 2004) (**Figure 2C-F**). Thus, even during these early stages, myocardial fates appear to be imparted to a population of cells within this marginal zone (Lee et al., 1994; Stainier et al., 1993). However, transplantation experiments using blastula staged cells indicate cells will adopt a new fate when placed in a new location, suggesting these cells are plastic in nature, often referred to as progenitor cells (Lee et al., 1994; Stainier et al., 1993). At the conclusion of gastrulation, the cardiac progenitors have migrated to two parallel populations of cells on either side of the midline in the anterior portion of the lateral plate mesoderm (LPM), where the ventricular cells are more medially positioned and the atrial cells are more laterally positioned (Keegan et al., 2004) (**Figure 2G -H**). Eventually, these two populations of cells will coalesce at the midline of the embryo, due to migration of the entire LPM, to form a heart cone, where ventricular and atrial populations will form the inner and outer cardiac cone, respectively (Keegan et al., 2004; Yelon et al., 1999) (**Figure 2I**).

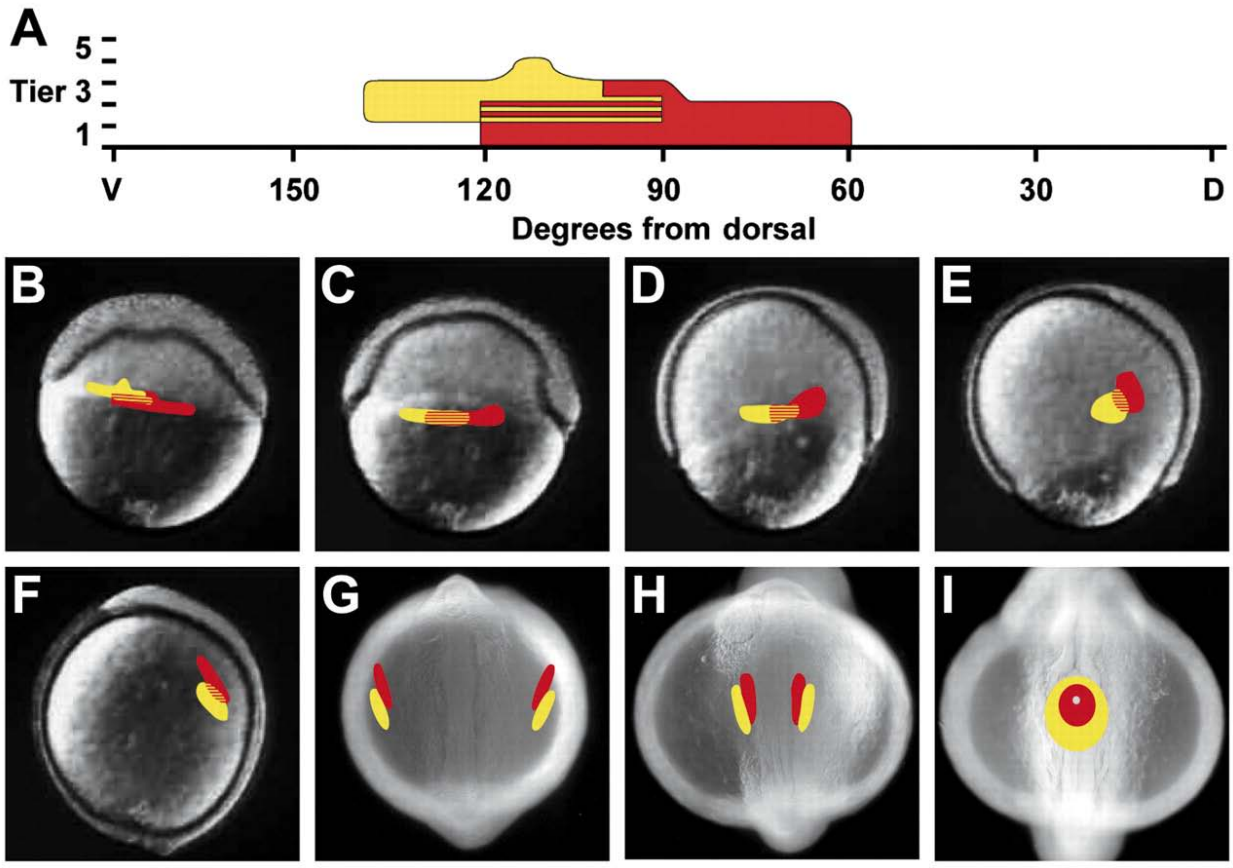

Figure 2: Model of Early Myocardial Morphogenesis.

The spatial organization of myocardial progenitor cells during early zebrafish heart development indicates orderly migration and segregation based upon heart chamber progenitor cells. **(A-I)** Schematic representation of regions of ventricular myocardial progenitors (red) and atrial myocardial progenitors (yellow). **(A)** Zones containing both populations are indicated by red and yellow stripes. **(B-F**; lateral views, dorsal to the right**)** As development progresses from 40% epiboly **(B)**, shield **(C)**, 70% epiboly **(D)**, 85% epiboly **(E)**, and tailbud stage **(F)**, two distinct populations of myocardial progenitor cells can be identified. **(G-I**; dorsal views, anterior toward the top**)** Later in development, these progenitor cells are found within the LPM at 5 somites **(G)**, 15 somites **(H)**, and 22 somites **(I)**, when the heart tube begins to form (Figure from Keegan et al., 2004; used with permission).

Although fate mapping studies indicate where myocardial progenitor cells are at early developmental stages, this does not indicate specifically when myocardial specification occurs. But as early as somitogenesis, the LPM already has an elaborate gene expression pattern, specifically along the anterior-posterior axis (**Figure 3**). *gata4*, a transcription factor shown to be required for proper heart development, is expressed in two bilateral populations of cells located in a large portion of the anterior LPM (ALPM) (Ho and Kimmel, 1993; Kuo et al., 1997; Molkentin et al., 1997). The expression pattern of *nkx2.5*, a homeodomain containing transcription factor also considered a marker for precardiac mesoderm, is co-expressed within a subpopulation of *gata4*-expressing cells, more posterior and medial in orientation (**Figure 3**) (Chen and Fishman, 1996; Evans, 1999; Lee et al., 1996; Lyons et al., 1995; Serbedzija et al., 1998; Yelon et al., 1999). The expression of another precardiac helix-loop-helix transcription factor gene, *hand2*, also overlaps with *gata4* expression in the ALPM, but is restricted to more posterior and lateral regions (Angelo et al., 2000; Yelon et al., 2000). Conversely, a population of cells most anterior in the ALPM expresses the transcription factor *scl*, known to be required for the formation of endothelial lineages, blood, and vessel (**Figure 3**) (Gering et al., 1998; Liao et al., 1998).

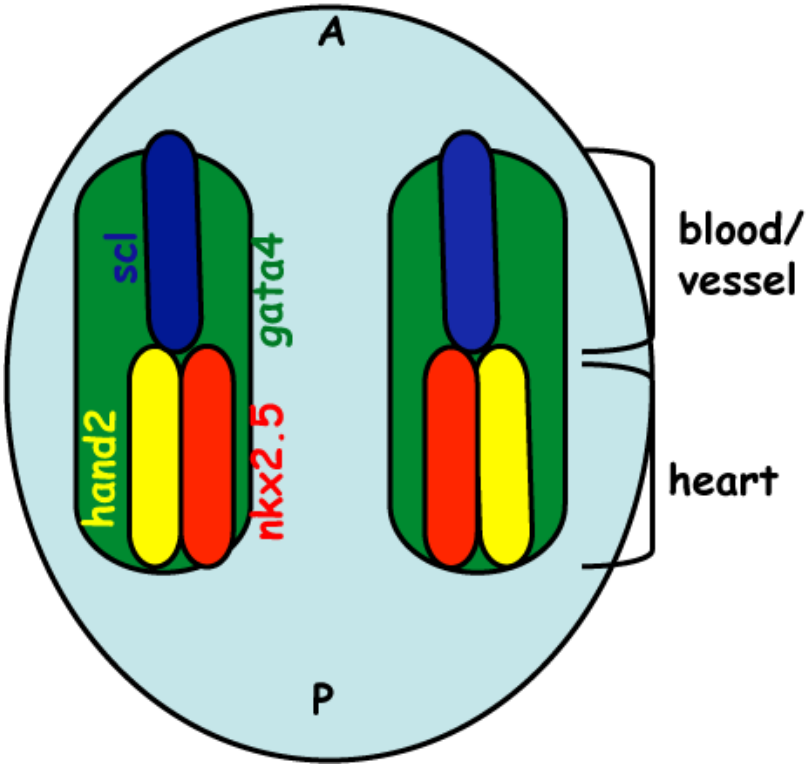

Figure 3: Expression Patterns within the ALPM of a Somitogenesis Stage Zebrafish Embryo.

A dorsal view schematic of a somitogenesis stage zebrafish embryo, where anterior is toward the top (denoted by 'A') and posterior is toward the bottom (denoted by 'P'). *gata4* expression outlines the entire ALPM. The ALPM can be further subdivided based on expression patterns of other transcription factors. Cells located posterior in the ALPM expressing *nkx2.5* (medially) and *hand2* (laterally) will give rise to heart progenitor cells, while ALPM cells located more anterior, expressing *scl*, will give rise to blood and vessel lineages.

The availability of zebrafish mutants and the ease of manipulating signaling pathways in zebrafish have revealed the importance of these transcription factors on cardiac progenitor cells. For example, a zebrafish mutant, *swirl*, contains a mutation in the *bmp2b* gene. In addition to altered dorsoventral axis patterning, as is expected when modulating BMP signaling, homozygous mutants also have a severe reduction in *nkx2.5*-expressing precardiac mesoderm. This gross lack of precardiac mesoderm early in development eventually leads to embryonic

death due to the formation of a deformed heart (Kishimoto et al., 1997; Reiter et al., 2001). Furthermore, another mutant, Oep (*one eyed pinhead*), a critical cofactor in Nodal signaling expressed both maternally and zygotically in zebrafish, also exhibits heart defects (Gritsman et al., 1999; Zhang et al., 1998). Mutant embryos lacking only zygotic *oep* have a drastic reduction of *nkx2.5* expression during somitogenesis. Later in development this early signaling defect results in altered myocardial differentiation, specifically in the ventricle. Cardia bifida, the result of the two heart progenitor populations remaining separate and not forming a single heart tube, is a common phenotype among these mutants (Reiter et al., 2001).

Using zebrafish, mouse, and *Drosophila* mutants, the link between FGF signaling and early heart development has been described. In *Drosophila*, mutants in FGF receptor 1 (*Fr1* or *heartless*) display a lack of cell fate organization in several lineages, including the heart and dorsal somatic cells (Gisselbrecht et al., 1996). This defect is likely a result from the failure of the mesoderm to spread over the ectoderm and receive patterning signals. Heart precursor cells cannot be identified in these embryos including *nkx2.5* (tinman), and heart-specific genes are not even expressed (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997). In mice, due to the early embryonic lethality of $fgfr1^{-/-}$ mice, $fgfr1^{-/-}$ embryonic stem (ES) cells are examined for their potential to differentiate into cardiomyocytes *in vitro*. Less than 10% of the embryoid bodies from ES cells formed clusters of pulsating cardiomyocytes in $fgfr1^{-/-}$ when compared to greater than 90% observed in $fgfr1^{+/-}$ embryoid bodies. Accordingly, $fgfr1^{-/-}$ embryoid bodies lack expression of early cardiac transcription factors *nkx2.5* and *d-hand* (Dell'Era et al., 2003). In zebrafish, *fgf8* expression can be detected in *nkx2.5*-expressing precardiac mesoderm and neighboring cells during early somitogenesis stages. The zebrafish *fgf8* mutant (*ace*) exhibits a loss of cardiac precursors due to a decrease in *nkx2.5* and *gata4*

expression (Araki and Brand, 2001; Reifers et al., 1998). Furthermore, Fgf8-soaked beads near the ALPM can slightly expand the expression domain of *nkx2.5* in *ace* mutants (Reifers et al., 2000). From these experiments, it was concluded that FGF signaling is critical for early heart development.

1.3.5.2 FGF Signaling during Late Heart Development

Following cardiac fusion in the developing zebrafish heart, the cardiac cone extends, and by 24 hours post fertilization (hpf), has converted into a linear heart tube (Yelon et al., 1999). As the myocardial tube extends, discrete ventricle and atrial ends form, each containing a subset of genes that are chamber-specific. For example, *atrial myosin heavy chain* (*amhc*) is only expressed in the atrium, while *ventricular myosin heavy chain* (*vmhc*) is only expressed in the ventricle (Yelon et al., 1999). The heart tube will begin to beat immediately after formation, driving circulation with regular contractions by 24hpf (Warren and Fishman, 1998). Between 24 and 48hpf, the linear heart tube will bend, causing a distinct division between the ventricle and atrium, creating an S-shaped heart (Chen et al., 1997; Chin et al., 2000). Due to this looping, the ventricle now lies to the right and dorsal of the atrium, and the two-chambered heart of the zebrafish adult is now formed.

Since chamber-specific markers are known, several studies have examined the effect of altered signaling on each individual chamber of the heart. Cells within each chamber can be counted, and experiments have indicated that about 205 cardiomyocytes exist in the looped heart, with the larger ventricle containing slightly more cells (115 cardiomyocytes) compared to the smaller atrium (90 cardiomyocytes) (Marques et al., 2008; Marques and Yelon, 2009). Marques and Yelon (2009) recently investigated the roles of BMP signaling on the lineages of each of the chambers of the zebrafish heart. Mutation in the type I BMP receptor *alk8* resulted in a

reduction of cardiomyocytes, but this reduction is restricted only to the atrium. Conversely, increasing BMP signaling within the embryo increases the total cardiomyocytes with a more pronounced increase within the atrial portion of the heart. In addition to mutations causing changes in chamber/heart size, shape is also largely affected by altering signaling during heart development. The *heart and soul* mutation (*has*) was originally described to have a small heart phenotype (Stainier et al., 1996). Further analysis determined this smaller size was not due to a reduced number of cardiomyocytes, but rather to a gross malformation of the heart tube. The atrium, which normally lies posterior to the ventricle, surrounds the ventricle in *has* mutants (Fishman and Chien, 1997; Yelon et al., 1999).

FGF signaling has also been shown to play an important role in these later stages of heart development. In addition to the role of *fgf8* in cardiac precursor development (see 1.3.2.1), *fgf8* is more specifically required for proper ventricle formation in later stages. In *ace* embryos, the resulting ventricle is greatly diminished, in addition to lacking proper heart looping (Reifers et al., 2000). This is consistent with the predominant expression of *fgf8* in the ventricle. Furthermore, an allelic series of mouse mutants hypomorphic for *Fgf8* display left-right asymmetry defects in addition to hypoplastic right ventricle and outflow tract, indicating the importance of the specific dosage of FGF8 protein (Abu-Issa et al., 2002). Consistent with the differential effect of FGF signaling on each of the chambers of the heart, exogenous FGF2 or FGF4 in chick embryos promotes ventricle-specific gene expression (VMHC1) and decreases atrial-specific gene expression (AMHC1) (Lopez-Sanchez et al., 2002). Most recently, Marques et al. (2008) determined that FGF signaling initially regulates heart size and chamber proportionality during cardiac specification. Later in heart development, FGF signaling refines the ventricle by regulating the cell number after differentiation. Thus, this single pathway can

act to coordinate organ size and proportion. This was further confirmed in our lab, where we hyper-activated FGF signaling in zebrafish during development. We found that the overall heart size was greatly expanded, specifically in the ventricle upon hyper-activation of FGF signaling. Importantly, it was also concluded that the size of the heart is sensitive to the temporal increase in FGF signaling, where increasing FGF signaling at early somite stages causes the greatest increase in heart size (Molina et al., 2009a). Thus, not only is the amount of FGF signaling important for the size and proportion of the heart, but also the temporal distribution of FGF signaling during development is critical for proper cardiac formation.

A number of zebrafish mutants exhibiting defects in cardiac looping have been described (Bisgrove et al., 2000; Chen et al., 1997; Chin et al., 2000). In some cases, mutant hearts were looped in the opposite direction, where the ventricle is positioned to the left of the atrium. These mutants are thought to have defects in the initial assignment of the embryonic left-right (L/R) axis. In other mutants, hearts fail to loop, and remain as a straight heart. In these less severe phenotypes, defects could arise from molecular mechanisms that allow the heart to interpret L/R cues (Bisgrove et al., 2000; Chen et al., 1997; Chin et al., 2000). Interestingly, several of these mutations also cause defects in L/R morphogenesis of endodermal organs, such as the liver and gut, suggesting a common mechanism for generating all asymmetries (Bisgrove et al., 2000; Chen et al., 1997; Chin et al., 2000).

1.3.6 FGF Signaling in Breaking Lateral Symmetry in Zebrafish

Most vertebrates outwardly appear bilaterally symmetric, however internal asymmetries exist along the L/R axis. This is revealed by the asymmetric placement of organs along the midline, such as the heart, liver, and stomach. Exactly how this asymmetry is established during early

embryogenesis is still under debate, but it is accepted that the L/R axis is defined after both the dorsal-ventral (D/V) and anterior-posterior (A/P) axes (Capdevila et al., 2000; Takaoka et al., 2007). The initial evidence for L/R patterning in all vertebrates is asymmetric gene expression in the LPM, which will eventually lead to proper organ laterality in fish, frog, chick, and mouse (Bisgrove and Yost, 2001; Burdine and Schier, 2000; Speder and Noselli, 2007).

1.3.6.1 Breaking Symmetry in a Developing Organism

The initial break in symmetry has been thought to involve a leftward flow of extra-embryonic fluid around the embryonic node at the tip of the primitive streak that would transport molecules to act as 'handed' determinants (Brown et al., 1991; Brown and Wolpert, 1990). For example, nodal flow is thought to push molecules located around the node to one side of the embryo. Members of the TGF-β family, such as Nodal, Lefty1, and Lefty2 are thought to be asymmetrically expressed on the left side of the embryo due to this flow (Meno et al., 1997; Meno et al., 1996; Zhou et al., 1993). Nodal regulates its own expression in a positive feedback loop and activates *Lefty2* expression on the left side of the lateral plate mesoderm. Lefty2 then acts as a feedback inhibitor of Nodal (Adachi et al., 1999; Norris and Robertson, 1999; Saijoh et al., 1999). Lefty1 has been found to function as a midline barrier that prevents left-side-specific signaling from crossing the midline. *Lefty1* mutant mice have bilateral expression of *Lefty2* and *Nodal*, and have pulmonary left-isomerism (Meno et al., 1998). The Nodal signal is further mediated by a homeobox transcription factor, Pitx2, to further drive situs specific morphogenesis, although many of the downstream targets of Pitx2 are not yet known (Campione et al., 1999; Pagan-Westphal and Tabin, 1998; Piedra et al., 1998; Yoshioka et al., 1998). Pitx2 is responsible for generating left-sided morphology of several visceral organs. For example,

Pitx2-null mutant mice display right isomerism in the lung, with each lung containing four lobes (Gage et al., 1999; Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999).

1.3.6.2 The Role of Cilia in Breaking Symmetry

A link between cilia and L/R specification has been suspected since the discovery of Kartagener syndrome, a rare human genetic disorder identified by situs inversion accompanied by a loss of motility of respiratory cilia and sperm flagella (Afzelius, 1976). Furthermore, nodal flow is impaired in mouse mutants that show situs defects, supporting the necessity for nodal flow in L/R determination. Further investigation determined these mice lacked the primary cilia found within the node, and consequently the nodal flow was absent (Marszalek et al., 1999; Nonaka et al., 1998; Takeda et al., 1999). It has been shown that cilia within the mouse node rotate in a clock-wise direction and are tilted toward the posterior. Therefore, the flow above the individual cilia is rotational, but the rightward swing of the cilium close to the cell surface retards the fluid. Thus, the net flow at the node is from right to left (Nonaka et al., 2005; Okada et al., 2005). This nodal flow is considered to be conserved among *Xenopus*, medaka, and rabbit (Blum et al., 2007; Okada et al., 2005; Schweickert et al., 2007), and has been proposed as the process that breaks L/R symmetry for all vertebrates (Nonaka et al., 1998; Nonaka et al., 2005; Okada et al., 2005).

1.3.6.3 Kupffer's Vesicle is a Ciliated Organ that Breaks Symmetry in Zebrafish

In zebrafish, a transient ciliated organ called Kupffer's vesicle (KV) is derived from the dorsal forerunner cells (DFC), and the current dogma assumes that KV is analogous to the mouse node (Cooper and D'Amico, 1996; D'Amico and Cooper, 1997; Essner et al., 2002). DFCs are initially formed in the dorsal germ ring prior to gastrulation, and migrate attached to the surface epithelium. During somitogenesis, these cells coalesce into a single rosette-like epithelial

structure that will differentiate into KV with a ciliated lumen (Oteiza et al., 2008). However, the fluid dynamics inside KV is somewhat different than the mouse node. The ciliated surface within the mouse node is relatively flat, while the KV is shaped more as a sphere, with cilia projecting both from the dorsal roof and the ventral floor (Amack et al., 2007; Kreiling et al., 2007). In both the floor and the roof, the cilia are posteriorly pointed and rotate clockwise when viewed apically. Microinjection of beads into the KV indicated that the cilia cause a net circular flow, but the local flow differs in direction depending on the location within the vesicle. The plane of the circular net flow is tilted within the KV, and thus cells in the anterior-dorsal region experience a local dominant leftward flow (Okabe et al., 2008). Thus, it can be hypothesized that the net flow in the zebrafish KV is analogous to flow in the mouse node in terms of L/R patterning even though the ciliated structures have differing architectures. Studies have shown that altering cilia within the KV of developing zebrafish embryos affect L/R asymmetry. A KV-specific knock-down of *left-right dynein-related1* (*lrdr1*), a motor protein critical for cilia movement in zebrafish, results in randomization of the situs of the heart and gut.

1.3.6.4 The Importance of FGF Signaling in Asymmetry

Recent reports implicate FGF signaling in playing a major role in cilia number and length, and thus L/R asymmetry. Under normal conditions, it was observed that in the mouse node, local microvilli would release membranous parcels, termed ‘nodal vesicular proteins’ (NVPs) that consists of lipophilic granules sheathed by an outer membrane. These NVPs would be released and flow down the stream of nodal flow. Finally, the NVPs were fragmented by ciliated surfaces into several smaller particles in proximity to the left wall (Tanaka et al., 2005). When treating the embryos with an FGF receptor inhibitor, SU5402, the release of NVPs was silenced, indicating the importance of FGF signaling in L/R asymmetry. In a recent report from Hong and

Dawid (2008), two FGF target genes, *ier2* and *fibp1*, were shown to be involved in laterality determination. Knocking down these genes individually or in combination significantly decreased the amount of cilia found within the KV. Furthermore, this phenotype can be rescued by injection of *ier2* and *fibp1* mRNA. In addition, Neugebauer et al. (2009) provides evidence that FGF signaling regulates cilia length in a variety of epithelia. By knocking down expression of *fgfr1* only in the DFCs, the expression of *spaw* (the zebrafish homologue of Nodal) was completely randomized and this phenotype was accompanied with significantly shorter cilia. At mid-gastrula stages, global knockdown of *fgfr1* decreased monocilia length found in the kidney and otic vesicle as well. In addition, it was found that *foxj1*, a transcription factor required for proper ciliogenesis, was decreased in these embryos (Neugebauer et al., 2009). Previous studies have implicated FGF ligands Fgf8 and Fgf24 as signaling through FGFR1. In addition to *fgf8* and *fgf24* having overlapping expression patterns in and around DFCs and KV in zebrafish, *fgf24* mutants (*ikarus*) and *fgf8* mutants (*ace*) display L/R patterning defects (Draper et al., 2003; Fischer et al., 2003; Scholpp et al., 2004; Zhang et al., 2006). A current model has been designed based on this data, whereby Fgf8 and Fgf24 ligands will signal through FGFR1, which will activate transcription factors (such as *foxj1*) that will regulate intraflagellar transport genes to maintain proper motile cilia (**Figure 4**)(Neugebauer et al., 2009).

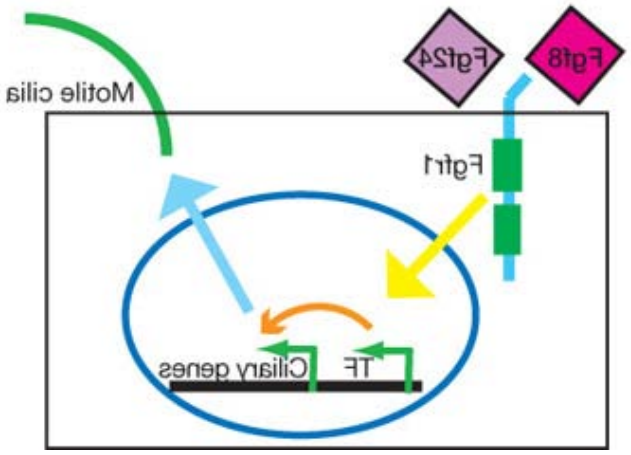

Figure 4: FGF Signaling Controls Proper Cilia Formation in KV.
Proposed mechanism whereby FGF signaling controls the formation of motile cilia. FGF ligands signal through FGFR1, activating downstream transcription factors (TF). These transcription factors will activate ciliary genes to maintain proper cilia formation (Figure from Neugebauer et al., 2009; used with permission).

1.4 THE FAMILY OF ETS TRANSCRIPTION FACTORS

The ETS-domain family of eukaryotic transcription factors consists of over 30 members found in a diverse group of organisms ranging from sponges to humans (Degnan et al., 1993; Laudet et al., 1993). Originally identified through sequence homology with the *v-ets* oncogene encoded by the E26 (**E t**wenty-**s**ix) erythroblastosis virus in chickens, the ETS family of transcription factors has been shown to be important for cell proliferation, differentiation, and migration (Kobberup et al., 2007; Wasylyk et al., 1998). Importantly in humans, chromosomal translocations that generate ETS fusion proteins have been identified as the causative agent for myelogenous leukemia and Ewing's sarcoma (Golub et al., 1995; Golub et al., 1994; May et al., 1993). Furthermore, ETS factors are overexpressed in oncogene-induced mouse mammary tumors, and

expression of an inhibitor form of an ETS factor reduces the number and size of tumors (Shepherd et al., 2001).

All ETS family members contain the ETS domain, an 85 amino acid winged-helix-turn-helix (wHTH) domain that, with the exception of one member, GABPα, binds DNA as a monomer. This domain binds to a 5'-RGAA/T-3' core DNA sequence. Nucleotides flanking this core site influence binding affinity and the specificity of an ETS family member for that particular site (Graves and Petersen, 1998). More specifically, helix α3 recognizes the GA core site while the surrounding β-hairpin and loop make multiple contacts to nucleotides flanking the GA core. Thus, through structural studies, it is suggested DNA contacts coupled with the sequence-dependent DNA structure combine to give individual innate ETS protein specificity (Kodandapani et al., 1996; Mo et al., 1998; Mo et al., 2000). In addition, the low specificity and binding affinity of ETS proteins to individual target sites is compensated for with the interaction of neighboring proteins. For example, Elk-1 ETS factors will commonly interact with SRF proteins. Only through this interaction will Elk-1 proceed to bind to and induce c-fos promoter activation (Buchwalter et al., 2004; Shore and Sharrocks, 1994). ETS proteins are thought to act as either transcriptional activators or repressors, and many are targets of signal transduction pathways. Biologically, ETS-domain transcription factors appear to have distinct roles in regulating differentiation and proliferation during embryonic development and in the adult organism.

1.4.1 The Sub-family of PEA3 ETS Transcription Factors

ETS transcription factors can be further sub-classified based on the high amino acid conservation of the ETS DNA binding domain and the conservation of other domains and motifs. For

example, the PEA3 subfamily of ETS transcription factors contains significant sequence similarity within the ETS domain (>95% amino acid identity), a 72 amino acid N-terminal transcriptional activation domain, and a short C-terminal domain. This subfamily consists of three members, Polyomavirus enhancer activator 3 (PEA3/ETV4), Er81, and ETS-related molecule (ERM). These proteins are phosphorylated via activated ERKs, a key component of the FGF signaling pathway, and Protein kinase A, which will then facilitate interactions with DNA to induce gene expression (O'Hagan et al., 1996; Oikawa and Yamada, 2003; Sharrocks, 2001). Therefore, FGF activity is eventually relayed into the regulation of downstream target gene expression due to the modification of PEA3 ETS factors via activated ERKs. For example, ERK2 has been shown to directly phosphorylate GST-Erm proteins. Furthermore, the RAS/MAPK pathway *in vivo* can increase Erm reporter gene activation (Janknecht et al., 1996).

Interestingly, through domain mapping studies specific motifs have been defined within the Erm amino terminus that inhibit Erm from binding to target DNA sequences (Laget et al., 1996). Previous *in vitro* studies indicate in resting cells that Erm is folded in an inactive state, but upon ERK phosphorylation undergo a conformational change, allowing the ETS domain to be exposed to bind DNA and regulate gene expression (Laget et al., 1996; O'Hagan et al., 1996; Oikawa and Yamada, 2003; Sharrocks, 2001)(**Figure 5 A&B**). ERKs, being serine threonine kinases, are likely to phosphorylate PEA3 ETS factors at these residues (O'Hagan et al., 1996) but the specific sites of phosphorylation, in addition to how these factors activate gene transcription, is still unknown.

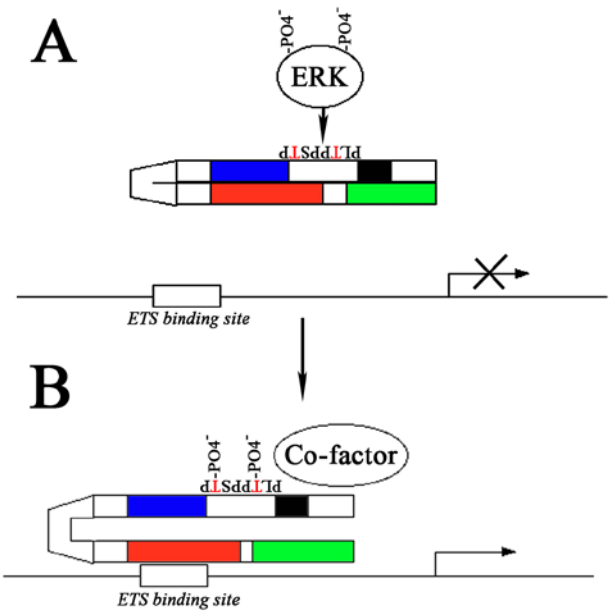

Figure 5: A Model of PEA3 ETS Transcription Factor Regulation.

(A) PEA3 proteins are in a folded confirmation where the DNA binding domain (red) is interacting with the inhibitory domain (blue) to prevent binding to the promoter of downstream targets. **(B)** Phosphorylation by ERK at threonine/serine sites induces a conformational change, allowing the DNA binding domain to be freed and bind to ETS binding sites on promoters, activating transcription of downstream targets. The black and green boxes represent the conserved N-terminal and C-terminal domains, respectively.

To determine the importance of PEA3 factors in developmental processes, all PEA3 members have been inactivated via gene knock-out techniques in mice. Single knock-outs of Erm, Pea3, or Er81 resulted in developmentally normal mice surviving to adulthood, although mice were mutant in motor neuron differentiation and spermatogonial stem cell renewal (Chen et al., 2005; Haase et al., 2002; Hippenmeyer et al., 2005; Livet et al., 2002). This lack of gross developmental defects can be explained due to the overlapping expression pattern of ETS factors. Redundancy may allow for compensation of individual deletion of ETS genes, whereby knocking out only one PEA3 ETS factor will not have an overall large effect on development.

More recent studies in mice used conditional knockouts of two ETS transcription factors, Etv5 and Etv4 (the ortholog of zebrafish Pea3) to examine the effects of eliminating two ETS transcription factors in specific tissues. Inactivating Etv4/5 in the mouse limb bud causes preaxial polydactyly. This was determined to be a result of overexpression of sonic hedgehog (Shh) signaling, indicating a role for PEA3 ETS factors and FGF signaling by inhibiting *Shh* expression (Zhang et al., 2009). Another mouse study generated a compound $Etv4^{-/-}$ and $Etv5^{+/-}$ mouse knock-out, in which the mice showed a complete absence of kidney formation (Lu et al., 2009). These two experiments indicate the redundancy of this family of transcription factors, whereby expression of two family members must be reduced to generate gross phenotypic effects. Alternative studies with these factors depend upon the generation of dominant negative or constitutive active constructs. These studies have indicated the importance of PEA3 factors in both relaying and restricting FGF signaling in the chick somites (Brent and Tabin, 2004) and relaying FGF signaling in mouse lung outgrowth (Liu et al., 2003).

In zebrafish, *erm, pea3,* and *Er81* developmental expression has been described (Kudoh et al., 2001; Munchberg et al., 1999; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001; Roussigne and Blader, 2006). *pea3* and *erm* are spatially restricted to the mesoderm and anterior lateral plate mesoderm (ALPM) in the developing embryo, a region where cardiac progenitor cells have been fate mapped. These areas are spatially consistent with *fgf3* and *fgf8* expression, alluding that these transcription factors are important within the FGF signaling pathway (Munchberg et al., 1999; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). Furthermore, manipulating the amount of FGF signaling an embryo will receive can alter the expression of *pea3* and *erm* (Munchberg et al., 1999; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). In contrast to *pea3* and *erm* expression, *Er81* is not limited to domains

where *fgf* ligands are localized and suppression of FGF signaling does not affect *Er81* expression (Roussigne and Blader, 2006). Thus, *Er81* has probably evolved to serve a separate function in zebrafish that is distinct from mouse and *Xenopus laevis* orthologs (Chotteau-Lelievre et al., 2001; Munchberg and Steinbeisser, 1999; Roussigne and Blader, 2006).

1.4.2 Etv5 is a Member of the PEA3 ETS Factors

From a random *in situ* hybridization screen in zebrafish, genes that exhibited similar expression patterns to *fgf3* and *fgf8* throughout embryonic development were identified. Genes displaying this similar expression pattern were classified into the FGF syn-expression group (Kudoh et al., 2001; Tsang et al., 2002; Tsang et al., 2004). Another factor, *etv5* (ETS-variant 5), was identified that was classified into the FGF syn-expression group. Performing a sequence alignment, Etv5 was placed into the PEA3 subfamily of ETS transcription factors due to the high conservation of the amino-terminal acidic domain, the DNA binding inhibitory domain, the ETS DNA binding domain, and the C-terminal region. In mouse and human, only one *Etv5/Erm* gene has been identified (Liu et al., 2003). In zebrafish, due to genome duplication, two ETV5/ERM genes exist, *etv5* and *erm* (also known as *etv5a* and *etv5b)* , in addition to *Er81* and *pea3* (Kudoh et al., 2001; Roussigne and Blader, 2006).

Expression analysis was performed to determine the expression pattern of *etv5* during development. Beginning at two hours after initiation of zygotic transcription, expression of *etv5* was similar to that of *fgf3* and *fgf8*. During blastula stages, expression is within the germ ring, where cardiac progenitors are located. At gastrula stages, *etv5* is also located in the organizer region, an area with high levels of FGF signaling (Furthauer et al., 2002; Furthauer et al., 2004; Keegan et al., 2004; Tsang et al., 2002; Tsang et al., 2004). Later during development, *etv5* is

localized to the anterior nervous system, cardiac progenitor regions in the ALPM, the MHB, and the retina (**Figure 6 A-E**). This expression pattern of *etv5* is highly overlapping with *erm* and *pea3*, suggesting redundant functions among these factors during development (Munchberg et al., 1999; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001).

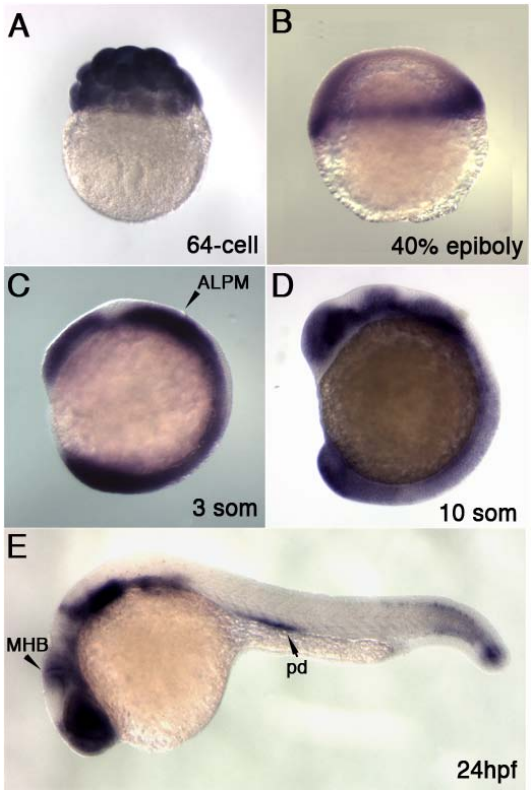

Figure 6: Zebrafish Etv5 Expression.

Stages are indicated in the lower right corner. **(A)** The initial expression of *etv5* is as a maternal transcript, but by 40% epiboly **(B)** is restricted to the germ ring, an area also expressing *fgf3* and *fgf8*. **(C, D)** By somitogenesis stages, *etv5* is restricted to the ALPM, anterior regions of the nervous system, and the tail bud. **(E)** At 24hpf, *etv5* is found in the MHB, retina, otic vesicles, pronephric ducts (pd), and tail bud.

In addition, FGFs were shown to regulate *etv5* expression during zebrafish development. By injecting *fgf8 mRNA* into zebrafish embryos, FGF signaling can be experimentally altered. Under these circumstances, expansion of *etv5* expression resulted at gastrula stages **(Figure 7A,B)**. Conversely, over-expression of *dusp6* (also known as *mkp3*), a molecule that inhibits MAPK signaling, suppresses *etv5* at shield stage **(Figure 7 C,D)**. Taken together, these experiments indicate *etv5* reacts similarly to changes in FGF signaling during development as does *erm* and *pea3*, further indicating that *etv5* is a member of the PEA3 subfamily of ETS transcription factors (Munchberg et al., 1999; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001).

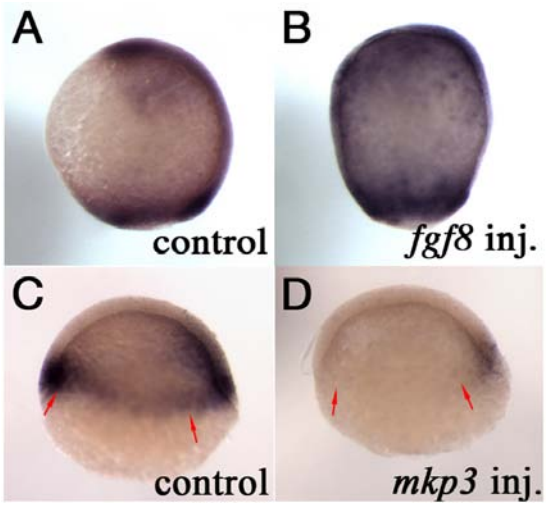

Figure 7: Expression of *etv5* is Regulated via FGF Signaling.
(A,B) *fgf8* overexpression results in expansion of *etv5* at gastrula stages. **(C,D)** Conversely, blocking FGF signaling mediated via a feedback inhibitor *dusp6*, *etv5* expression is inhibited, as indicated by the red arrows.

In my thesis work, I focused on the role of PEA3 ETS transcription factors in FGF signaling and embryogenesis in the zebrafish. Through the generation of constitutively active Etv5 constructs, I determined that the over-expression of *etv5* induced expression of the FGF-regulated transcripts *dusp6* and *sef*. Conversely, decreasing expression of PEA3 transcription factors by antisense morpholino injections disrupted the MHB development, heart formation, and L/R patterning, similar to phenotypes observed in fish and mouse mutants of FGF signaling. In addition, I determined the essential DNA sequences required for FGF-mediated induction of *dusp6* using luciferase reporter assays. Electrophoretic Mobility Shift Assays (EMSAs) showed that the Etv5 ETS DNA binding domain can directly bind to a putative ETS site within the *Dusp6* promoter, and mutating this site eliminated binding. Finally, in collaboration with Anne Moon at the University of Utah, we show that the orthologous region of the mouse *Dusp6* promoter is regulated by PEA3 and Erm via conserved ETS sites and is bound by PEA3 *in vivo* in tissues undergoing active FGF signaling during mouse embryogenesis. These results indicate the importance of ETS factors in relaying FGF signals during development and provide insights as to how FGF target genes are regulated by them.

1.5 AIMS OF DISSERTATION RESEARCH

In an effort to determine the transcription factors responsible for regulating FGF responses in zebrafish, the aims of my thesis work have been three-fold: 1) to determine the post-translational mechanisms that regulate ETS transcription factors, 2) to analyze the role of ETS transcription factors in development, and 3) to determine how ETS transcription factors regulate gene expression.

Aim 1 – *Analyze the post-translational regulation of* ETS *factors to modulate* FGF *signaling*

FGF activity regulates gene expression through the modification of PEA3 ETS transcription factors. *In vitro* studies have shown that ERK2 can directly phosphorylate GST-Erm fusion proteins and that activation of the RAS/MAPK pathway can increase reporter gene activation by Erm (Janknecht et al., 1996). In addition, mapping studies have indicated that Erm has an inhibitory region within the amino terminus that prevents the ETS domain from binding to a target DNA sequence. After ERK2 phosphorylation, a conformational change releases this inhibition and reveals the ETS DNA binding domain (Laget et al., 1996). As a result, the model of PEA3 activity can be described as these proteins being natively folded in an inactive state, where the inhibitory region is in contact with the DNA binding domain. Upon activation by ERK phosphorylation, a conformational change occurs that allows the ETS DNA binding domain to be accessible for DNA binding (Laget et al., 1996). Since ERKs are members of the serine threonine kinase family, I hypothesize that PEA3 ETS factors are likely to be phosphorylated at these residues.

Aim 2 – *Role of* ETS *transcription factors in* FGF-*regulated developmental processes*.

Two approaches have been used to determine the role of ETS genes in development, including gain- and loss-of-function studies. Fusion constructs utilizing the VP16 transactivation domain were used in over-expression studies, while antisense morpholino oligonucleotides (MOs) targeting specific ETS factors were employed to knock down expression. In the over-expression experiments, I focused on the expression of downstream FGF targets, while in MO-injected embryos I examined downstream FGF targets in addition to proper formation of the brain, ear, and heart. These aspects of development are altered in zebrafish *fgf8* mutants. Similar

types of defects recognized in the *EtsMO*-injected fish can indicate the importance of ETS transcription factors in FGF-regulated developmental processes.

Aim 3 – *Characterization of* ETS *transcription factor binding*

A key to understanding FGF signaling is to identify the cis-regulatory elements in FGF-target genes. To address this, I have generated deletion luciferase reporter constructs within the *Dusp6* promoter to determine the essential DNA sequences that are required for FGF-mediated induction of luciferase activity. By using these constructs in *Xenopus* animal caps assays, I can measure luciferase protein activity just 6 hours after zygotic transcription initiation, thus allowing direct measurement of the activation of *Dusp6* by FGFs. The luciferase constructs were injected in the presence or absence of FGFs. In addition, EMSAs and ChIP assays were utilized to determine direct binding of ETS factors to a specific region of the *Dusp6* promoter.

2.0 MATERIALS AND METHODS

2.1 ZEBRAFISH MAINTENANCE

Danio rerio were maintained at 28.5^{o}C on a 14hour light/10 hour dark cycle. Wildtype (Oregon AB*), *ace*, *Tg(Cmlc2:DsRednuc)*, and *Tg(Dusp6:d2GFP)*^{pt6} were used for experiments. Embryos were staged as described by Kimmel et al. (1995) and Brand et al. (1996).

2.2 RNA INJECTIONS AND IN SITU HYBRIDIZATIONS

These procedures were performed as described previously (Tsang et al., 2000) with the following modifications for RNA injections: Wildtype AB* zebrafish embryos were injected with *etv5* (100pg), *etv5:VP16* (75pg), *etv5:EnR* (150pg), or mutated forms of *etv5; etv5:T135D, etv5:T139D, etv5:S142D* (50pg) at the 1-2 cell stage. All mutations within Etv5 were generated as described in the Quick-Change II Site-directed Mutagenesis Kit (Stratagene) using the primers:

5’-GGGTTCAAGCCCATTGGACCCTCCCTCGGACCCC-3’ (forward *etv5:T135D*),

5’-GGGGTCCGAGGGAGGGTCCAATGGCTTGAACCC-3’ (reverse *etv5:T135D*),

5’-ACTCCTCCCTCGGACCCCGTCTCCCCATGT-3’ (forward *etv5:T139D*),

5’-ACATGGGGAGACGGGGTCCGAGGGAGGAGT-3’ (reverse *etv5:T139D*),

5’-CCCTCGACGCCCGTCGACCCATGTGTCCCCAGC-3’ (forward *etv5:S142D*),

5’-GCTGGGGACACATGGGTCGACGGGCGTCGAGGG-3’ (reverse *etv5:S142D*).

Zebrafish embryos were fixed with 4% paraformaldehyde and followed by methanol storage. Fixed embryos were analyzed by whole mount *in situ* hybridization using the protocol described previously (Kudoh et al., 2001). The following antisense riboprobes were generated in this study; *etv5* (Kudoh et al., 2001), *erm* (Munchberg et al., 1999), *pea3* (Brown et al., 1998), *fgf3* (Kiefer et al., 1996), *fgf8* (Reifers et al., 1998), *dusp6* (Kawakami et al., 2003; Tsang et al., 2004), *sef* (Tsang et al., 2002), *bmp4* (Hwang et al., 1997), *chordin* (Schulte-Merker et al., 1997), *pax2a* (Krauss et al., 1991), *her5* (Bally-Cuif et al., 2000), *amhc* (Berdougo et al., 2003), *vmhc* (Yelon et al., 1999), *nkx2.5* (Chen and Fishman, 1996), *gata4* (Reiter et al., 1999), *scl* (Liao et al., 1998), *spaw* (Long et al., 2003) and *hand2* (Yelon et al., 2000).

2.3 IMMUNOFLUORESCENCE

Whole mount immunofluorescence was performed to detect monocilia in Kupffer’s vesicle as described (Yamauchi et al., 2009). Monoclonal acetylated tubulin primary antibody (1:1000; Sigma) and Cy3-conjugated anti mouse IgG secondary antibody (1:500; Jackson ImmunoResearch Laboratories) was used in this study. Images were acquired on an Olympus Fluoview1000 instrument using an UplanSapo 20X (NA 0.75) objective. Data was acquired using the FV10-ASW software and z-stacks compressed using ImageJ software (NIH).

2.4 ANTISENSE MORPHOLINO INJECTIONS

Morpholino oligos (MOs) were designed and purchased from Gene Tools (www.gene-tools.com):

etv5MO sequence, 5'-ATCCGTCCATGTCACCTGGGTCTTC-3';

etv5/ermMO sequence, 5'-TGCTGGTCATAAAATCCGTCCATGT-3';

fgf8MO sequence, 5'-GAGTCTCATGTTTATAGCCTCAGTA-3';

and *ContMO* sequence, 5'-CCTCTTACCTCAGTTACAATTTATA-3'.

From Open Biosystems (www.openbiosystems.com):

ermMO sequence, 5'-AACCCATCCATGTCGCTTGCTTCTC-3';

pea3MO sequence, 5'-ATCCATGCCTTAACCGTTTGTGGTC-3';

ContMO (10ng), *fgf8MO* (1-3ng), single *EtsMO* (5ng), *2EtsMO* (2.5ng of each *EtsMO*), or *3EtsMO* (1.3ng of each *EtsMO*) were injected into the yolk of 1-8 cell stage AB* or *Tg(d2EGFP)*^{pt6} embryos as described (Molina et al., 2007).

2.5 GENERATION OF *DUSP6* REPORTER CONSTRUCTS

Zebrafish *Dusp6* promoter sequences were PCR amplified and directionally cloned into the pENTR Gateway vector (Invitrogen) with the following primers:

Forward primers: 5Kb: 5'- CACCGACCGGTAGTGAATTTTGATTTGAAC-3';

4Kb: 5'-CACCGGCCTAGTCGGCACTCAAACCAGTGA-3';

3Kb: 5'-CACCACTGTGGCATTACAGTGACAGGCCCG-3';

2Kb: 5'-CACCCTGCGCAGAAGTTCACTTAGACAGTG-3';

1Kb: 5'-CACCCCACACTGAACTGAGCTAAACTGAAC-3';

Reverse primer: *Dusp6* Rev: 5'-GGTACCGTGAGACCTTAAAACTGCGG-3'.

The promoter sequences were verified and subcloned into a Gateway-modified pGL3-promoter (Promega) by the Gateway System (Invitrogen). Pea3A and Pea3B mutant reporter constructs were generated using QuickChange II with:

MutA: 5'- CACTCGCACTCCTCCGGCCGTCCCGTGAAGCGCCTCTCG-3';

MutB: 5'-CCGCTGATCCGGCGCGGCCCGTCCTTTCCGTTTTTGTG-3',

A 782bp fragment of DNA 5' to the mouse *Dusp6* gene was amplified by PCR and cloned into pGL3-Promoter. The primers were:

5'-ccctggtaccGTACCGTTGGATTAGCATTTAACACTTCGT (sense, UCSC genome browser Chr10: chr10:98,725,230)

5'- ccctagatctAGTCTAGCGGCTCTTAATCCTC (anti-sense, UCSC genome browser Chr10: 98,726,011)

Upper-case letters indicate genomic sequence. Amplification was performed with Pfx Platinum polymerase (Invitrogen) and 0.1µg mouse DNA (C57Bl/6) under manufacturer conditions. The single reaction product was purified by Qiaquick spin column (Qiagen), digested with Acc65I and BglII (New England Biolabs), and cloned into pGL3-Promoter. Mutagenesis of the conserved putative PEA3/ERM binding sites 1-3 was performed with a Quick-Change Lightening Kit (Agilent) with mutagenic oligonucleotides designed by the manufacturer and plasmids were sequenced.

2.6 BCI TREATMENT

Ten embryos were placed into each well of a 24-well plate in 200 μl of E3 (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$), and a 0.5% (v/v) DMSO solution was added along with BCI [(E)-2-**b**enzylidene-3-(**c**yclohexylamino)-2,3-dihydro-1H-**i**nden-1-one] at 10 μM. Embryos were incubated in this compound from 1 somite or 8 somite stages until 24hpf, then washed and incubated in E3 until 48hpf and fixed. BCI (also known as NSC150117) was identified as a compound that enhances fluorescence in treated transgenic embryos. Each experiment was repeated four times to show the reproducibility of the assay.

2.7 MAMMALIAN CELL AND *XENOPUS* EXPLANT CULTURES AND LUCIFERASE ASSAYS

Human 293T cells (ATCC) were grown at 37°C, 5% CO_2 in DMEM (GIBCO) containing 10% FBS, 100U/ml penicillin and streptomycin. Cells were seeded into 24-well plates and were 70–80% confluence at the time of transient transfection using FuGene6 (Roche). Each well received 100ng of pGL3-Control luciferase reporter, 10ng of pRL-TK vector (Promega) encoding *Renilla* luciferase and increasing amounts of *Pea3* (Open Biosystems, Clone ID: 3854349) or *Erm* (Open Biosystems, Clone ID: 4036564) expression vector. The total amount of DNA in each well was adjusted to 310ng with pcDNA3. The cells were harvested after 48h using Passive Lysis buffer (Promega).

Xenopus laevis 2-cell stage embryos were injected with *fgf8* RNA (25pg), *Dusp6:luc* construct (125pg), and pCMV-Renilla (50pg) into each cell. In other experiments, *etv5* (50pg),

etv5:VP16 (30pg) or *etv5:T135D* (40pg) was injected in place of *fgf8* RNA. Animal caps were dissected at stage 8.5 and cultured for 6h. Animal cap lysates were prepared from a population of four animal caps. In both cell and frog assays, luciferase activities were determined using Dual Luciferase Reporter Assay System (Promega). The data were normalized by calculating the ratio of firefly luciferase to *Renilla* luciferase. A two-sample equal variance T-test using a two-tailed distribution was applied to analyze the data for statistical significance.

2.8 ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSA)

5’ labeled biotinylated and standard oligonucleotides (Midland Certified) 5’-GTTTGTTTGCACTCCGCT-3’ (forward) and 5’-TTTGTCATTCACAAAAAC-3’ (reverse) were used to generate biotinylated and competitor PCR amplified *Dusp6* promoter. For oligonucleotide competition assays, 18mer Pea3B site competitors were generated:

5’-TCCGGAGCG-GAAATTCCT-3’ (forward)

5’-AGGAATTTCCGCTCCGGA-3’ (reverse).

A random sequence competitor oligonucleotide was generated using:

5’-CCCTCGACGCCCGTCGACCCATGTGTCCCCAGC-3’ (forward)

5’-GCTGGGGACACATGGGTCGACGGGCGTCGAGGG-3’ (reverse).

GST-Etv5-ETS DNA binding domain protein was expressed in bacteria (BL21) cells (Invitrogen) and batch purified with GST-sepharose beads (Amersham). Biotin labeled DNA was incubated with Etv5-ETS protein (10ug) and resolved on a 6% native polyacrylamide gel. The products were transferred to a nylon membrane, cross-linked by a UV Stratalinker (Stratagene) and detected with streptavidin-HRP (Pierce). Competition assays were performed

with unlabeled oligos or PCR-amplified *Dusp6* promoter at 1-500 fold molar excess to labeled probes.

2.9 CHROMATIN IMMUNOPRECIPITATION (CHIP) ASSAY IN MOUSE

Chromatin was immunoprecipitated using a variation of the protocol described at http://www.cellsignal.com. Briefly, 100 E9.5 mouse embryos pharyngeal arch regions 2–6 were dissected, minced into fine slurry, cross-linked in 2% formaldehyde for 30 min, washed with ice-cold PBS, pelleted, and re-suspended in 1% NP-40 non-denaturing lysis buffer with protease inhibitors (Roche, 1-836-153). The chromatin DNA was digested with micrococcal nuclease (New England Biolabs, M0247S) for 5 min to generate fragments ranging from 150 to 900 bp and diluted in ChIP buffer with protease inhibitors. Two percent of the diluted supernatant was kept as input control. Sample was incubated at 4 °C overnight with antibody against Pea3 (Etv4) (Santa Cruz, sc-113 X). A normal mouse IgG (Santa Cruz, sc-2025) was used as negative control.

Complexes were precipitated with Dynabeads® Pan Mouse IgG (Invitrogen, 110-41). Immunoprecipitated chromatin was washed, eluted in elution buffer, incubated at 65 °C for 8 h, and treated with proteinase K. DNA was purified by phenol extraction and ethanol precipitation. Two different *Dusp6* primer sets were employed to determine if the putative PEA3 binding sites in the *Dusp6* promoter were enriched in chromatin immunoprecipitated with anti-ETV4 antibody relative to distant sequences at this locus: the "*Dusp6* prom" set amplifies the highly conserved region of the mouse *Dusp6* promoter:

5′ AGTGCCCTGGTTTATGTGC 3′ (chr10: 98,725,599-98,725,618, sense),

5′ CGGGAGGAAGGAGAAAGAA 3′ (chr10: 98,725,599-98,725,618, anti-sense).

The "*Dusp6* neg" negative control primer set amplifies a non conserved region 5′ to the *Dusp6* gene:

5′ AAGGCCGAGGAAAAGACTTC3′ (chr10: 98,721,292-98,721,311, sense),

5′ ACCCGTGTTACTGGAGATCG′ (chr10: 98,721,426-98,721,445, anti-sense).

An additional negative control primer set was employed that amplifies a region upstream of *β*-actin gene:

5′ GTGCTTAAGAGTCCACTATGAGGG3′ (sense),

5′ TCCACTCGCAATCATATACTTAGG3′ (anti-sense).

Equal quantities of input and immunoprecipitated DNA samples were subject to PCR (35 cycles), electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

To quantitate the enrichment of the Dusp6 promoter region in the ChIPed region, quantitative PCR was performed in the Bio-Rad iCycler IQ™ Multicolor Real-Time PCR Detection System using 25 µl IQ™ SYBR® Green Supermix (Bio-Rad, 170-8882), 250 nM of each primer and 2 µl of immunoprecipitated and input DNA samples. The amplification ramp included an initial hold of 5 min at 94 °C, followed by a three step cycle consisting of denaturation at 94 °C (30 s), annealing at 57 °C (30 s) and extension at 72 °C (30 s); the amplification fluorescence was read at the end of the cycle. The Ct values and standard deviations were analyzed using a method modified from that of SuperArray Biosciences: (http://www.workingthebench.com/search/label/chromatin%immunoprecepitation). This protocol correctly propagates the standard errors in the PCR data and permits quantitation of the *Dusp6* target amplicon in the ETV4 ChIPed sample relative to that ChIPed by a nonspecific

antibody (normalized to the actin negative control amplicon to control for total DNA quantity in each sample), and expressed as a percent of amplicon detected in the input sample. The enrichment was nearly 7-fold and this was highly reproducible over multiple ChIP experiments with this tissue.

3.0 ANALYSIS OF POST TRANSLATIONAL REGULATION OF ETS FACTORS TO MODULATE FGF SIGNALING

3.1 INTRODUCTION

Several members of the mouse PEA3 ETS proteins have been characterized, providing some evidence for the role of these transcription factors in FGF signaling and development (Brent and Tabin, 2004; Liu et al., 2003; Mao et al., 2009; Zhang et al., 2009). In Aim 1 of my thesis research, I wanted to validate that these factors are responsive to FGF signaling in zebrafish, and thus are effectors in this specific pathway. In addition, although ETS factors have been shown to be activated via phosphorylation (Janknecht et al., 1996; Laget et al., 1996), the specific phosphorylation sites on ETS factors remain unknown. Aim 1 examines putative phosphorylation sites on Etv5 and Pea3 and the role of these sites on ETS activation and FGF signaling.

3.1.1 PEA3 ETS Factors have Similar Expression to One Another and to FGF Ligands

A phylogenetic analysis of selected PEA3 ETS factors in mouse, human, and zebrafish indicate that the newly identified zebrafish Etv5 is most similar to zebrafish Erm based on amino acid substitutions (**Figure 8 A**). In zebrafish, due to genome duplication, there are two individual factors, Erm and Etv5, while in human and mouse, only one factor exists, ERM/ETV5 (Kudoh et

al., 2001; Roussigne and Blader, 2006)(**Figure 8 A**). Amino acid alignment between the three PEA3 ETS transcription factors in zebrafish shows high sequence conservation, especially within the DNA binding domain, acidic domain, and carboxyl region (**Figure 8 B**). In addition, the expression pattern of *etv5*, *erm*, and *pea3* is similar throughout zebrafish development. Furthermore, the expression pattern of two *fgf* ligands, *fgf3* and *fgf8*, are similar to that of *etv5*, *erm*, and *pea3* throughout development, indicating a potential importance of ETS factors in FGF signaling. An example of these overlapping expression patterns during somitogenesis stages is indicated in **Figure 8C-G**. These findings are akin to what has been described for *pea3* and *erm* regulation by FGF signaling, further indicating that PEA3 ETS genes are regulated in a similar manner (Munchberg et al., 1999; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). In contrast to these three PEA3 ETS members, the other family member in zebrafish, *Er81*, has an expression pattern not limited to the domains where *fgf* ligands are expressed, and altering FGF signaling does not affect *Er81* expression (Roussigne and Blader, 2006). It is likely that *Er81* has evolved in zebrafish to serve a separate function that is distinct from its orthologs in mouse and *Xenopus laevis* (Chotteau-Lelievre et al., 2001; Munchberg and Steinbeisser, 1999; Roussigne and Blader, 2006).

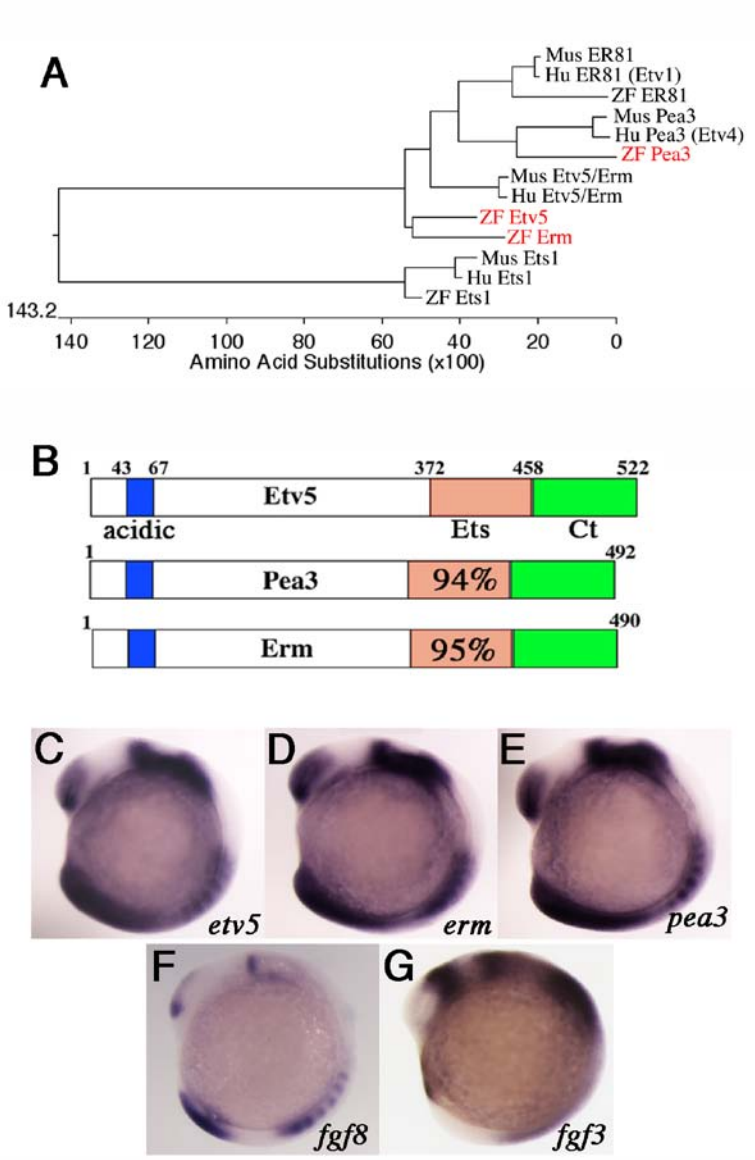

Figure 8: Comparative Expression of PEA3 ETS Transcription Factors during Zebrafish Development.

(A) Phylogenetic alignment of selected zebrafish (red), mouse, and human PEA3 ETS factors. **(B)** Diagram of structural features of PEA3 ETS proteins showing the conserved acidic region (acidic), ETS DNA binding domain (Ets), and carboxyl region (Ct). Percent identities between Etv5 ETS domain to Pea3 and Erm are listed. **(C-G)** Lateral views at 10-somite stage. *In situ* probes indicated on the bottom right corner. PEA3 ETS transcription factors have overlapping expression patterns and are similar to that of *fgf3* and *fgf8* ligands.

3.2 ECTOPIC EXPRESSION OF ALTERED FORMS OF ETV5 RESULTS IN MISEXPRESSION OF FGF TARGET GENES

To determine the function of *Etv5* during development, expression constructs were generated for ectopic expression experiments by subcloning the *Etv5* open reading frame into the pCS2+ plasmid. This plasmid allows for generation of *mRNA* suitable for microinjection studies in zebrafish embryos. Previous findings indicated that ETS factors contain an auto-inhibitory domain that prevents binding to the promoter, however, upon RAS/MAPK signaling, the inhibition is released and promoter binding can occur (Greenall et al., 2001; Laget et al., 1996; O'Hagan et al., 1996)(**Figure 5**). Due to this, it was hypothesized that overexpression of full-length Etv5 alone would not be active in this assay, unless stimulated by RAS/MAPK signaling.

To overcome the activity of the auto-inhibitory domain, a VP16 fusion construct was generated. Herpes simplex virus-encoded protein VP16 is a potent activator that can control transcription of early viral genes through the interaction of host factors (Wilson et al., 1997). VP16 is a strong activation domain that functions in many organisms when tethered to independent DNA binding domains (Cousens et al., 1989; Sadowski et al., 1988; Triezenberg et al., 1988). Based on this property, fusion of VP16 with DNA binding domains of transcription factors can be used as a model to investigate transcription activation. In this case, VP16 transcriptional activation motif was fused to the Etv5 ETS DNA binding domain, generating a constitutively active form of Etv5 (*Etv5:VP16*) (**Figure 9**).

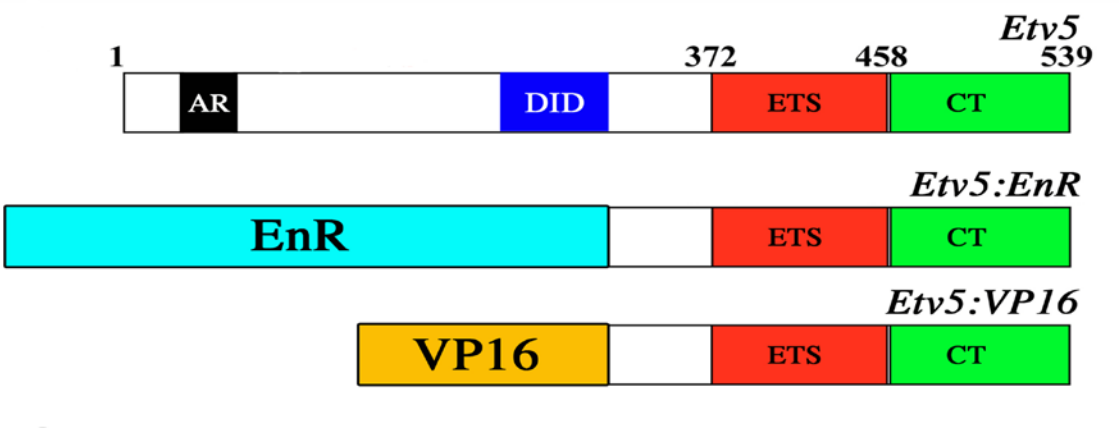

Figure 9: Etv5 Expression Constructs.
Diagram of Etv5, indicating the acidic region (AR), DNA inhibitory domain (DID), ETS binding domain (ETS), and carboxyl region (CT) represented as colored boxes. Engrailed Repressor and VP16 constructs generated by fusion to the binding domain are represented below the full length Etv5.

Injection of full-length *etv5 mRNA* at the 1-cell stage (100-300pg) did not have any affect on development, as indicated by *in situ* hybridizations at shield stage, during the start of gastrulation. When compared to uninjected embryos, the expression of FGF target genes *dusp6* **(Figure 10A-B,Q)** and *sef* **(Figure 10E-F,Q)** was not affected upon injection of full-length *etv5*. In addition, since FGF signaling has been implicated in dorsal-ventral polarity (Furthauer et al., 2004; Schmid et al., 2000; Tsang et al., 2004), a dorsally expressed gene, *chordin* (*chd*), and a ventrally expressed gene, *bmp4*, were also examined. In full-length *etv5*-injected embryos, no changes occurred in dorsal-ventral patterning when compared to uninjected embryos. The expression patterns of *chd* and *bmp4* were similar in these embryos (**Figure 10I-J**,**M-N,Q**).

To determine if PEA3 ETS factors can regulate the expression of downstream FGF targets, I employed gain-of-function studies using *etv5:VP16*. Injection of *etv5:VP16* (75pg) at the 1-cell stage dramatically induced *dusp6* and *sef* at shield stage (**Figure 10 C,G,Q**). In

conjunction with these results, embryos exhibited drastically dorsalized phenotypes, indicated by altered expression of *chd* and *bmp4* **(Figure 10K,O,Q)**. The *etv5:VP16*-induced phenotypes are identical to activation of the FGF pathway by *fgf8* mRNA injections **(Figure 7)**. These results indicate that the fusion of the Etv5 ETS domain to the VP16 transactivation motif is sufficient to generate a constitutively active form of Etv5, and altering Etv5 expression has an impact on FGF signaling.

The lack of gross developmental phenotypes in *Erm* or *Pea3* knock-out mice suggests that these genes are functionally redundant (Chen et al., 2005; Laing et al., 2000). In zebrafish, since *etv5*, *erm*, and *pea3* have overlapping expression patterns throughout development **(Figure 8)**, and these genes appear to be regulated by FGF signaling **(Figure 7)**, therefore redundant functionality may also be present here. One approach to study the function of PEA3 ETS factors and to circumvent redundancy has been to generate a dominant negative construct. Previously, PEA3 ETS factors have been described to function as activators and ectopically expressing an engrailed repressor (EnR) fusion construct in the mouse lung and in chick somites yielded phenotypes opposite of constitutively active versions (Brent and Tabin, 2004; Liu et al., 2003). Engrailed (En) is a powerful *Drosophila* repressor homeodomain protein required for proper segmentation and posterior identity (Jaynes and O'Farrell, 1991). It has been shown to efficiently repress the activity of a variety of transcription factors. Two separate domains within En are responsible for a majority of the activity, ultimately resulting in transcriptional repression through histone deacetylation and modification of chromatin structure (Chen et al., 1999; Jimenez et al., 1997; Tolkunova et al., 1998; Vinters et al., 1999).

A dominant negative version of Etv5 was constructed by fusion of the Etv5 ETS domain to the Engrailed transcriptional repressor domain, *etv5:EnR* **(Figure 9)**. Injection of *etv5:EnR*

(150pg) into 1-cell stage embryos dramatically diminished *dusp6* and *sef* at shield stage, completely opposite to the phenotypes generated by ectopic expression of *etv5:VP16* (**Figure 10D,H,Q**). This suggests that ectopic expression of *etv5:EnR* blocks FGF signaling. In conjunction with these results, embryos exhibited drastically ventralized phenotypes, indicated by misexpression of *chd* and *bmp4* **(Figure 10L,P,Q)**. These results suggest that this construct is acting in a dominant negative fashion as has been described with Pea3 and Erm Engrailed fusion constructs in chick and mouse (Chen et al., 2005; Laing et al., 2000). Thus ectopic expression of *etv5:EnR* is sufficient to block FGF signaling and PEA3 ETS genes are required to relay transcriptional events mediated by FGF signals.

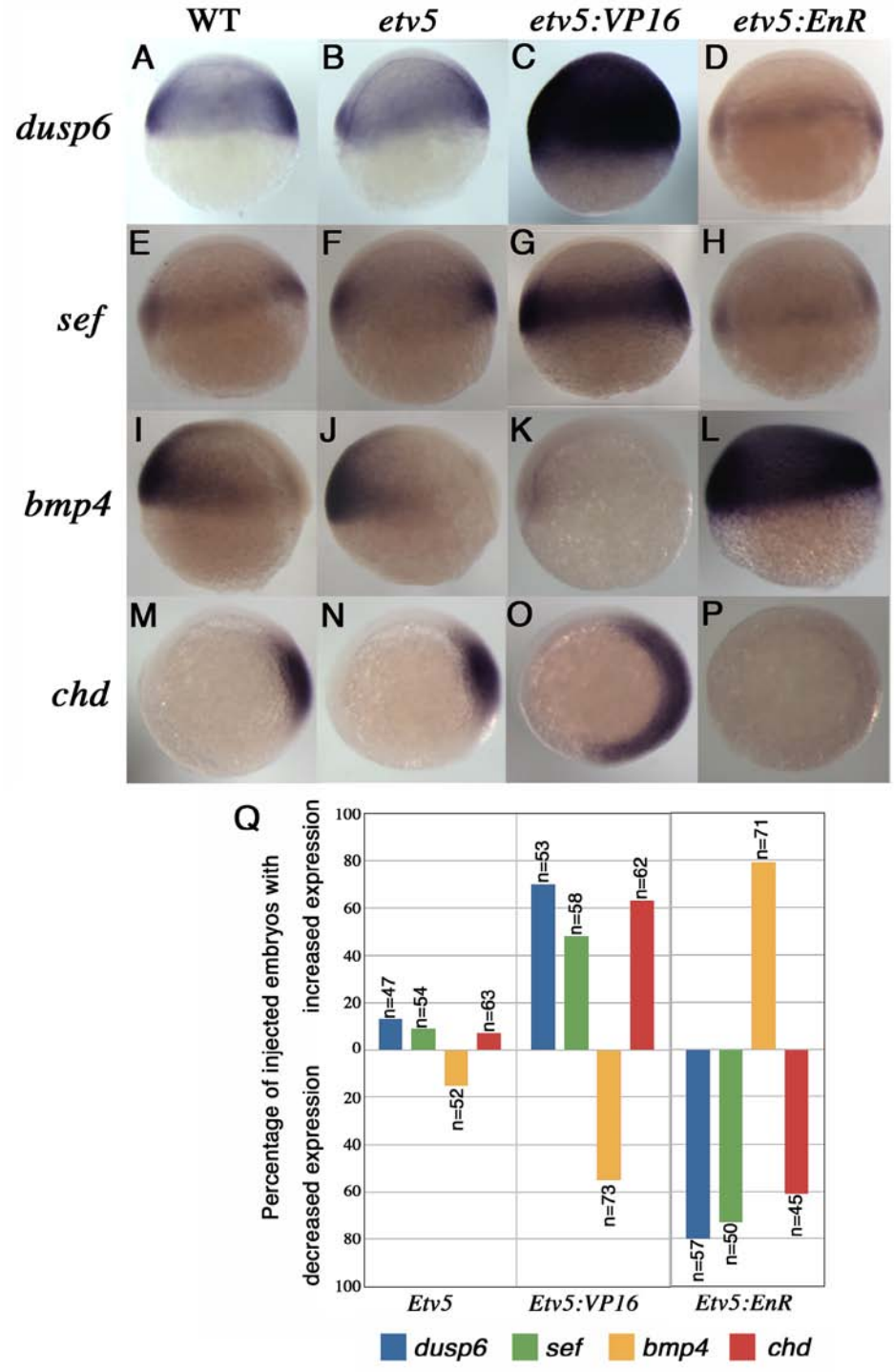
WT
etv5
etv5:VP16
etv5:EnR
A
B
C
D
dusp6
E
F
G
H
sef
I
J
K
L
bmp4
M
N
O
P
chd
Q
Percentage of injected embryos with
increased expression
decreased expression
100
80
60
40
20
0
20
40
60
80
100
n=47
n=54
n=52
n=63
n=53
n=58
n=73
n=62
n=57
n=50
n=71
n=45
Etv5
Etv5:VP16
Etv5:EnR
dusp6
sef
bmp4
chd

Figure 10: Etv5 Functions as a Positive Effector in FGF Signaling.

(A-L) Lateral views and **(M-P)** animal views at shield stage. *In situ* probes and constructs injected are indicated on the left and above, respectively. Expression of downstream targets of FGF signaling, *dusp6* and *sef*, are unaltered in etv5-injected embryos **(A,B,E,F)**, but were induced by *etv5:VP16* mRNA injections **(C,G)**. Ectopic expression of dominant negative (*etv5:EnR*) mRNA blocks *dusp6* and *sef* expression **(D,H)**. Expression of *bmp4* and *chordin* was unchanged in *etv5*-injected embryos **(I,J,M,N)**. A dorsalized phenotype was noted in *etv5:VP16*-injected embryos **(K,O)** and a ventralized phenotype upon *etv5:EnR* mRNA injections **(L,P)**. **(U)** Frequency of phenotypes elicited by microinjection of mRNAs indicated.

3.3 ETV5 IS PHOSPHORYLATED VIA ERK ON SPECIFIC RESIDUES TO RELAY FGF SIGNALS

The above results suggest Etv5 is non-functional when over-expressed in the native full-length form, and must be activated in some way in order to function. This is similar to previous observations that PEA3 and ERM contain auto-inhibitory domains that prevent these factors from binding to DNA (Janknecht et al., 1996; Laget et al., 1996). Since previous evidence has indicated PEA3 ETS factors play a role in FGF signaling in mice (Brent and Tabin, 2004; Liu et al., 2003; Mao et al., 2009; Zhang et al., 2009), this suggests that Etv5 must be activated by post-translational mechanisms such as ERK phosphorylation **(see F igure 1)**. Two previous experiments *in vitro* have shown the importance of ERK phosphorylation on ETS factor function (Laget et al., 1996; O'Hagan et al., 1996), but an *in vivo* analysis of ERK phosphorylation in zebrafish, along with the identities of specific phosphorylated residues on zebrafish ETS factors, has yet to be determined.

Scanning the Etv5 protein sequence using ScanSite and Netphos2.0 software, three ERK signature phosphorylation motifs were identified. Two putative threonine sites (T135 and T139) and one putative serine (S142) site were found in Etv5 in regions that are similar to the consensus ERK phosphorylation site (PXS/TP)(Gonzalez et al., 1991; Songyang et al., 1996). These putative threonine/serine phospho-acceptor sites are absolutely conserved across species, supporting they are likely to play an important role in Etv5/Erm function **(Figure 11)**. Since these regions of sequence are identical in Etv5 and Erm in zebrafish, these are likely to be the same phospho-acceptor sites for both family members. To determine the importance of these sites, I generated mutations in each of them to aspartic acid, which would mimic the introduction of a negative charged phosphate on the sites (*etv5:T135D*; *etv5:T139D*; *etv5:S142D*)**(Figure 11)**.

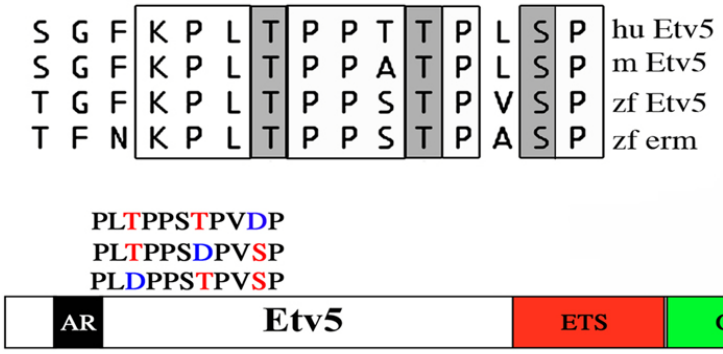

Figure 11: Putative ERK Phosphorylation Sites on Etv5/Erm.
Alignment of conserved putative ERK phosphorylation sites in Etv5/Erm. Constitutively active Etv5 constructs with threonines and serines mutated to aspartic acid are also indicated.

Regardless of which conserved site was mutated, injection of any of the phospho-acceptor mutations (50pg) at the 1-cell stage dramatically induced *dusp6* and *sef* at shield stage (**Figure 12A-D,F-I,U**). In conjunction with these results, embryos exhibited drastically

dorsalized phenotypes, indicated by expansion and suppression of *chd* and *bmp4* expression, respectively **(Figure 1 2K-N,P-S,U)**. The introduction of a double mutation, in which both threonines are mutated to aspartic acid in Etv5 (*Etv5:2T>D*), also induced downstream targets and caused a dorsalization phenotype **(Figure 1 2E,J,O,T,U)**. These phospho-acceptor-induced phenotypes indicate ETS factors can function as a protein that hyperactivates FGF signaling, and a single mutation on any of these 3 conserved serines/threonines was sufficient to generate a constitutively active Etv5. Thus, I have confirmed Etv5 functions as a transcriptional activator in FGF signaling, and identified 3 key residues that control Etv5 activity.

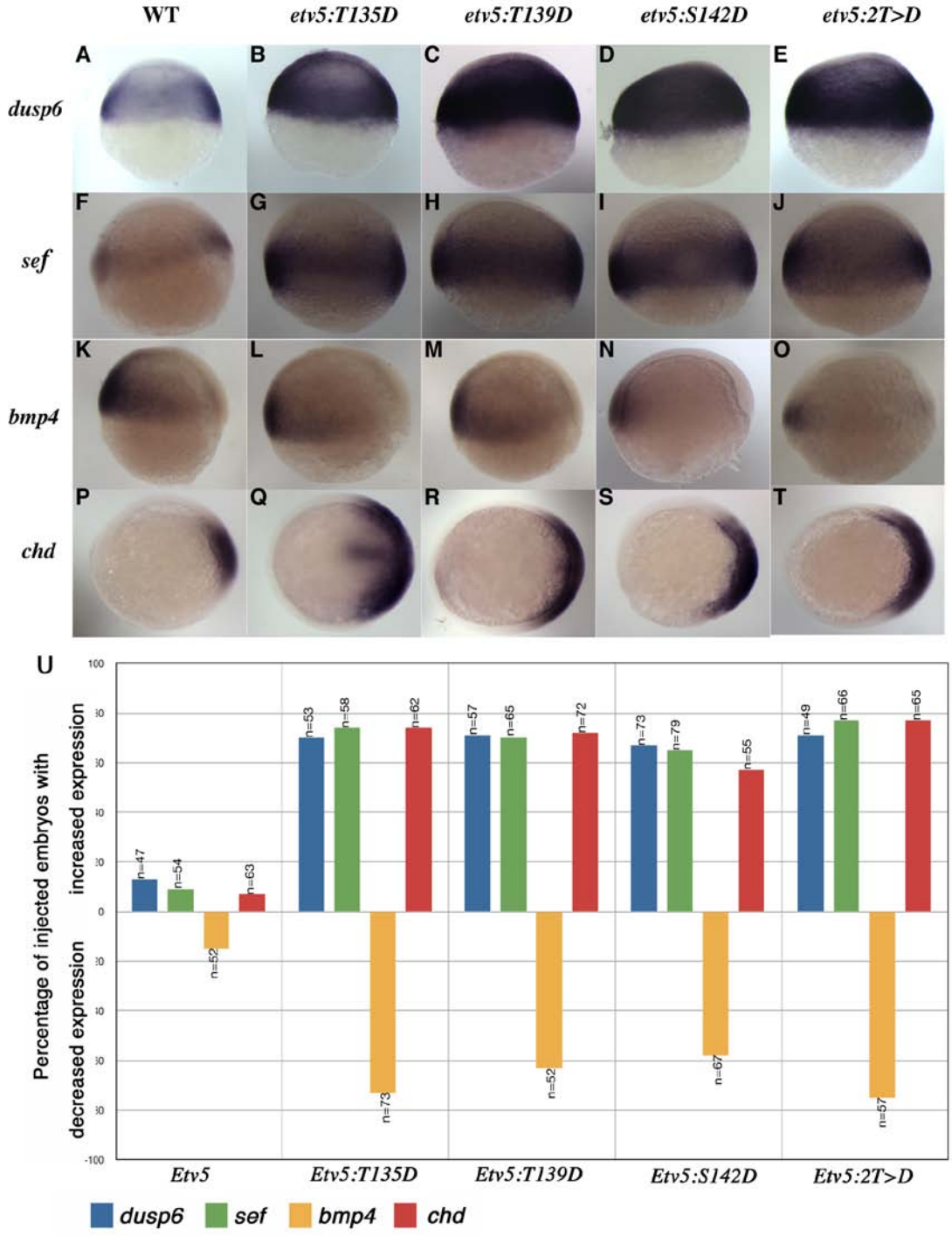
WT
etv5:T135D
etv5:T139D
etv5:S142D
etv5:2T>D
dusp6
sef
bmp4
chd
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
Percentage of injected embryos with
increased expression
decreased expression
100
-100
n=47
n=54
n=52
n=63
n=53
n=58
n=73
n=62
n=57
n=65
n=52
n=72
n=73
n=79
n=67
n=55
n=49
n=66
n=57
n=65
Etv5
Etv5:T135D
Etv5:T139D
Etv5:S142D
Etv5:2T>D
dusp6
sef
bmp4
chd

Figure 12: Any One of Three Conserved Residues is Sufficient to Activate Downstream Targets of FGF Signaling.

(A-O) Lateral views and **(P-T)** animal views at shield stage. *In situ* probes and constructs injected are indicated on the left and above, respectively. Expression of downstream targets of FGF signaling, *dusp6* and *sef* were induced by mRNA injections of *Etv5:T135D* **(A,B,F,G)**, *Etv5:T139D* **(C,H)**, and *Etv5:S142D* **(D,I)**. Mutation of two of these residues (*Etv5:2T>D*) displayed similar expansion **(E,J)**. Expression of *bmp4* and *chordin* indicates a dorsalized phenotype was noted in all phospho-acceptor forms **(K-T)**. **(U)** Frequency of phenotypes elicited by microinjection of mRNAs indicated.

3.4 PEA3 IS PHOSPHORYLATED ON A SPECIFIC SERINE RESIDUE TO RELAY FGF SIGNALING

Although Etv5 and Erm have similar conserved residues that are responsive to phosphorylation **(Figure 11 and 12)**, less sequence conservation is apparent between Etv5/Erm and Pea3 outside of the ETS DNA binding domain. Similar methods were used to find potential ERK phosphorylation sites using ScanSite and NetPhos2.0 within Pea3. A conserved serine residue (S100) was found contained in an ERK phosphorylation consensus sequence (PXS/TP) in Pea3 (Gonzalez et al., 1991; Songyang et al., 1996). Using site-directed mutagenesis, this residue was mutated to aspartic acid to generate a phospho-mimic version of Pea3 (*Pea3:S100D*). Injection of this construct into 1-cell stage embryos suppressed *bmp4* expression, while *chd* expression was expanded at shield stage. This dorsalized phenotype is again reminiscent of an overabundance of FGF signaling within the embryo, suggesting that this PEA3 ETS family member also plays a role in transcribing the FGF signal **(Figure 13)**. In addition, this provides

evidence that I have identified at leat one site within Pea3 that appears to be sensitive to ERK phosphorylation.

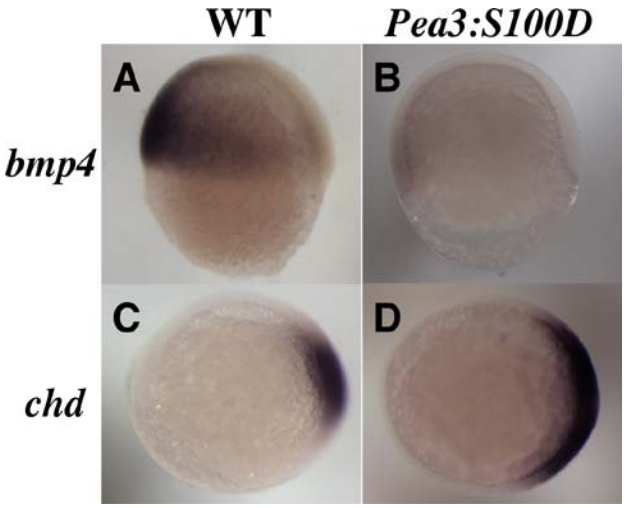

Figure 13: One residue in Pea3 is sufficient to regulate FGF signaling effects.
(A,B) Lateral views and **(C,D)** animal views at shield stage. *In situ* probes and constructs injected are indicated on the left and above, respectively. Mutation of one residue in Pea3, S100, to a phospho-mimic form (*Pea3:S100D*) results in a dorsalized phenotype, with expression of *bmp4* reduced **(A,B)** and expression of *chordin* expanded **(C,D)**. This phenotype is similar to overexpressing *fgf8* in embryos.

3.5 DISCUSSION

3.5.1 PEA3 ETS Factors are Transcriptional Activators of FGF Signaling

In Aim 1, I showed that PEA3 ETS factors function to mediate the FGF pathway transcriptional response in zebrafish. In agreement with previous studies in mice (Brent and Tabin, 2004; Liu et al., 2003; Mao et al., 2009; Zhang et al., 2009), ETS factors are transcriptional activators of FGF signaling in zebrafish. Injection of a constitutively active form of Etv5 (*etv5:VP16*) in embryos

showed an expansion of FGF target domains, *dusp6* and *sef*. In addition, *chd* was expanded and *bmp4* reduced in these embryos, an indication of dorsalization. This is reminiscent of overexpression of *fgf8* in embryos, another indication that ETS factors relay FGF signals. Conversely, a dominant negative form of Etv5 (*etv5:EnR*) diminished downstream FGF targets when injected into embryos. Ventralized phenotypes were also apparent in these embryos by analysis of *bmp4* and *chd*.

3.5.2 PEA3 ETS Factors are Activated Via ERK Phosphorylation

In addition, I have identified three conserved residues (T135, T139, S142) in Etv5 that are located outside of the ETS DNA binding domain and are part of the ERK consensus sequence (PXS/TP). Mutations of these sites to generate phospho-mimics activated downstream FGF targets when injected into embryos. Since high levels of amino acid sequence homology exist between Etv5 and Erm, these sites are likely to be phosphorylated in both Etv5 and Erm. Interestingly, phospho-mimic mutations in any one of these three residues results in large expansion of downstream FGF targets, indicating that all three sites may be direct targets of ERK phosphorylation. Injection of either threonine (135 or 139) mutants resulted in the same level of expansion of *dusp6* and *sef*, suggesting that each site elicits a complete response, and no single site is preferentially favorable to another. This was the first study to indicate specific ERK phosphorylation sites on ETS factors that can activate downstream FGF targets *in vivo*.

Previous studies have indicated that FGF-dependent activation of ERK2 was essential to induce Dusp6 under cell culture conditions (Ekerot et al., 2008). Further *in vivo* approaches examined a member of the Ets subfamily of ETS transcription factors, Ets1, in sea urchins to determine how this protein is activated. In this study, high levels of ERK were localized within

cells that will give rise to the urchin skeleton, termed primary mesenchyme cells (PMCs)(Rottinger et al., 2004). Ets1 was found to be a putative target for ERK phosphorylation, containing a single consensus site for phosphorylation via ERK. Overexpression of *ets1* increases the amount of PMCs, and this effect is inhibited when treating embryos with ERK inhibitor. Furthermore, mutating the consensus ERK binding sequence in Ets1 to aspartic acid or alanine resulted in constitutively active or dominant negative affects, respectively (Rottinger et al., 2004). Combining my data from Aim 1 with this data, it can be concluded that ETS transcription factors in several subfamilies and in multiple organisms are activated in the same way, via ERK phosphorylation. Interestingly, only one ERK phosphorylation site was identified on Ets1, while in Etv5, I have identified three putative ERK phosphorylation sites, all of which appear individually and collectively to regulate FGF downstream targets.

3.5.3 Other Modes of Post-Translational Modification in ETS Factors

Since Etv5 contains multiple sites for ERK phosphorylation, it is likely that other types of post-translational modifications are used in combination to temporally and spatially control activities of PEA3 ETS transcription factors. Recent studies indicate Small Ubiquitin-like MOdifier proteins (SUMO proteins) can control the activity of Pea3 and Erm (Degerny et al., 2005; Guo and Sharrocks, 2009). Sumoylation has been shown to impart repressive properties on transcriptional regulatory proteins (Gill, 2005; Girdwood et al., 2004), but has also been associated with transcriptional activation (Liang et al., 2004; Terui et al., 2004; Vrieseling and Arber, 2006). It was found that in the case of Pea3, SUMO modification takes place both *in vivo* and *in vitro* on multiple SUMO binding sites (ψKXEXXSP) within the N-terminal region. Importantly, this sumoylation of Pea3 was promoted by the activation of the ERK/MAPK

pathway, thus the same pathway may control two types of post-translational modifications, phosphorylation and sumoylation. Sumoylation of Pea3 results in ubiquitination and destabilization of the protein. This destabilization is required to control the activation of downstream promoters (Guo and Sharrocks, 2009). These results fit well with conclusions from Aim 1. When Etv5 phospho-mimics are injected into embryos, a strong, global activation of downstream targets occurs (**Figure 12 and 13**). In addition, multiple ERK phosphorylation sites are found on Etv5 that each can elicit a strong response (**Figure 1 2**). Due to the powerful response induced from ERK phosphorylation, it is likely that other post-translational modifications, such as sumoylation, are required to regulate the activities of PEA3 ETS transcription factors throughout development.

4.0 THE ROLE OF ETS TRANSCRIPTION FACTORS IN FGF-MEDIATED DEVELOPMENTAL PROCESSES

4.1 INTRODUCTION

Results from over-expression assays (Aim 1) suggest that PEA3 ETS transcription factors play a crucial role in relaying FGF signals during development. However, these results do not address the requirement for these factors in developmental processes. To ascertain the requirement for specific ETS factors during development, antisense Morpholino oligonucleotide technology (MO) was utilized in zebrafish. Antisense MOs are commercially generated oligonucleotides (GeneTools, Inc) that are customized to target the AUG codon of a specific mRNA. Annealing of the MO to the mRNA prevents ribosomal binding, blocking mRNA translation, and thus generation of the specific protein is knocked-down. The morpholine rings that make up the MO backbone, instead of deoxyribose rings, prevent nucleases and other enzymes within the cell from recognizing and breaking down the MO. These molecules can freely diffuse between the cytosol and the nucleus (www.gene-tools.com). Antisense MOs have been extensively utilized to knock-down gene function in zebrafish and *Xenopus* embryos (Heasman et al., 2000; Nasevicius and Ekker, 2000).

Specifically, since ETS factors are thought to function in FGF signaling, I examined FGF-mediated developmental processes in zebrafish. Due to the established large role of FGF

signaling on MHB formation (Blak et al., 2007; Blak et al., 2005; Crossley et al., 1996; Martinez et al., 1999; Trokovic et al., 2005; Walshe and Mason, 2000) and a critical role in early and late zebrafish heart development, (Araki and Brand, 2001; Marques and Yelon, 2009; Molina et al., 2009a; Reifers et al., 1998; Reifers et al., 2000), I examined to role of ETS factors in these processes.

4.2 GENERATION OF ANTISENSE MORPHOLINOS SPECIFICALLY TARGETED TO EACH INDIVIDUAL PEA3 ETS TRANSCRIPTION FACTOR

To determine the requirement of PEA3 ETS factors for FGF-mediated developmental processes, antisense MOs were generated that inhibited translation of members of the PEA3 ETS genes. Since sequence conservation and similar expression patterns during development suggest that the three ETS factors may perform redundant or overlapping functions, it was imperative that each MO generated was specific for only one PEA3 ETS family member. Each antisense MO targeted the AUG codon of *etv5*, *erm*, or *pea3*, and one MO targeted both *etv5* and *erm* (*etv5/ermMO*), given the high degree of identity between these two genes. To confirm MO specificity, 2-cell stage embryos were co-injected with MOs and a construct containing the 5' UTR of an ETS factor fused to enhanced green fluorescent protein (EGFP)**(Figure 14A)**. All combinations of MO and EGFP construct were injected into embryos, and were screened at shield stage for the presence of EGFP. A representation of the *etv5MO* specificity analysis is shown in **Figure 14B**. After co-injection of *etv5MO* and *etv5:EGFP*, EGFP expression was eliminated in these embryos showing the MO was functioning properly. Furthermore, this MO did not inhibit EGFP expression in other *5'UTR Ets:EGFP*-injected embryos, indicating the

specificity of the *etv5MO* **(Figure 14B)**. At shield stage, each individual MO suppressed expression of its respective Ets:EGFP fusion protein and not the other related constructs (**Figure 14C**), indicating that each MO was effective and specific.

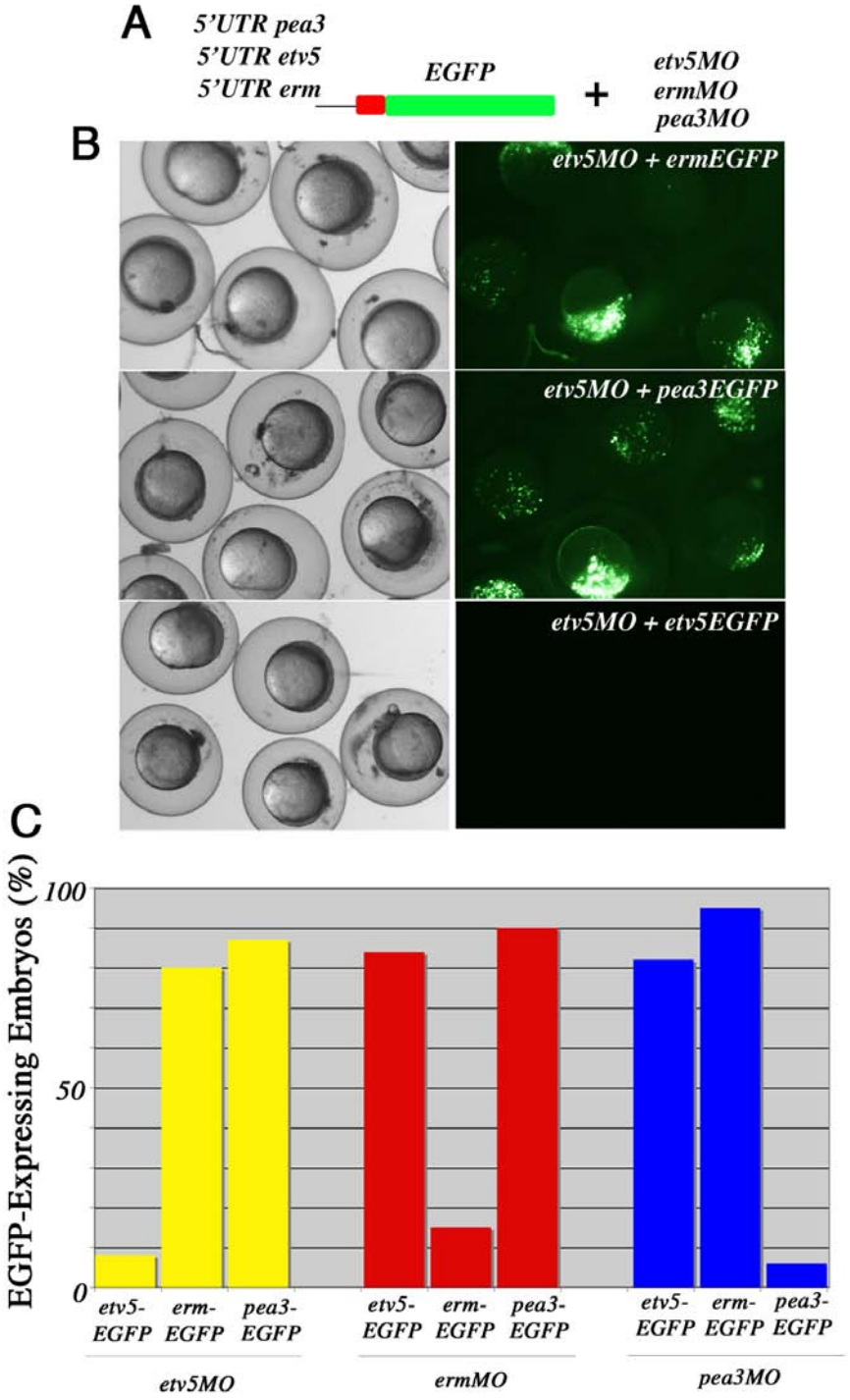

Figure 14: Specificity of PEA3 ETS Transcription Factor MOs.

(A) Experimental design to determine the specificity of each of the PEA3 ETS MOs. **(B)** Brightfield live images of shield stage embryos on the left and EGFP expression on the right. The construct and MO injected are indicated in the upper right corner. Embryos co-injected with the *5'UTR erm:EGFP* DNA and *etv5MO* at 2-4 cell stages or with *5'UTR pea3:EGFP* DNA and *etv5MO* at 2-4 cell stages expressed *EGFP*. In contrast, over 90% of embryos microinjected with *5'UTR etv5:EGFP* DNA and *etv5MO* did not show GFP expression, indicating the efficiency of *etv5MO*. **(C)** A graphical representation of all possible combinations of ETS MO and Ets:EGFP, supporting that each MO is specific for the knock-down of only one PEA3 ETS family member.

4.3 PEA3 ETS FACTORS FUNCTION REDUNDANTLY AS TRANSCRIPTIONAL MEDIATORS OF FGF SIGNALING

Microinjection of *etv5MO* into a 2-cell stage embryo resulted in minor effects on *dusp6* or *sef* expression at shield stage **(Figure 15A,B,E,F,Q)**. This is in agreement with previous mouse studies. Single knock-outs of *Erm, Pea3,* or *Er81* results in developmentally normal mice surviving to adulthood, although mice were mutant in motor neuron differentiation and spermatogonial stem cell renewal (Chen et al., 2005; Haase et al., 2002; Hippenmeyer et al., 2005; Livet et al., 2002). This lack of gross developmental defects could be explained due to the overlapping expression pattern of ETS factors. Redundancy may allow for compensation of individual deletion of ETS genes, whereby knocking out only one PEA3 ETS factor will not have an extensive effect on development. Due to this effect, I performed microinjections targeting two ETS factors, Etv5 and Erm (*2EtsMO*). It is important to note, that the total concentration of MO injected with the *2EtsMO* was identical to the *etv5MO* injections. The

knock-down of both *etv5* and *erm* resulted in a mild suppression of these FGF target genes (**Figure 15C,G,Q**). Furthermore, the concerted depletion of all three factors dramatically diminished both *dusp6* and *sef* expression, even with lower concentrations of MO targeting individual ETS factors **(Figure 15D,H,Q)**. Similar results were observed with *bmp4* and *chd* expression at shield stage. MO injections against a single family member had no impact on *bmp4* or *chd* expression (**Figure 15I,J,M,N,Q)**, however injections targeting multiple members of PEA3 ETS factors, such as *etv5* and *erm* (termed *2EtsMO* throughout this dissertation), or injections targeting all three members (termed *3EtsMO* throughout this dissertation) resulted in a disruption of dorso-ventral patterning **(Figure 15 K,L,O,P,Q)**. The expansion of *bmp4* concomitant with the suppression of *chd* expression indicates a ventralized phenotype, a common result of reduced FGF signaling. These results indicate that not only are PEA3 ETS factors important in mediating FGF signaling, but also act in a redundant manner.

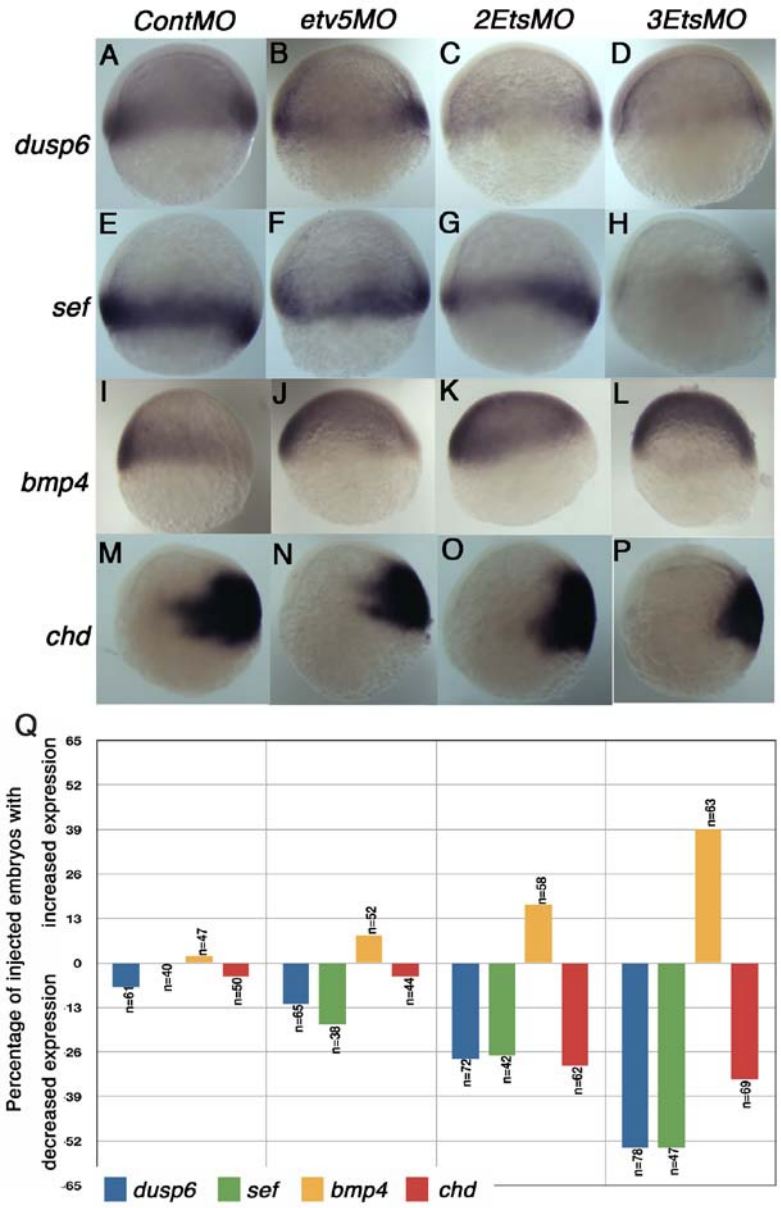

Figure 15: PEA3 ETS Factors Function Redundantly to Mediate FGF Signaling.

(A-P) Lateral views at shield stage. *In situ* probes and MO injected are indicated on the left and above, respectively. Expression of downstream targets of FGF signaling, *dusp6* and *sef* remained similar to *ContMO* upon *etv5MO* injection **(A,B,E,F)**, but were reduced when multiple ETS family members being knocked-down **(C,D,G,H)**. Expression of *bmp4* and *chordin* indicates a ventralized phenotype, but only after multiple family members have been target via MOs **(I-P)**. **(U)** Frequency of phenotypes elicited by microinjection of MOs indicated.

4.3.1 PEA3 ETS Factors are Critical in Establishing the MHB in Zebrafish

Due to the known importance of FGF signaling on the formation of the MHB (Blak et al., 2005; Crossley and Martin, 1995; Heikinheimo et al., 1994; Trokovic et al., 2005; Walshe and Mason, 2000), expression patterns of genes critical for the proper formation of the MHB were analyzed after MO injection. Embryos were injected with MOs at the 2-cell stage and *in situ* hybridizations were performed at 28hpf, a timepoint when the MHB region of the brain is properly formed. Overall phenotypic morphology of the MHB was also evaluated. This analysis determined the importance of ETS transcription factors in a specific FGF-mediated developmental process.

4.3.1.1 Expression Patterns of MHB Genes are Reduced in *EtsMO*-injected Embryos

Targeted depletion of a single family member or two family members did not result in discernable disruption of the expression of two MHB critical genes, *her5* and *pax2a* **(Figure 16A-C,E-G,M)**. In contrast, the knock-down of all three family members resulted in substantial reduction in *her5* and *pax2a* expression, indicating a disruption in MHB formation **(Figure 16D,H,M)**. Interestingly, expression of *dusp6* is even more sensitive to the knock-down of ETS factors, having almost completely reduced expression when only knocking down two factors **(Figure 16I-M)**. Although *dusp6* is expressed in other domains at 28hpf, the most dramatic reduction of expression in MO-injected embryos occurred within the MHB region. These results collectively indicate the importance of ETS factors in regulating gene expression within the MHB.

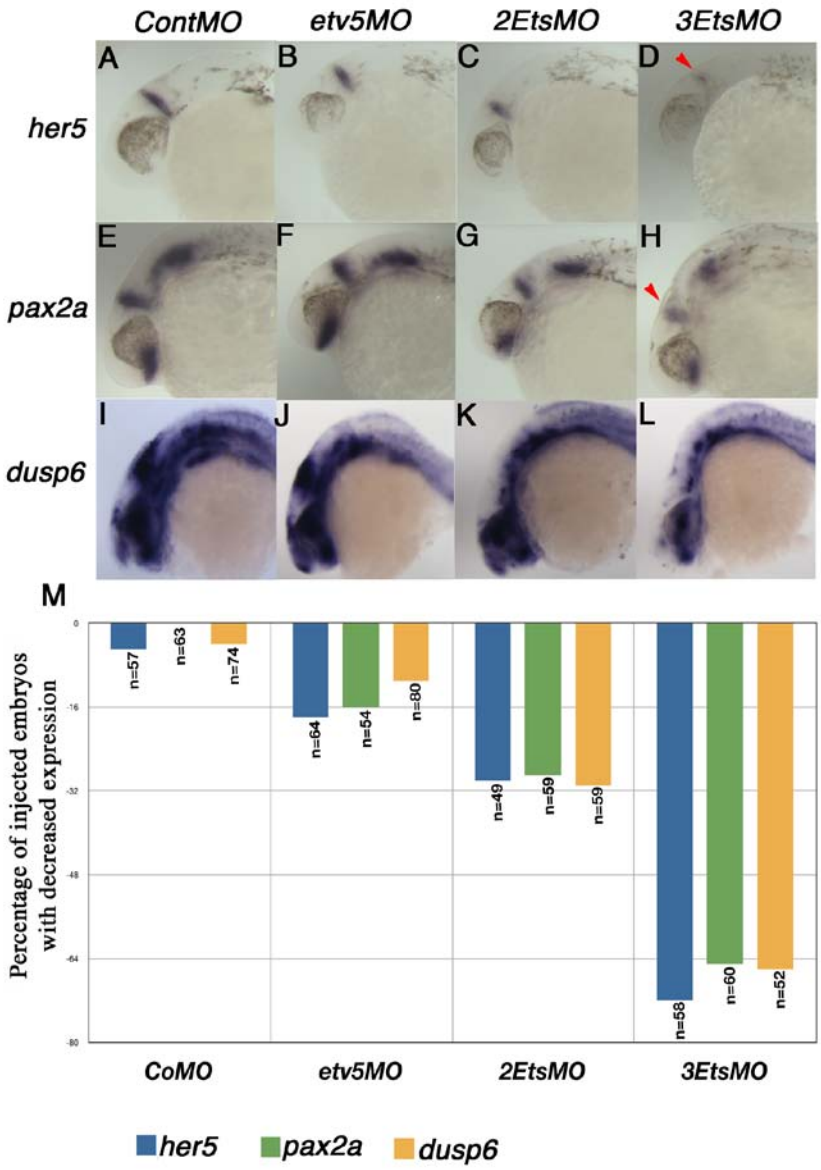

Figure 16: PEA3 ETS Factors are Required to Regulate Gene Expression within the MHB. **(A-L)** Lateral views of 28hpf embryos. *In situ* probes and MOs injected are indicated on the left and above, respectively. Expression of genes localized within the MHB are unaltered in *etv5MO*-injected embryos **(A,B,E,F,I,J)**. *2EtsMO* mildly reduces *her5* and *pax2a* expression **(C,G,M)**, while greatly reducing *dusp6* expression within the MHB region **(K,M)**. *3EtsMO*-injected embryos have reduction in all MHB markers examined **(D,H,L,M)**. **(M)** Frequency of phenotypes elicited by microinjection of MOs indicated. Red arrowheads mark loss of gene expression within the MHB.

4.3.1.2 PEA3 ETS Factors are Required for MHB formation

Due to the large reduction of gene expression within the MHB upon knock-down of PEA3 ETS factors **(Figure 16)**, a phenotypic analysis was then used to examine the overall formation of the MHB in the absence of ETS factors. Upon *etv5MO* injections, a well-formed MHB can still be seen clearly separating the midbrain from the hindbrain **(Figure 17A ,B)**. After injection of *2EtsMO*, the MHB structure breaks down drastically **(Figure 17C)** in 23% of embryos, and this phenotype is intensified in *3EtsMO*-injected embryos **(Figure 17D)**, apparent in 63% of embryos. These defects are similar to the *fgf8* zebrafish mutant, *acerebellar* (*ace*)(Picker et al., 1999; Reifers et al., 1998), further indication of the importance of these factors in the FGF pathway. These results reiterate that PEA3 ETS transcription factors are functionally redundant, and are necessary in an FGF-mediated developmental event, the formation of the zebrafish MHB.

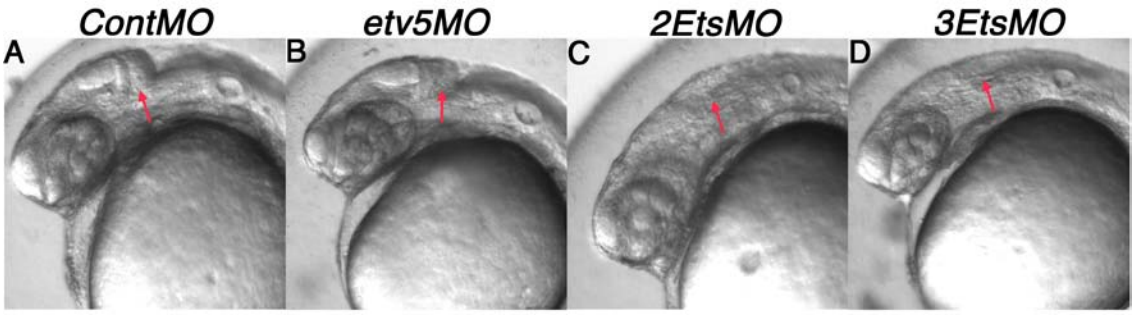

Figure 17: PEA3 ETS Factors are Required for Proper MHB formation.
(A-D) Brightfield lateral views of 28hpf embryos. **(A)** Injection of *etv5MO* does not disrupt MHB formation when compared to *ContMO*. MHB breakdown is apparent in *2EtsMO*-injected embryos **(C)** and is further intensified in *3EtsMO*-injected embryos **(D)**. Red arrows mark the MHB region.

4.3.1.3 *Ets* Morphant Phenotypes are rescued with ETS Factors

To confirm that the MHB phenotypes observed were the specific result of *EtsMO*-injections, rescue experiments were performed. Since the MOs have strong sequence specificity prior to and including the AUG codon region of each of these factors, tagged mRNA versions in which the tag is located just upstream of the the AUG codon should be resistant to MO recognition, and thus knockdown of the protein is inhibited. When performing co-injections of *3EtsMO* with MO-resistant *etv5* (*HAetv5*) or *pea3* (*HApea3*) mRNA, the MHB phenotype of the *3EtsMO* was rescued (**Figure 18A,B,D,E**; 73% rescued with *HAetv5*, n=71 and 66% rescued with *HApea3*, n=44). Furthermore, the co-injection of *HAetv5* and *HApea3* mRNA together did not result in an additive or synergistic rescue of *3Ets* morphants, suggesting that either gene can compensate for the loss of the other (**Figure 18F**; 69% rescue, n=56). These results provide further evidence indicating the redundant nature of this family of factors. Rescue attempts with random mRNA, such as *GFP*, did not rescue the *3EtsMO* MHB phenotype **(Figure 18C)**. To further examine this phenotypic rescue on the mRNA level, *in situ* results indicate induced expression of *dusp6* upon *HAetv5/HApea3* injection in *3Ets* morphants **(Figure 18G-I)**. These data further exemplify that PEA3 ETS factors function redundantly and are required in the FGF-mediated developmental process of MHB formation.

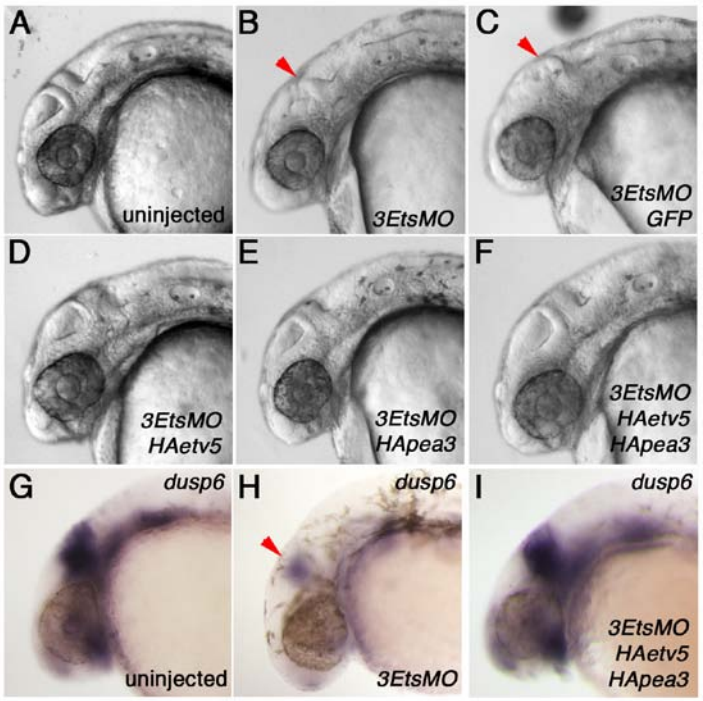

Figure 18: PEA3 ETS Factors rescue the MHB Phenotype of *3Ets* morphants.
(**A-I**) Lateral views of 28hpf embryos. Injection of antisense MOs targeting *pea3*, *erm* and *etv5* (*3EtsMO*) resulted in MHB defects (**B**) as compared to uninjected controls (**A**). *3EtsMO*-induced MHB defects can be rescued by co-injection of *HAetv5* and/or *HApea3* mRNA (**D-F**) but not by *GFP* mRNA (**C**). Expression of *dusp6* is diminished in *3EtsMO*-injected embryos (**G,H**). Co-injection of *HAetv5* and *HApea3* rescued *dusp6* expression (**I**). Red arrowhead marks loss of MHB in *MO*-injected embryos.

4.3.2 A Role for PEA3 ETS Factors during Cardiac Development

In mice, altering gene dosage of *Fgf8* or the source of Fgf8 protein demonstrated a role for *Fgf8* in cardiovascular development (Abu-Issa et al., 2002; Frank et al., 2002; Macatee et al., 2003; Meyers and Martin, 1999; Park et al., 2006). More recently, conditional ablation of *Fgf8* and *Fgf receptors* with different Cre driver lines revealed that Fgf8 regulates expression of *Pea3* and *Erm* in heart precursors and is required for outflow tract formation (Ilagan et al., 2006; Park et al.,

2006; Park et al., 2008). These studies determined the temporal and spatial role for Fgf8 derived from both the endoderm and mesoderm for proper morphogenesis of the heart, and showed that Fgf8 is required for proliferation and survival of cardiac progenitors in mice (Ilagan et al., 2006; Park et al., 2006). In addition, FGF signaling in zebrafish is necessary to specify and maintain cardiac progenitor cells during somitogenesis stages (Marques et al., 2008; Molina et al., 2009a; Molina et al., 2009b; Reifers et al., 2000). These studies lead to the analysis of the roles that PEA3 ETS factors may play in these processes.

4.3.2.1 PEA3 ETS Factors are Spatially and Temporally Located to Play a Role in Cardiac Development

Based upon previous fate mapping studies in zebrafish, heart precursor cells are within the anterior lateral plate mesoderm (ALPM) during early somitogenesis stages. These cells can be identified in two subpopulations, running parallel and on either side of the notochord from the anterior to posterior region (Lee et al., 1994; Stainier et al., 1993). For PEA3 ETS factors to play a role in maintaining cardiac progenitors, they must also be within the ALPM during early somitogenesis stages. *In situ* analysis of the three ETS factors at 8-somite stage shows expression of each of these factors within the ALPM region **(Figure 19)**. All three factors appear to have the same level of expression within this region, indicating they all may be important in this process. Due to this expression pattern, ETS factors may play a role in maintenance of cardiac progenitors and early heart development.

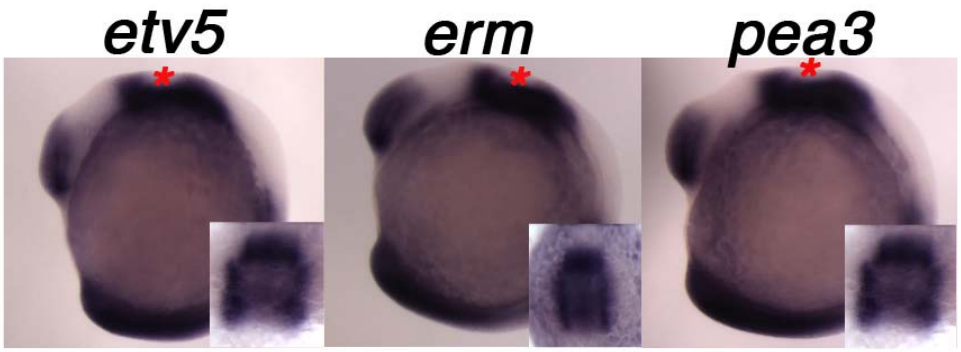

Figure 19: PEA3 ETS Factors are Expressed within the ALPM during Early Somitogenesis.

Lateral views of 8-somite stage embryos. *In situ* analysis indicates *etv5*, *erm*, and *pea3* expression within the ALPM during 8-somite stage, where cardiac progenitors are located. The inserts show a dorsal view of the magnified ALPM only (identified by the red asterisk), indicated by the two parallel subpopulations of cells.

4.3.2.2 A Role for PEA3 ETS Factors Maintaining Cardiac Progenitors

Injection of *etv5MO* mildly reduced expression of a cardiac specific transcription factor, *nkx2.5* (**Figure 20A,B,Y**). However, injection of *2EtsMO* or *3EtsMO* markedly reduced this cardiac population, a phenotype that was similar to *fgf8MO*-injected embryos or in *ace* mutants (**Figure 20C-F,Y**). *Gata4*, a gene expressed throughout the entire ALPM, was also reduced in both *2EtsMO*- and *3EtsMO*-injected embryos, and was comparable to *fgf8*-deficient embryos (**Figure 20G-L,Y**) (Draper et al., 2001; Marques et al., 2008; Serbedzija et al., 1998). I next analyzed expression of *hand2*, a marker for lateral cardiac progenitors in the ALPM (Yelon et al., 2000). Complimentary to the observed reduction of *nkx2.5*$^{+}$ cells, *hand2* expression was also reduced with the knock-down of multiple PEA3 factors or in *fgf8*-deficient embryos (**Figure 20M-R,Y**). As has been seen earlier, these factors appear to function redundantly to maintain the cardiac

progenitor populations, where the most dramatic changes are seen in *2EtsMO*- and *3EtsMO*-injected embryos.

Within the ALPM, an important interplay between the hematopoietic/vascular cells and cardiac progenitors exists to maintain the size of both populations (Keegan et al., 2004; Schoenebeck et al., 2007). I analyzed expression of *scl*, an endothelial transcription factor, in *3EtsMO*-injected embryos to determine if these factors play a role in endothelial lineages within the ALPM. Knock-down of PEA3 ETS factors resulted in an expansion of *scl* expression at the 10-somite stage (**Figure 2 0S-V,Y**). Most striking was that the depletion of *etv5* alone was sufficient to expand *scl* populations, indicating that this precursor population is particularly sensitive to Etv5 activity (**Figure 20T**). Similar MO injections against only *erm* or *pea3* had no effect on *scl* expression (data not shown), indicating the importance of a single, specific ETS family member, *etv5*, in restricting endothelial domains within the ALPM. Injection of *2EtsMO* or *3EtsMO* elicited stronger expansion of hematopoietic and vessel lineages as also noted in *fgf8*-depleted or *ace* embryos, implicating that the loss of FGF signaling expanded endothelial lineages (**Fig. 4W-Y**). Taken together, these results indicate that *fgf* ligands signal through ETS factors within the process of early heart development. ETS factors then play a pivotal role in maintaining cardiac progenitor identity. When these factors are knocked down, cardiac progenitors cannot be properly maintained, allowing an adjacent population of cells, expressing *scl*, to have an increased expression domain.

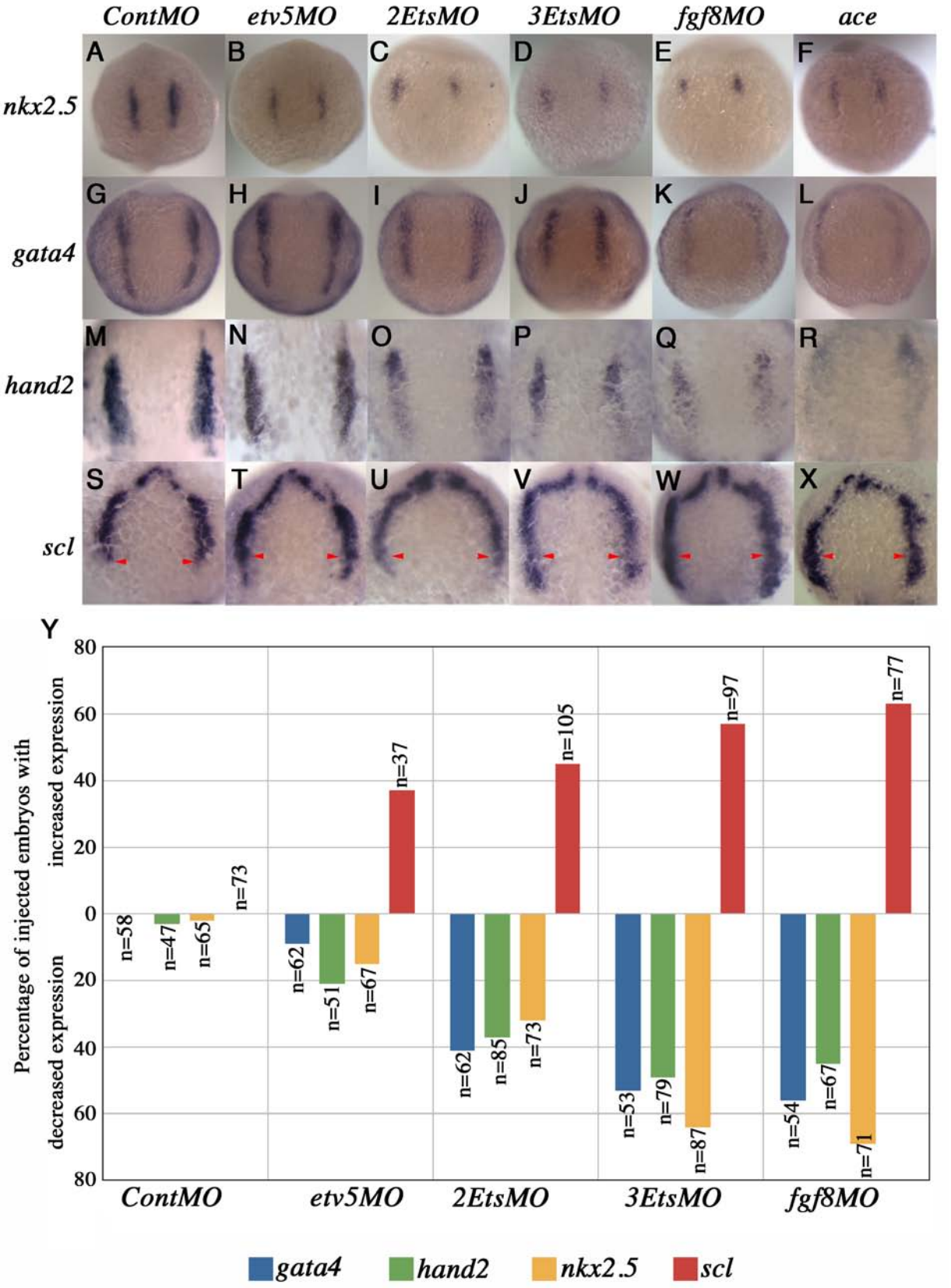
ContMO
etv5MO
2EtsMO
3EtsMO
fgf8MO
ace
nkx2.5
gata4
hand2
scl
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
Percentage of injected embryos with
increased expression
decreased expression
80
60
40
20
0
20
40
60
80
n=58
n=47
n=65
n=73
n=62
n=51
n=67
n=37
n=62
n=85
n=73
n=105
n=53
n=79
n=87
n=97
n=54
n=67
n=71
n=77
ContMO
etv5MO
2EtsMO
3EtsMO
fgf8MO
gata4
hand2
nkx2.5
scl

Figure 20: PEA3 ETS Factors are Required to Maintain Cardiac Progenitors.
(**A-X**) Dorsal views at 10-somite stage. *In situ* probe listed on left and MO above. (**A-D**) *nkx2.5* expression was reduced as multiple PEA3 ETS factors were knocked down. This was similar to *fgf8MO* knock-down and in *ace* mutants (**E-F**). (**G-J;M-P)** *gata4* expression (**G-J**) and *hand2* expression (**M-P**) were also reduced after PEA3 ETS depletion (**J,P**). A similar phenotype was observed in *fgf8MO* knock-downs and in *ace* mutants (**K,L,Q,R**). (**S-V**) Dorsal view of *scl* expression indicated expansion of endothelial lineages in *EtsMO* injections (**T-V**) as indicated by arrowheads that mark the caudal limit in uninjected embryos (**S**). Similar results were noted in *fgf8MO* and *ace* embryos suggesting that FGF signaling is required to maintain cardiac progenitors and to limit endothelial lineages to the rostral ALPM (**W,X**). (**Y**) Graph providing quantitative data for MO experiments.

4.3.2.3 The Role of PEA3 ETS factors in Late Heart Development

Since ETS factors were critical in maintaining cardiac progenitors, I next determined if the loss of cardiac progenitors at early somitogenesis stages resulted in later heart defects. I assayed expression of two specific cardiac differentiation markers at 24hpf, *ventricular myosin heavy chain* (*vmhc*) for ventricular tissue, and for atria, *atrial myosin heavy chain* (*amhc*) (Berdougo et al., 2003; Yelon et al., 1999). In *etv5MO*-injected embryos, *amhc* was expressed in a population of cells just below the left eye (**Figure 21 B**), resembling observations in *ContMO*-injected embryos (**Figure 2 1A**). In the *2EtsMO* and *3EtsMO*-injected embryos, diffuse *amhc* staining was noted at the midline, suggesting disruption of heart tube morphogenesis (**Figure 21C,D**). In *ContMO*- and *etv5MO*-injected embryos, *vmhc* expression outlined the heart tube as it "jogged" to the left (**Figure 2 1E,F**). However in *2EtsMO*- or *3EtsMO*-injected embryos, ventricle morphology was altered and was predominantly located at the midline between the eyes (**Figure 21G,H**). Thus, by disruption of cardiac maintenance earlier in development when knocking down ETS factors, late heart defects are apparent. Due to a decreased population of cardiac cells

during early heart development upon *EtsMO* injections, when these cell migrate to form the heart tube, they may not be capable to migrate correctly. This lack of cells and cell-cell contacts may be the cause of the lack of migration of the heart tube toward the left eye at 24hpf. Again, it can be noted that no alteration in phenotype in either *amhc*- or *vmhc*-expressing cells is apparent until multiple ETS factors are knocked down.

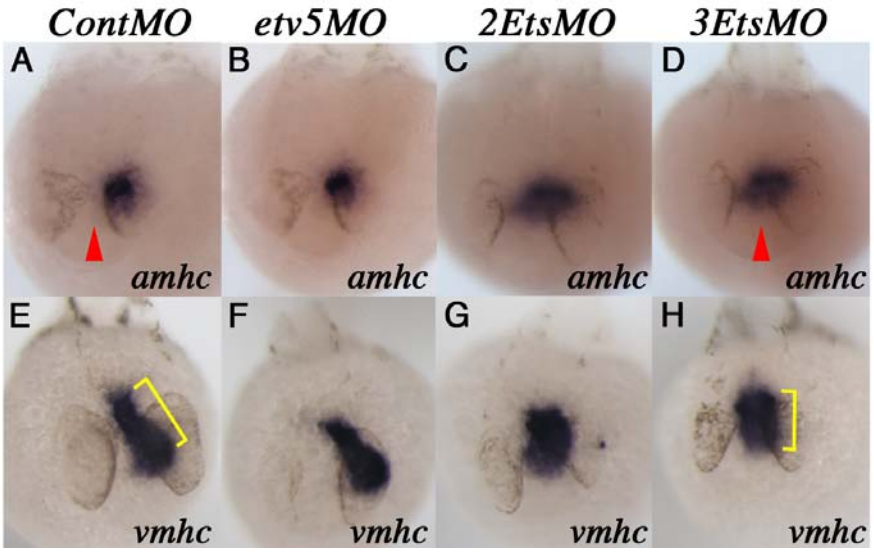

Figure 21: PEA3 ETS Factors are Required for Late Heart Development.
(**A-H**) *In situ* hybridization showing cardiac gene expression at 24hpf. Knock-down of multiple PEA3 ETS factors altered *amhc* (**D**; red arrowhead indicates midline of embryo) and *vmhc* expression (**H**; cell population and spatial arrangement in yellow bracket).

4.3.2.4 FGF Signaling is Necessary for Proper Heart Size and Looping

One of the final stages in embryonic heart development is cardiac looping. At 48hpf, the linear heart tube will begin to bend to cause a distinct division between the ventricle and atrium, creating an S-shaped heart (Chen et al., 1997; Chin et al., 2000). Due to this looping, the ventricle now lies to the right and dorsal of the atrium, and the two-chambered heart of the

zebrafish adult is now formed. In some cases, zebrafish with heart mutations will have hearts looped in the opposite direction, where the ventricle is positioned to the left of the atrium. This is commonly referred to "L-looping", since the mutant heart resembles the letter 'L', whereas wildtype hearts undergo what is called "D-looping", the final heart shape after looping resembling the letter 'D'. These mutants are thought to have defects in the initial assignment of the embryonic left-right (L/R) axis. In other mutants, hearts fail to loop, and remain as a straight heart. In these less severe phenotypes, defects could arise from molecular mechanisms that allow the heart to interpret L/R cues (Bisgrove et al., 2000; Chen et al., 1997; Chin et al., 2000). Interestingly, several of these mutations also cause defects in L/R morphogenesis of endodermal organs, such as the liver and gut, suggesting a common mechanism for generating all asymmetries (Bisgrove et al., 2000; Chen et al., 1997; Chin et al., 2000).

Previous studies examined the role of FGF signaling on heart size and looping during late heart development, but most of these studies examined heart defects due to decreasing FGF signals (see Section 1.3.5.2). Recently in our lab, using a transgenic zebrafish chemical screen, a small molecule inhibitor of Dusp6 was identified: (E)-2-**b**enzylidene-3-(**c**yclohexylamino)-2,3-dihydro-1H-**i**nden-1-one (BCI) (Molina et al., 2009a). BCI treatment blocked Dusp6 activity and enhanced FGF target gene expression in zebrafish embryos. This small molecule could now be used to examine the effects of hyper-activation of FGF signaling upon late heart development. Treating embryos at 40% epiboly for eight hours with 10μM BCI showed a drastic increase in heart size at 48hpf when looking at both *vmhc* **(Figure 22A,B)** and *cardiac myosin light chain 2* (*cmlc2*), marking myosin in all cardiac cells **(Figure 22C,D)**.

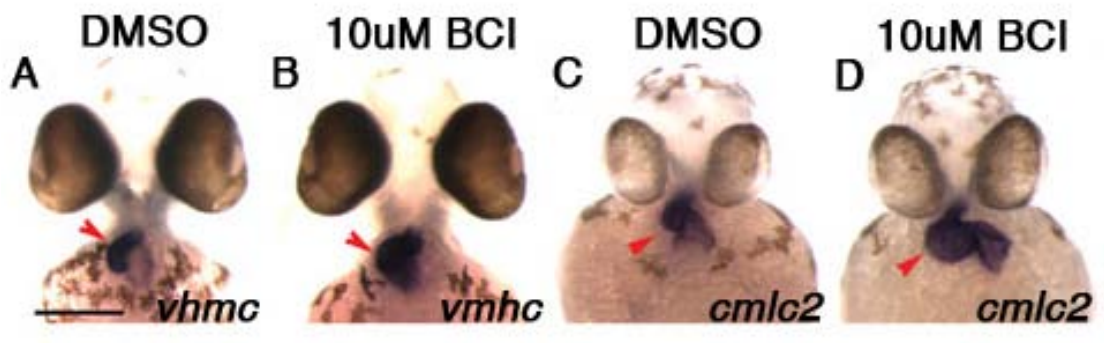

Figure 22: Hyper-activation of FGF Signaling with BCI Increases Cardiac Size.
(A-D) *In situ* hybridization showing cardiac gene expression at 48hpf. *vmhc* **(A,B)** and *cmlc2* **(C,D)** expression were drastically increased upon treatment of BCI for eight hours. Red arrowheads indicate heart region. Scale bar, 250 μM.

Examining this effect of hyper-activation of FGF signaling more closely, it was determined that the developmental time period when embryos are treated with BCI has unique impacts on heart development. When treating embryos with BCI early in somitogenesis (1 somite stage) through 24hpf, the heart greatly increases in size, indicated by an increase in *cmlc2* expression **(Figure 23 A,B)**. However, when hyper-activating FGF signaling during mid-somitogenesis (8 somite stage), the heart does not change in size, but the looping of the heart is reversed. Instead of the common D-looping phenotype **(Figure 23C)**, embryos are displaying a reversed, L-looping phenotype **(Figure 23D)**.

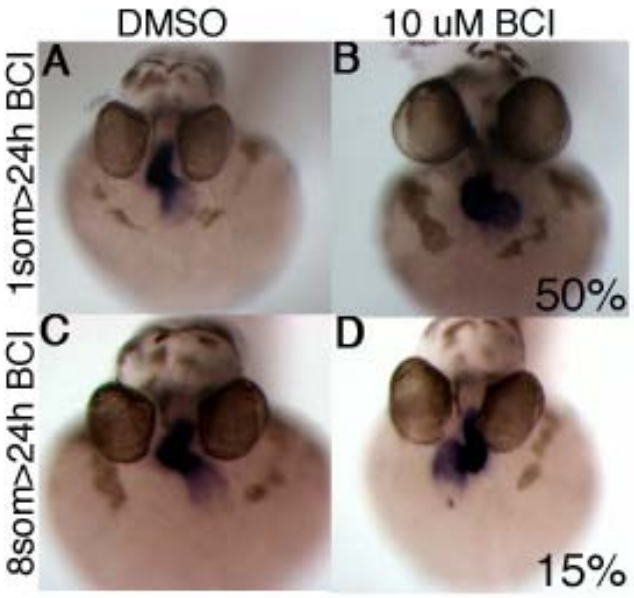

Figure 23: Late Cardiac Development is Sensitive to the Timing of FGF Hyper-activation. **(A-D)** *In situ* hybridization showing cardiac gene expression at 48hpf. *cmlc2* expression indicates a large increase in heart size when FGF signaling is hyper-activated during early somitogenesis **(A,B)**, while heart looping is effected when FGF signaling is hyper-activated during mid-somitogenesis **(C,D)**.

4.3.2.5 Heart Looping is Regulated by PEA3 ETS Factors

Previous studies examining embryos with decreased FGF signaling (Abu-Issa et al., 2002; Lopez-Sanchez et al., 2002; Marques et al., 2008; Molina et al., 2009a; Reifers et al., 2000) or increasing FGF activity (Molina et al., 2009a) displayed late heart defects. Thus, it appears that a proper level of FGF signaling must be maintained during development to form a proper heart. Too little or too much FGF signaling can result in mispatterning of the heart, such as heart looping defects. Since altered FGF signaling was shown to have an impact on heart looping, I next examined if FGF signals were transcribed through PEA3 ETS factors to have an effect on heart looping events. If so, altering expression of ETS factors should cause heart looping defects. To examine if the knock-down of ETS factors affect heart looping, *in situ*

hybridizations with *cmlc2* expression at 48hpf were performed. Since *cmlc2* is expressed within the entire heart, looping of the heart is easily visible. Disruption of cardiac looping in *3EtsMO*-injected larvae was identified, resulting in either non-looping straight hearts or reversed looping hearts **(Figure 24A-D**; non-looping, straight heart phenotypes not shown**)**. The instances of hearts with altered looping increases as multiple ETS factors are knocked down **(Figure 24E)**. These data suggested that PEA3 ETS factors are required for proper heart morphogenesis. Thus, ETS factors are likely to relay signals from Fgf8, Fgf24 and Fgf4, all ligands shown to be necessary to establish proper L/R asymmetry (Neugebauer et al., 2009; Yamauchi et al., 2009).

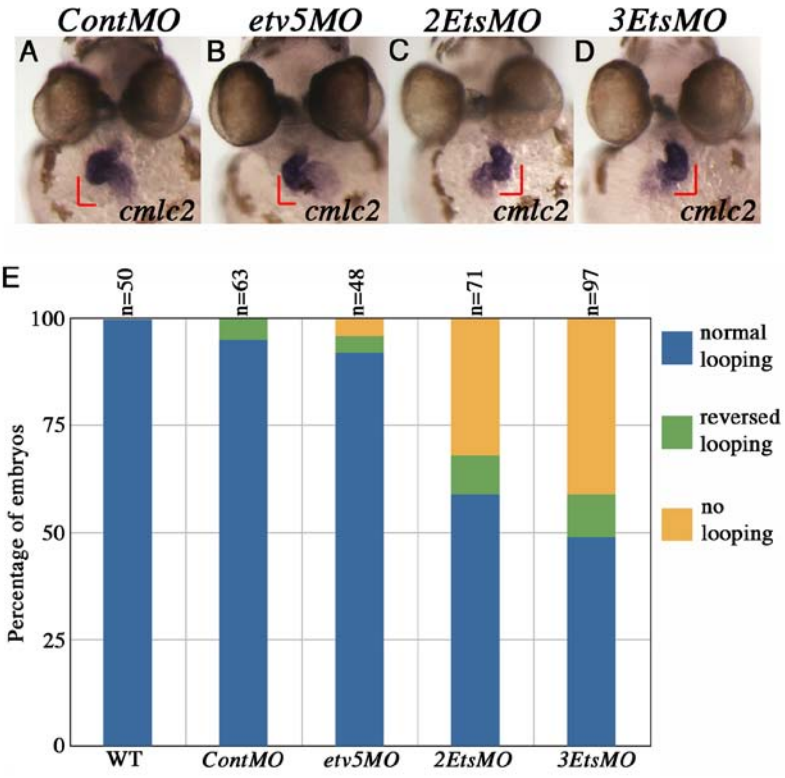

Figure 24: Proper Cardiac Looping Requires PEA3 ETS Factors.

(A-D) *In situ* hybridization showing cardiac gene expression at 48hpf. Cardiac looping was severely affected in *2EtsMO*- **(C)** and *3EtsMO*-injected embryos **(D)** as shown by expression of *cmlc2*. (**E**) Quantification of effects on heart looping in MO-injected embryos.

4.3.3 PEA3 ETS Factors Function in L/R Asymmetry

Given the L/R patterning defects observed in the knock-down of PEA3 ETS factors **(Figure 24)**, I Their role in asymmetry formation during development was next investigated. Even though outwardly symmetrical, internal asymmetries exist within the zebrafish, such as the sidedness of the gut and the directional looping of the heart. The initial break in symmetry is thought to be due to nodal flow (Brown et al., 1991; Brown and Wolpert, 1990), where the node region of the embryo gives bilateral symmetry and midline axis to the embryo. This break in asymmetry has been thought to involve a leftward flow of extra-embryonic fluid around the node that would transport molecules, which could act as a 'handed' determinant (Brown et al., 1991; Brown and Wolpert, 1990). For example, nodal flow is thought to push molecules located around the mouse node to one side of the embryo. In zebrafish, a transient ciliated organ called Kupffer's vesicle (KV) is thought to be analogous to the mouse node (Cooper and D'Amico, 1996; D'Amico and Cooper, 1997; Essner et al., 2002). Examining the effect, if any, of PEA3 ETS factors on KV formation and function may explain the L/R heart defects seen in *Ets* morphants **(Figure 24)**.

4.3.3.1 PEA3 ETS Factors are Not Required for KV Formation

Our lab has recently generated a transgenic reporter line, *Tg(dusp6:d2EGFP)*^{pt6}, that expresses *d2EGFP* in response to FGF signaling (Molina et al., 2007). To generate these transgenic fish, a 10Kb fragment that included the 5' untranslated sequence within exon I of Dusp6 was subcloned into a vector containing *d2EGFP*, a gene that encodes a destabilized green fluorescent protein that has a two hour half-life. Since *dusp6* is controlled by FGF signaling throughout development (Tsang et al., 2004), expression of *d2EGFP* is only present in FGF expression domains (Molina et al., 2007). Expression of *d2EGFP* is initiated as early as 4hpf within the

future dorsal region of the embryo, where *fgf3* and *fgf8* are initially expressed. At later stages, *d2EGFP* is detected within the MHB, pharyngeal endoderm, otic vesicle, hindbrain, and KV, structures that correlate with the expression of *fgf* ligands and their receptors. Injection of *3EtsMO* into *Tg(dusp6:d2EGFP)*^{pt6} embryos resulted in decreased GFP fluorescence within the MHB, rhombomere 4, and KV, suggesting that PEA3 ETS proteins are required for GFP reporter gene expression (**Figure 25A,B**). This was to be expected, since I had already determined PEA3 ETS factors are necessary to transcribe the FGF signal **(Figures 10, 12, and 13)**. However, even though GFP expression, and thus FGF signaling, was extinguished in the KV, the vesicle did form properly. This can be seen in brightfield images, where the spherical structure is completely formed in *3Ets* morphants (**Figure 25A',B'**). Therefore, FGF signaling and PEA3 ETS factors are not required for KV formation.

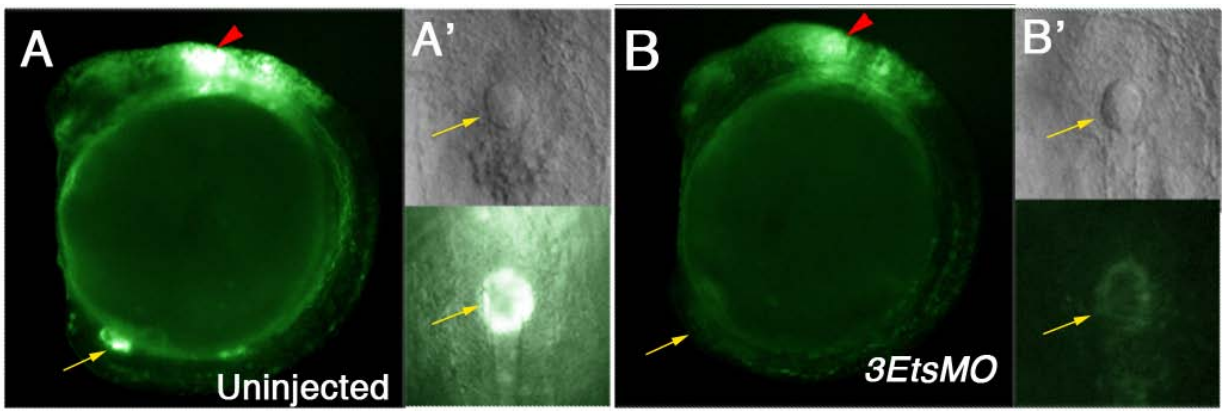

Figure 25: PEA3 ETS Factors are Not Needed to Properly Form KV.
(**A,B**) Lateral views of *Tg(dusp6:EGFP)*^{pt6} embryos at 8 somite stage. **(A',B')** Zoomed, ventral views of the spherical KV. **(A)** Wildtype embryos show *d2EGFP* expression within KV along with other FGF-expressing regions. **(A')** KV is formed properly and highly expresses *d2EGFP*. **(B)** Embryos injected with *3EtsMO* show grossly normal formation of Kupffer's vesicle **(B')**, even though FGF signaling was suppressed (**B,B'**). Red arrowheads indicate the MHB, a region of high FGF activity. Yellow arrows indicate KV.

4.3.3.2 Cilia Formation within KV is Sensitive to PEA3 ETS Factor Expression

L/R patterning defects are apparent in ETS-depleted embryos, even though KV appears to have developed properly. Since KV appeared to develop and form properly, I next analyzed the cilia found within KV. In both the KV floor and the roof, the cilia are posteriorly pointed and rotate clockwise when viewed apically. Microinjection of beads into the KV indicated that the cilia cause a net circular flow, but the local flow differs in direction depending on the location within the vesicle. The plane of the circular net flow is tilted within the KV, and thus cells in the anterior-dorsal region experience a local dominant leftward flow (Okabe et al., 2008). Studies have shown that altering cilia within the KV of developing zebrafish embryos affect L/R asymmetry. More specifically, knocking down FGF signaling has been shown to affect cilia formation (Hong and Dawid, 2009; Neugebauer et al., 2009; Yamauchi et al., 2009).

Analysis of cilia in the KV of *3EtsMO*-injected embryos revealed a significant decrease in cilia number (**Figure 26A ,B,E;** $p = 5.21 \times 10^{-8}$). In addition, expression of *foxj1a*, a transcription factor shown to be involved in ciliogenesis and localized in cells that give rise to the KV (Bonnafe et al., 2004; Brody et al., 2000), was reduced or absent in *3EtsMO*-injected embryos, implicating the importance of ETS transcription factors in cilia formation (**Figure 26C,D**; 33% with reduced *foxj1a* expression in *3EtsMO*-injected embryos, n=112). A recent study shows a similar decrease in *foxj1a* expression when *fgfr1* was depleted (Neugebauer et al., 2009).

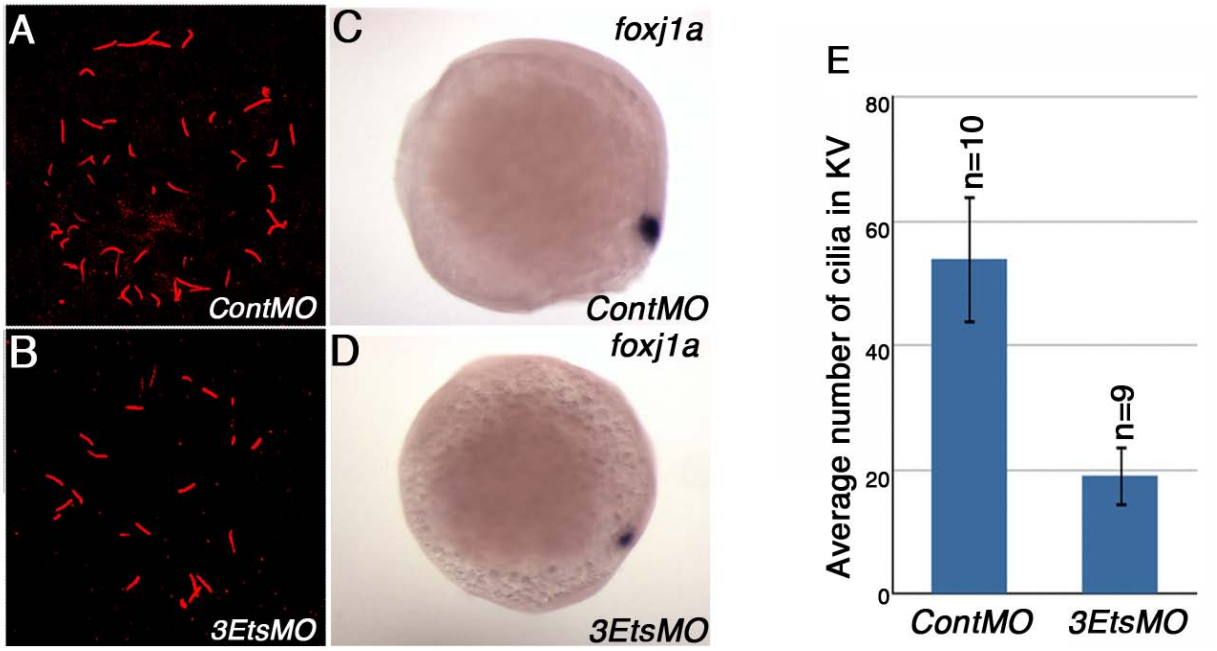

Figure 26: PEA3 ETS Factors are Required for Ciliogenesis in the KV.

(A,B) Confocal images of KV at 12 somite stage, ventral view. Cilia number in the KV was reduced in *3EtsMO*–injected embryo **(B)** compared to *ContMO*-injected embryos when analyzed using acetylated tubulin **(A)**. (**C,D**) *In situ* hybridization at 90% epiboly, lateral view. Expression of *foxj1a* is significantly reduced in *3EtsMO*-injected embryos **(D)** when compared to *ContMO*-injected embryos **(C)**. **(E)** Graph showing average number of cilia in *MO*-injected embryos. Error bars indicate the standard deviation.

4.3.3.3 Disrupting Cilia Formation Alters L/R Patterning in *3Ets* Morphants

Recent reports indicate the complexity of all cilia beating in varying ways, depending on the location within the KV, to cause a global leftward flow (Amack et al., 2007; Kreiling et al., 2007; Okabe et al., 2008). To determine if the lack of KV cilia within *3EtsMO*-injected embryos can be attributed to the L/R asymmetry defects in heart looping, *spaw*, a zebrafish Nodal gene, was analyzed. *spaw* is normally expressed in the left lateral plate mesoderm (Long et al., 2003) and is the first marker of asymmetry in the zebrafish. In *3EtsMO*-injected embryos, *spaw*

expression was completely randomized; often absent, bilateral, or right-sided. This is consistent with disruption of upstream initiation of the L/R cascade (**Figure 27A-D**). These L/R defects increase as multiple ETS factors are knocked down **(Figure 27E)**. Interestingly, knocking down Fgf8 only does not cause as drastic of an effect in L/R patterning as injection of *3EtsMO* **(Figure 27E)**. This may indicate that more ligands in addition to Fgf8 signal through ETS factors to play a role in L/R patterning. This finding agrees with a current model, whereby Fgf24 ligands in addition to Fgf8 ligands will signal through FGFR1 and activate transcription factors that will regulate intraflagellar transport genes to maintain proper motile cilia (**Figure 4**)(Neugebauer et al., 2009).

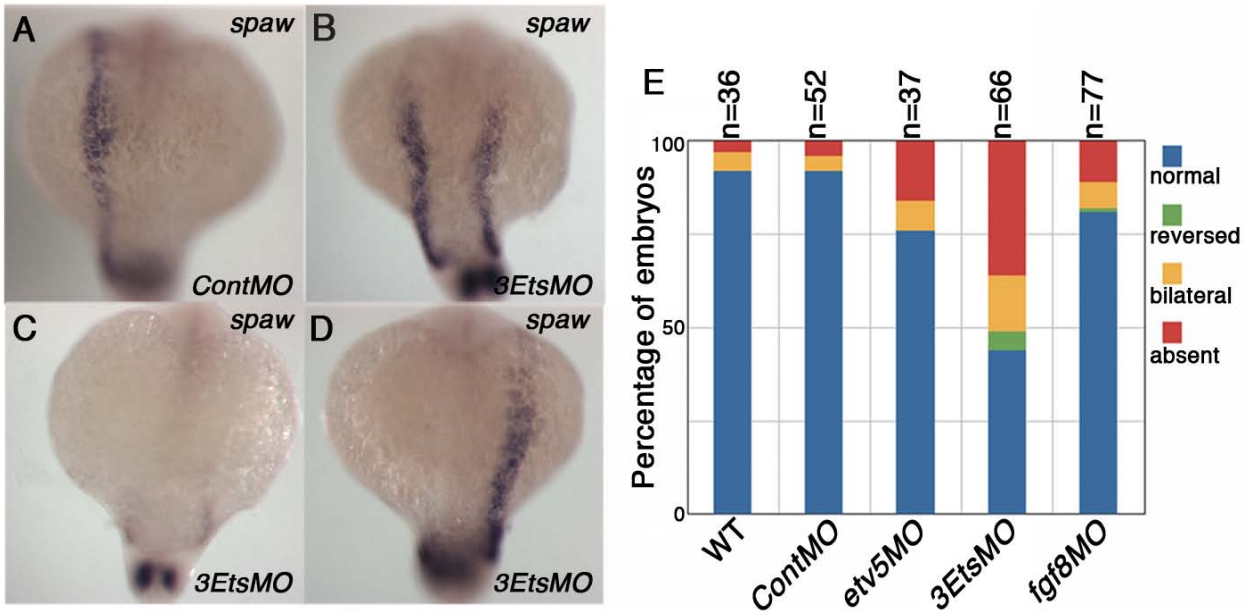

Figure 27: *spaw* Expression is Completely Randomized in *3EtsMO*-injected Embryos.
(A-D) *In situ* hybridization showing *spaw* expression at 18 somites, dorsal view. *spaw* expression is completely randomized in *3EtsMO*-injected embryos **(B-D)** when compared to *ContMO*-injected embryos **(A)**. Graph depicting L/R defects in PEA3 ETS- and Fgf8-depleted embryos.

4.4 DISCUSSION

4.4.1 The Role of ETS Factors in FGF-Mediated Developmental Processes

In Aim 2 I showed that PEA3 ETS factors function to mediate the transcriptional response of the FGF pathway in zebrafish. Antisense MO injections blocking the translation of *erm*, *etv5*, and *pea3* resulted in suppression of FGF target genes as well as a MHB defect in the zebrafish embryo. The importance of these ETS factors in heart development was revealed by the loss of cardiac progenitors in PEA3 ETS factor-depleted zebrafish embryos, consistent with a critical role for these factors downstream of FGFs in mice (Liu et al., 2003; Lu et al., 2009; Zhang et al., 2009). In zebrafish, experimental manipulations that expand cardiac progenitors negatively affected the blood and vessel lineages, and vice versa (Molina et al., 2009a; Molina et al., 2009b; Schoenebeck et al., 2007). Given that Fgf8 and PEA3 ETS factors play an important role in maintaining cardiac identity, I reasoned that the knock-down of PEA3 ETS factors decrease cardiac progentior populations. Indeed, the concerted depletion of PEA3 ETS factors resulted in a decrease in cardiac progenitors. This was concomitant with an expansion of blood and vessel progenitors as marked by expanded *scl* expression. Interestingly, one ETS factor, Etv5, was shown to be critical for proper *scl* expression, indicating that, although PEA3 ETS factors appear to function redundantly in most FGF-mediated developmental processes, this specific factor appears to be solely responsible for proper endothelial expression within the ALPM. Thus, FGF activity is required to maintain cardiac progenitor populations and suppress endothelial differentiation within the caudal domain of the ALPM **(Figure 28)**.

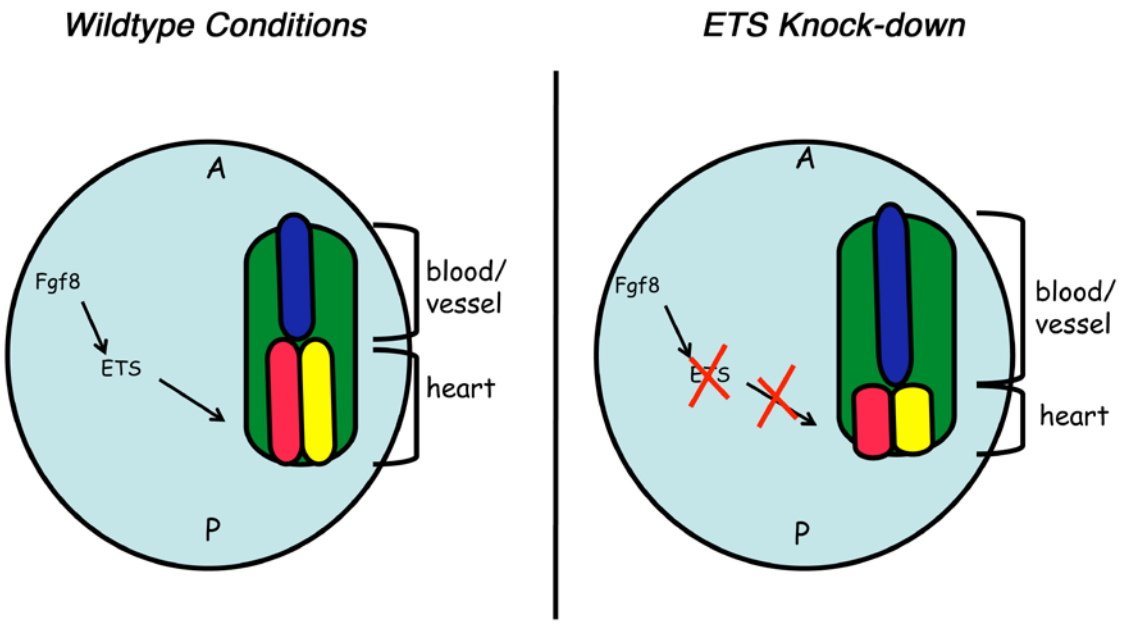

Figure 28: Model of ETS Factor Regulation on Cardiac Development.
Fgf lignds signal through PEA3 ETS factors that will maintain cardiac identity. When ETS factors are knocked down, this allows the blood and vessel populations to expand, while cardiac lineages are reduced.

Recent studies have highlighted a role for FGF signaling in L/R patterning of the zebrafish embryo (Albertson and Yelick, 2005; Hong and Dawid, 2009; Neugebauer et al., 2009; Yamauchi et al., 2009). The knock-down of either Fgf ligands or two effectors of the FGF pathway did not disturb formation of the KV, but did disrupt monocilia formation (Hong and Dawid, 2009; Neugebauer et al., 2009; Yamauchi et al., 2009). Similarly, in this study, depletion of PEA3 ETS factors did not disrupt KV formation, but cilia number was markedly reduced. This is responsible for the laterality defects such as alteration of *spaw* expression and both the cardiac jogging and looping defects. Interestingly, among these recent studies, the role of FGF signaling in proper monocilia formation within the KV has ranged from contributing to cilia length (Neugebauer et al., 2009; Yamauchi et al., 2009) to contributing to the total number of

cilia within the KV (Hong and Dawid, 2009). Due to multiple cilial phenotypes displayed when knocking down FGF signaling, it can be hypothesized that the specificity in patterning of KV monocilia may involve several Fgf ligands and/or transcription factors. In addition, it could also be hypothesized that Fgf ligands have morphogenic effects on cilia formation, whereby a certain level of FGF signaling must reached for proper cilial number, while another level of FGF signaling is necessary for proper cilial length. Within this study, I have demonstrated that the PEA3 ETS transcription factors relay the FGF signal to allow for formation of the proper number of cilia within KV.

4.4.2 Redundant Functions of PEA3 ETS Transcription Factors Throughout Development

Erm/Etv5 or *Pea3* gene knock-out approaches in mice resulted in developmentally normal animals that survived to adulthood, but developed motor neuron differentiation defects, spermatogonial stem cell renewal defects, and movement disorders (Arber et al., 2000; Chen et al., 2005; Livet et al., 2002). The lack of gross developmental phenotypes in these knock-out mice suggested that these genes may function redundantly in mediating FGF signaling. To circumvent redundancy among PEA3 factors, engrailed repressor fusion constructs were ectopically expressed in both mouse and chick embryos (Brent and Tabin, 2004; Liu et al., 2003). The repression of multiple PEA3 ETS family members using this technique overcame redundancy to show a role for these factors in somite and lung development (Brent and Tabin, 2004; Liu et al., 2003). Further evidence of their redundant function was shown by ectopic expression of a dominant negative version of *Etv5* in the mouse limb which resulted in shortened limbs and polydactyly (Mao et al., 2009). This was confirmed by the knock-out of both *Etv4* and

Etv5 in early mesodermal lineages that also resulted in limb and digit defects (Zhang et al., 2009). In the studies presented in this dissertation, antisense morpholinos allowed the targeted depletion of multiple members of the PEA3 family. In general, knocking down only one member of this family rarely resulted in developmental defects, but the knock-down of two or three members resulted in distinct phenotypes.

Functional redundancy within another ETS sub-family of transcription factors in zebrafish has also been described (Pham et al., 2007). This study examined four ETS family members expressed in the vasculature: *fli1*, *fli1b*, *ets1*, and *etsrp* (Pham et al., 2007). Using an antisense MO approach to knock down expression of all four genes, both vascular and hematopoietic development was disrupted, showing the importance of these genes for vessel sprouting and circulation. Interestingly, a hierarchy was observed, whereby the knock-down of *estrp* showed stronger phenotypes than a single knock-down of *fli1*, *fli1b,* or *ets1* (Pham et al., 2007). Similarly, a reduction of *etv5* was shown in our study to have a strong effect on the *scl* expression domain, indicating a hierarchy may also exist between members of the PEA3 ETS factors. In conclusion, we have defined the importance of PEA3 ETS transcription factors in mediating FGF signaling during development.

My data suggest that in some instances the knock-down of two PEA3 ETS proteins was sufficient to reveal a phenotype in a majority of the injected embryos, as noted with the expression of cardiac progenitor markers, implicating an overlapping function between these factors. Given that knock-down studies can be difficult to quantitate, a more effective means is to generate genetic nulls and determine the relative contribution of each PEA3 ETS factor to FGF signaling and development. One interesting observation that demonstrates ETS overlapping roles comes from a recent study in mouse kidney formation. Compound heterozygotes of two

factors (Etv4+/-; Etv5+/-) or Etv4 nulls resulted in a small percentage of embryos showing renal defects. In contrast, Etv4-/-; Etv5+/- mice exhibited a complete lack of kidneys, suggesting that a full compliment of Etv5 can function to restore normal kidney development, but in a reduced state, it cannot (Lu et al., 2009). Thus, gene dosage may play a critical role in how these factors bind to promoters and regulate transcription and development. A more detailed gene expression profiling and readout is required to correlate gene activity with developmental outcome.

5.0 CHARACTERIZATION OF PEA3 ETS TRANSCRIPTION FACTOR BINDING TO AN FGF DOWNSTREAM TARGET

5.1 INTRODUCTION

In the previous two aims, I have determined both how ETS factors are activated, and how their activation plays a role in FGF-mediated developmental processes. However, there is a lack of evidence that indicates PEA3 ETS factors directly regulate FGF-mediated gene transcription. In Aim 3, I will identify cis-regulatory elements within an FGF target gene, *Dusp6*, and illustrate how ETS factors bind to these elements within this promoter. To achieve this, luciferase reporter constructs containing the *Dusp6* promoter were generated to determine the essential DNA sequences that are required for FGF-mediated induction of luciferase expression. By using these constructs in a *Xenopus* animal cap assay, I can measure luciferase activity soon after the initial zygotic transcription takes place; providing a direct measure of promoter activation. This allows a direct analysis of transcription factor binding to a promoter to activate transcription in an *in vivo* system. In addition, Electrophoretic Mobility Shift Assays (EMSAs) were utilized to determine direct binding of ETS factors to a specific region of the *Dusp6* promoter. To further verify my conclusions, this aim will also contain data generated from a collaboration with Dr. Anne Moon at the University of Utah, where ETS transcription factor binding to the *Dusp6* promoter was analyzed in mouse to demonstrate evolutionary conservation. In addition, a ChIP

assay was performed to analyze this transcription factor binding to the promoter within mouse embryonic tissues.

5.2 USING *XENOPUS* ANIMAL CAP EXPLANTS TO DETERMINE THE LOCATION OF CIS-REGULATORY ELEMENTS WITHIN THE *DUSP6* PROMOTER

The process by which FGF signaling is relayed into a transcriptional response in development is not fully defined. Because *Dusp6* is a known target of FGF activity (Molina et al., 2007; Tsang et al., 2004), I focused on identifying cis-regulatory elements within the *Dusp6* promoter to determine if PEA3 ETS factors can directly regulate its transcription. Previously, transgenic zebrafish FGF reporter lines were generated in our lab (*Tg(dusp6:EGFP)*^{pt6}) and it was determined that a 10Kb sequence upstream of Exon 1 in *Dusp6* was sufficient to drive GFP reporter expression in an FGF-dependent manner (Molina et al., 2007). Thus, all of the elements required for transcription factor binding to this promoter are contained within this 10Kb region of sequence. To determine which area of the 10Kb region contains cis-regulatory elements, luciferase reporter constructs were generated by inserting varying lengths of *Dusp6* upstream sequence (1Kb to 10Kb) into a pGL3 luciferase reporter vector. These constructs were then used in *Xenopus laevis* explant cultures.

The *Xenopus* explant assay is an ideal and widely used system for analysis of transcription factor activation of a promoter *in vivo* (Casey et al., 1999; Friedle et al., 1998; Rebbert and Dawid, 1997; Rogers et al., 2009; Watanabe et al., 2002). The major advantage of this system is that luciferase protein activity can be measured just 6 hours after zygotic transcription initiation, thus allowing the direct measure of the activation of *Dusp6* by FGFs.

The luciferase reporter constructs were co-injected into the animal pole of 2-cell stage *Xenopus* embryos in either the presence or absence of *fgf8* mRNA. *pCMV-Renilla* was also co-injected to normalize for injection variability in the dual luciferase reporter assay system (Promega). The embryos were then cultured until Stage 8.5 (just prior to initiation of zygotic transcription) at which point the animal regions were explanted **(Figure 29)**. The explanted animal caps were cultured for 6 hours, then harvested and measured for luciferase activity. Due to the short length of the culture, the animal caps are naive to other signaling except for what has been injected into the embryo, thus a very direct effect of these injections can be measured. Important to note, lysates were prepared from a population of four animal caps, and several different lysates were analyzed during multiple injection sessions to account for variability between experiments.

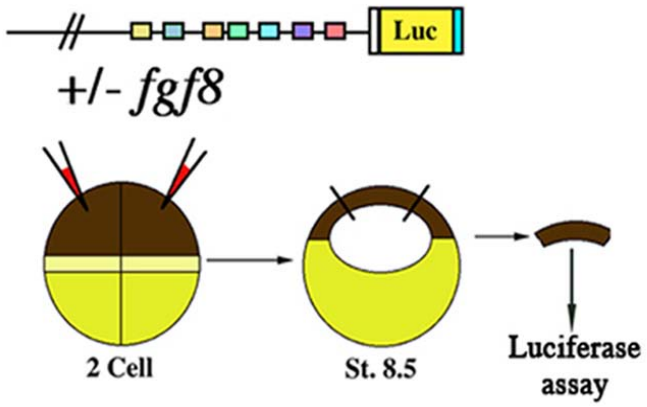

Figure 29: Diagram of *Xenopus laevis* Animal Cap Assay.
An overview of the protocol for *Xenopus* animal cap assays. A *Dusp6* promoter fragment (1Kb – 10Kb) with a luciferase reporter was injected (+/- fgf8) into each cell of a 2-cell stage *Xenopus* embryo. Just prior to zygotic transcription (Stage 8.5) the animal cap is removed and cultured for 6 hours. Lysate was made from four caps and analyzed for luciferase activity. The colored boxes on the luciferase construct represent potential regions where ETS factors may bind to the promoter.

5.3 FGF REGULATORY ELEMENTS WERE FOUND WITHIN A 2KB REGION OF THE *DUSP6* PROMOTER

In animal caps co-injected with *fgf8 mRNA*, an approximate 3-fold increase in luciferase reporter activity was detected in all *Dusp6* promoter constructs (10Kb – 1Kb) as compared to animal caps injected with *Dusp6* promoter constructs alone. This is a valid positive fold increase in animal cap assays based on previous studies (Casey et al., 1999; Friedle et al., 1998; Rebbert and Dawid, 1997; Rogers et al., 2009; Watanabe et al., 2002). This suggests each of these reporter constructs contain putative responsive elements that are sensitive to FGF signals and must be contained within a 1Kb region of the *Dusp6* promoter (n>4 for each construct; **Figure 30** shows typical results from 5Kb-1Kb *Dusp6* promoters). Since each promoter assay is a different injection experiment, comparisons cannot be made between different promoters, but trends can be evaluated based on luciferase induction of each promoter in the +/- of *fgf8*.

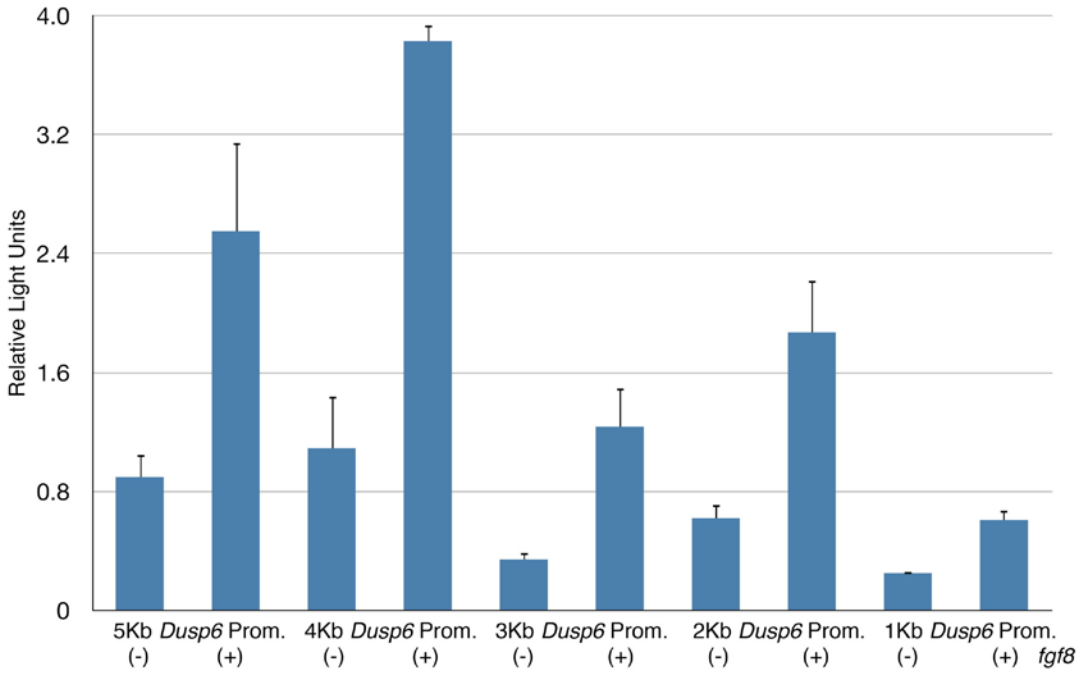

Figure 30: *fgf8* Activates the *Dusp6* promoter.

Luciferase activity measured with 5Kb-1Kb *Dusp6* promoter constructs in the presence or absence of *fgf8*. Each promoter was activated at least 2-3 fold in the presence of *fgf8*, indicating cis-regulatory elements are located within the 1Kb region of the *Dusp6* promoter. Error bars indicate standard deviation.

5.4 TWO PUTATIVE PEA3 ETS BINDING SITES LOCATED WITHIN THE 2KB REGION OF THE *DUSP6* PROMOTER REGULATE TRANSCRIPTION

Due to an increase in luciferase activity in the presence of *fgf8* upon injection of the 1Kb *Dusp6* promoter construct, it was hypothesized that a population FGF responsive elements are located within this 1Kb upstream promoter sequence. Using a bioinformatics approach to identify cis-elements within the *Dusp6* promoter that may be important for FGF regulated expression, I compared this 1Kb stretch of promoter region from several species (zebrafish, pufferfish, mouse, and human) based on sequence identity. Significant homology was identified within these vertebrate promoters within the 1Kb region, indicating that this non-coding sequence of DNA has been conserved through several hundred million years since their divergence **(Figure 31A)**. Further analysis revealed some of the conserved sequences represent known binding sites for transcription factors (http://www.ifti.org/cgi-bin/ifti/Tfsitescan.pl). From this approach, several putative transcription factor binding sites were identified within this region, including two PEA3 ETS binding sites (referred to Pea3A being the upstream site and Pea3B being the downstream site)**(Figure 31A)**. These sites were also described in the mouse *Dusp6* promoter (Ekerot et al., 2008).

To determine if these core ETS 5'-GGAA-3' recognition sequences represents important regulatory sites for FGF responsiveness, two new 2Kb luciferase constructs (*Pea3MutA* and *Pea3MutB*) were generated in which these putative sites were mutated (**Figure 31A**; sequence of mutated residues are indicated in red). Through these luciferase assays, it was determined that the Pea3B site, which contains three perfectly conserved core 5'-GGAA-3' motifs, was critical for the FGF-mediated induction of luciferase activity. Conversely, mutating the Pea3A site had no effect on luciferase activity **(Figure 31B)**. Of note, the sequences flanking this putative ETS binding site are also well conserved from zebrafish to human, suggesting that these regions may be potential binding sites for protein partners of Pea3 ETS factors.

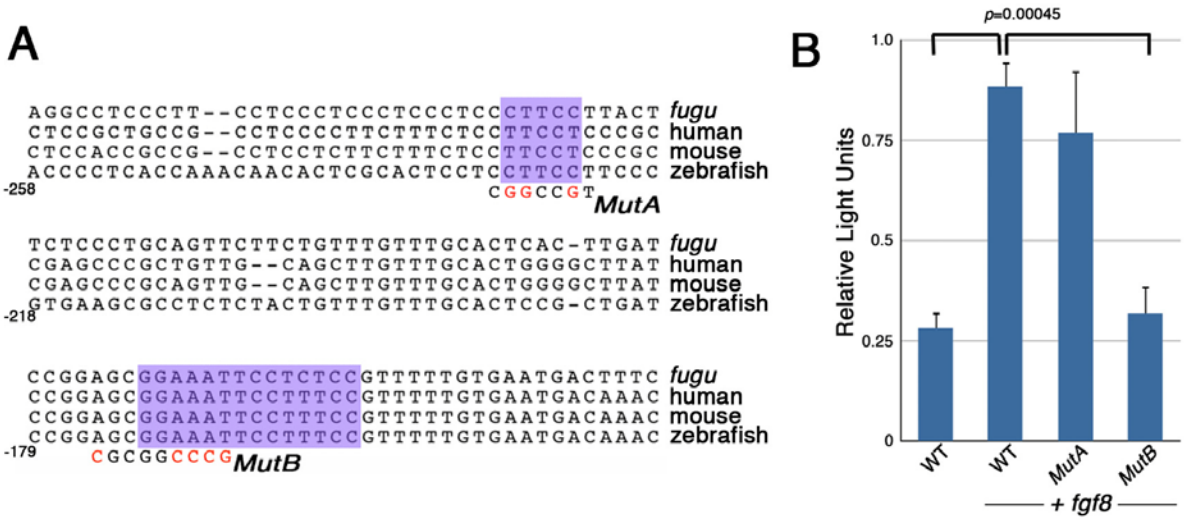

Figure 31: A Conserved Putative PEA3 ETS Binding Site within the *Dusp6* Promoter is Critical for FGF-Mediated Induction of Luciferase Activity.

(A) Alignment of the *Dusp6* promoter from several vertebrate species showing the conserved putative ETS binding sites (highlighted in purple) that were mutated, as shown in red below the sequence. **(B)** Luciferase activity measured with the 2Kb *Dusp6* promoter (WT) constructs in the presence or absence of *fgf8*, indicating the requirement for the Pea3B site (*MutB*). A two-sample equal variance T-test using a two-tailed distribution was applied to analyze for statistical significance. Error bars indicate standard deviation.

Since PEA3 ETS factors are expressed within the same temporal and spatial domains as *dusp6*, it is likely that these factors are direct regulators of *dusp6* expression *in vivo*. To test this, I co-injected *etv5:VP16* instead of *fgf8*, along with the 2Kb *Dusp6* reporter into the *Xenopus* animal pole and measured luciferase activity six hours after zygotic transcription. Ectopic expression of *etv5:VP16* resulted in a dramatic 5-fold induction of luciferase activity when compared to the injection of the *Dusp6* reporter construct alone **(Figure 32A)**. To confirm the hypothesis that *etv5:VP16* binds to Pea3 sites, I co-injected *etv5:VP16* and the Pea3Mut reporters and measured luciferase activity. As predicted, the integrity of the Pea3B site was important for *etv5:VP16* to activate luciferase expression **(Figure 32A)**. Furthermore, *etv5:T135D* increased luciferase activity and was dependent upon the Pea3B sequence (**Figure 32B**). These results indicate that FGF signaling directly regulates *Dusp6* transcription through the activity of PEA3 ETS factors in zebrafish. In addition, co-injection of *etv5* with the *Dup6* reporter did not increase luciferase activity **(Figure 32A ,B)**, further implicating the importance of post-translational modifications of *etv5* for transcriptional activity.

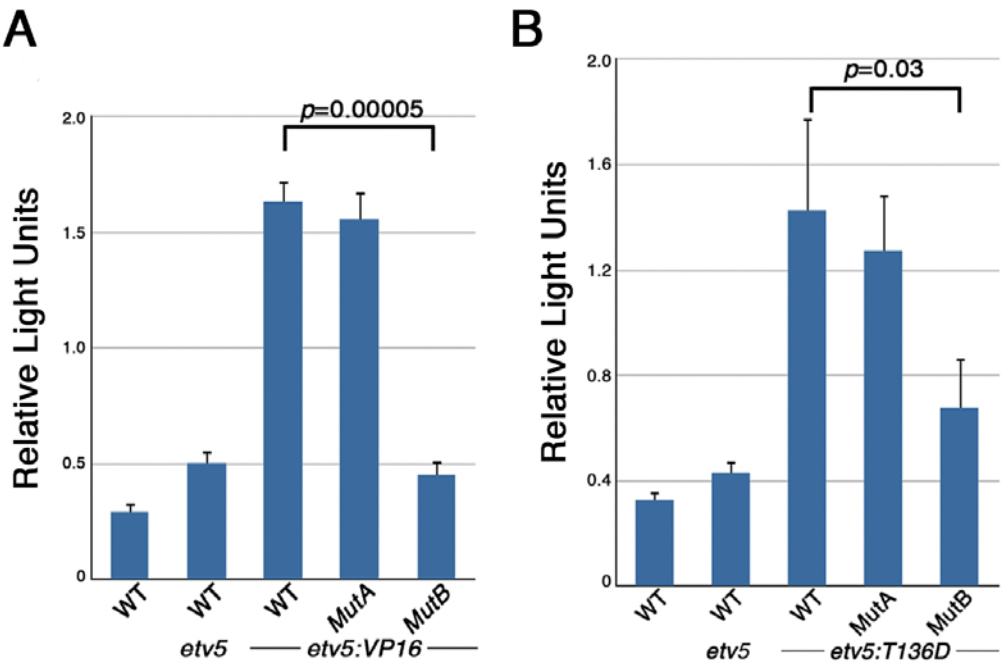

Figure 32: FGF Signaling Regulates *Dusp6* Transcription Through the Activity of PEA3 ETS Factors.

(**A**) *etv5:VP16* activated the *Dusp6* reporter (WT), and mutation of the Pea3B (MutB) site diminished this activity. (**B**) Ectopic expression of *etv5:T135D* activated the *Dusp6* reporter (WT), but not the mutant Pea3B reporter (MutB). A two-sample equal variance T-test using a two-tailed distribution was applied to analyze for statistical significance. Error bars indicate standard deviation.

5.5 PEA3 ETS FACTOR FUNCTION IS CONSERVED BETWEEN ZEBRAFISH AND MOUSE

In collaboration with Dr. Anne Moon at the University of Utah, mammalian PEA3 factors were tested to determine if they could drive reporter gene expression from the zebrafish promoter, indicating conservation between zebrafish and mouse PEA3 ETS factor function. 293T cells were co-transfected with Pea3 or Erm, the zebrafish *Dusp6* promoters, and *Renilla* control

expression plasmid to account for transfection variability. Both PEA3 and ERM were capable of inducing reporter gene expression controlled by the zebrafish promoter, and this was dependent on the Pea3B site **(Figure 33)**.

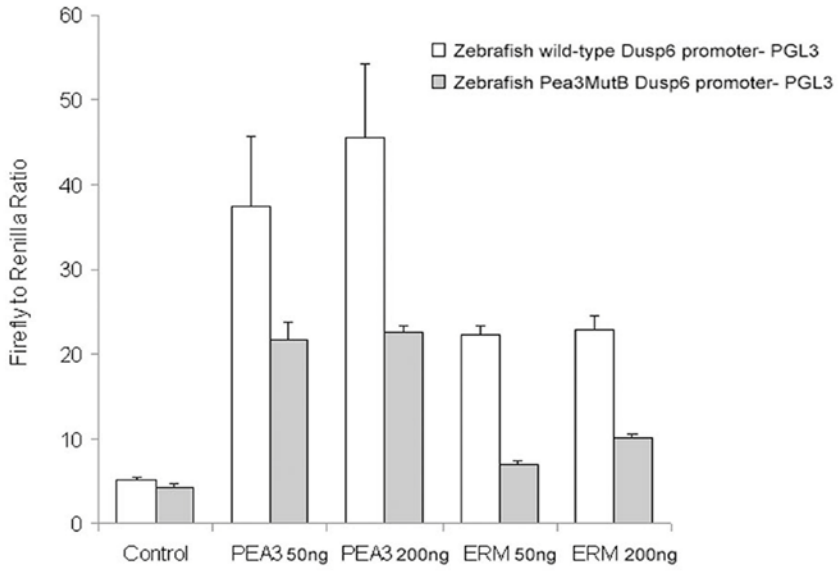

Figure 33: Conservation of PEA3 and ERM Function on Mouse and Zebrafish *Dusp6* Promoters.

Mouse PEA3 and ERM transactivate the zebrafish *Dusp6* promoter via a conserved putative PEA3/ERM binding site. HEK293 cells were cotransfected with the zebrafish 2Kb promoter in the PGL3 reporter and two concentrations of the mouse PEA3 or ERM expression plasmids. Both PEA3 and ERM significantly increased luciferase activity from the zebrafish promoter. The Pea3MutB diminished the response to PEA3 and ERM. Error bars indicate standard deviation.

Furthermore, the mouse *Dusp6* promoter was isolated and generated luciferase reporter constructs with 0.7Kb of upstream promoter sequence, where the critical putative Pea3 binding domain is located in zebrafish. Ectopic expression of mouse PEA3 or ERM transactivated the promoter, and this activity was also dependent on the exact Pea3B sequence found in the zebrafish *Dusp6* promoter **(Figure 34A,B)**. These results reveal the evolutionary conservation of

PEA3 ETS factors between mouse and zebrafish, and that proteins from both species can activate reporter genes from two diverse promoters.

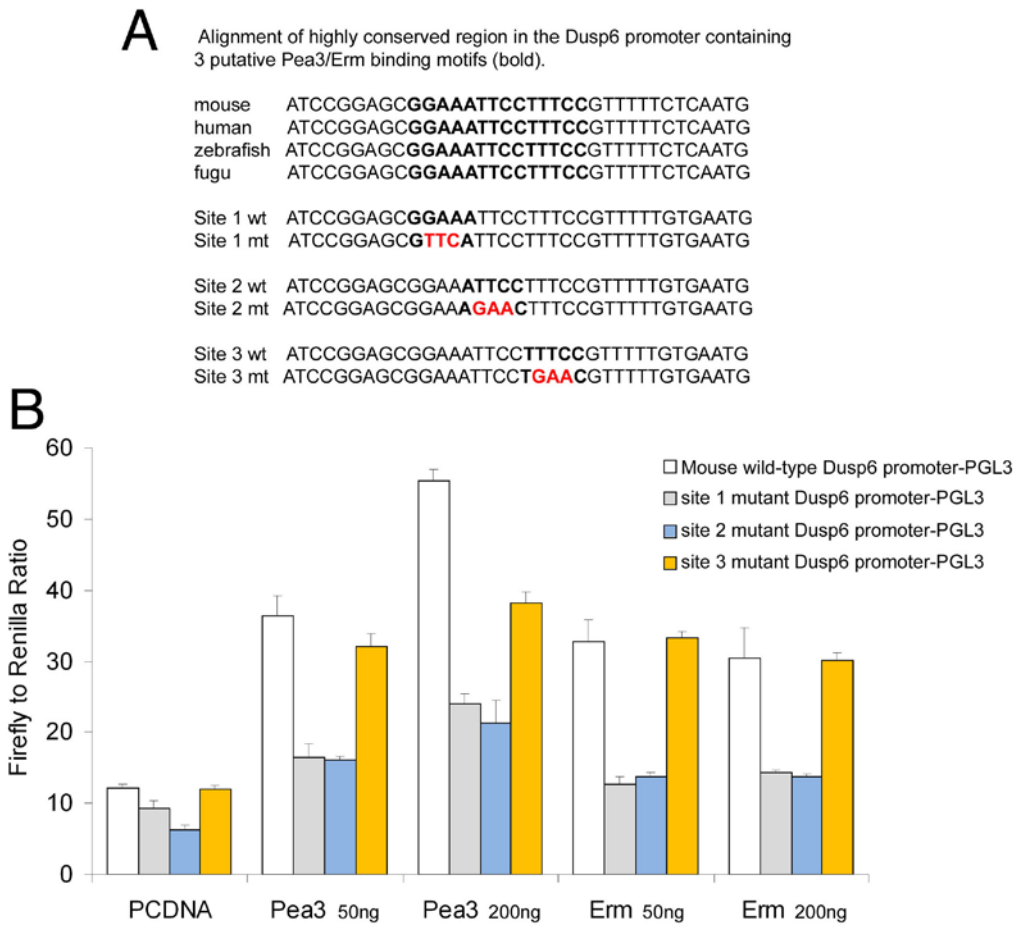

Figure 34: Requirement for the Conserved Pea3B Site in the Mouse *Dusp6* Promoter.
Transactivation of a 781bp fragment of the mouse *Dusp6* promoter by PEA3 and ERM is dependent on a region containing three consecutive putative PEA3/ERM core binding motifs (bold type). This region was also conserved in zebrafish (Pea3B). (**A**) Interspecies alignment and sequence of mutations (red type) generated in putative core motifs. (**B**) HEK293 cells were co-transfected with plasmids containing the 781bp *Dusp6* promoter luciferase reporter, the *Renilla* transfection control reporter, and *Pea3* or *Erm*. Maximal response to PEA3 requires all three 5' GGAA 3' (5' TTCC 3') core motifs, while response to ERM requires sites 1 and 2. Error bars indicate standard deviation.

5.6 PEA3 ETS PROTEIN DIRECTLY BINDS TO THE *DUSP6* PROMOTER

Xenopus animal cap luciferase assays indicated the requirement of a putative Pea3 binding site on the *Dups6* promoter to activate transcription *in vivo*, however evidence for direct transcription factor binding has not been reported. To demonstrate this, I used biotin-labeled electrophoretic mobility shift assays (EMSAs) to show direct binding of Etv5 Ets domain to the Pea3B site *in vitro*. To generate a purified Etv5 protein fragment, GST-Etv5-Ets DNA binding domain protein was expressed in bacteria (BL21) cells (Invitrogen) and batch purified with GST-sepharose beads (Amersham)(**Figure 35**).

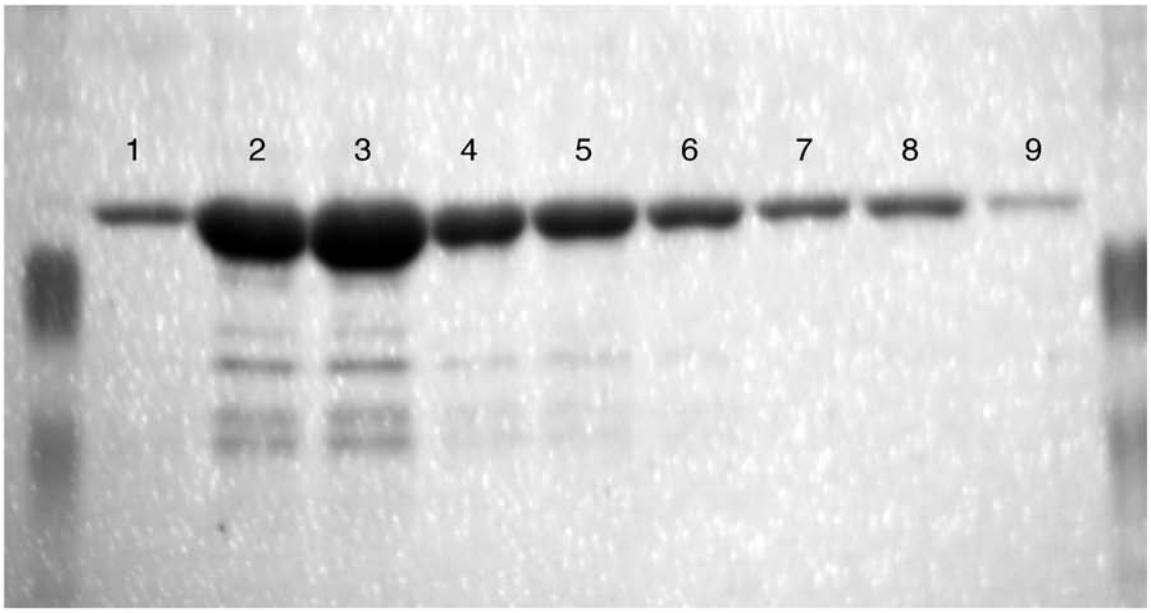

Figure 35: GST Purification of Etv5:Ets Binding Domain Protein.
An Etv5 protein fragment containing the entire Ets DNA binding domain was generated by expressing GST-Etv5-Ets DNA binding domain protein in BL21 cells and batch purified with GST-speharose beads. Elutes 1-8 are shown on an SDS PAGE gel, where elute 6 was used for the Etv5 protein in EMSA studies.

Biotin-labeled oligos that flank the Pea3B site (60bp) were synthesized and used to amplify this region by PCR (**Figure 36A**). Recombinant GST-Etv5:Ets domain protein interacted with the biotin-labeled *Dusp6* promoter DNA, causing a band shift (**Figure 36D**; lane 2). The bound DNA was competed by increasing amounts of non-biotin labeled promoter (**Figure 36D**; lanes 3-7). In addition, another more specific competitor was generated against only the Pea3B sequence. This Pea3B-18mer oligo (**Figure 3 6C**) also competed Etv5 ETS domain binding (**Figure 3 6D**; lanes 8-12), indicating that the direct binding of Etv5 to this promoter is at the specific putative Pea3B site. Conversely, a random sequence used as a control competitor did not compete with the biotin-labeled *Dusp6* fragment (**Figure 36D**; lanes 13-17). Furthermore, to verify the importance of the Pea3B site for Etv5 binding, a 60bp biotin-labeled Pea3B mutant *Dusp6* promoter was used in EMSAs (**Figure 3 6B**). Etv5:Ets protein did not shift the Pea3B mutant DNA (**Figure 36D;** lane 18,19), verifying that mutating this specific putative binding site within the promoter will not promote Etv5 binding.

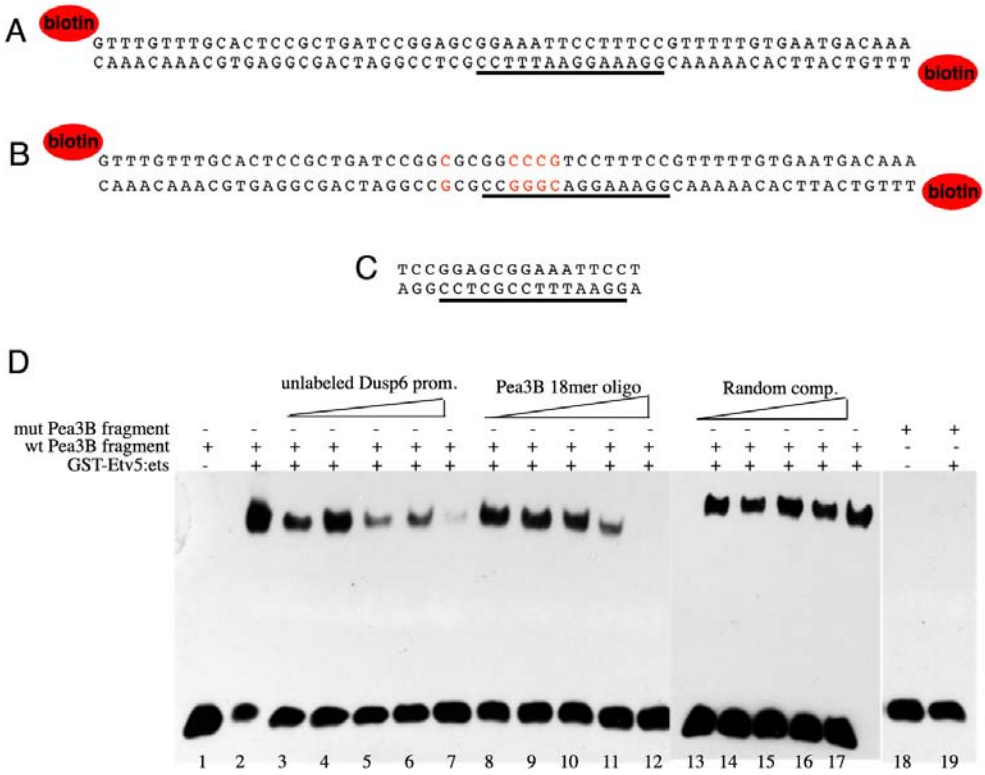

Figure 36: Etv5-Ets Domain Binds to the *Dusp6* Promoter.

(A) Biotin labeled PCR product. **(B)** Biotin labeled mutant Pea3B *Dusp6* promoter fragment, with the mutated residues identified in red. **(C)** Pea3B 18bp fragment used in competition assays. Underlined sequence represent the putative Pea3B binding site **(D)** EMSA using *Dusp6* promoter biotin fragment and GST-Etv5:ETS protein. The Etv5-ETS domain bound to the 60bp *Dusp6* promoter containing the Pea3B site (lane 2). This binding was competed with the non-biotin labeled 60bp *Dusp6* promoter (lanes 3-7) and the Pea3B 18bp fragment (lanes 8-12). A random oligonucleotide sequence did not compete this binding (lanes 13-17). Binding of Etv5-ETS to the Pea3B mutant *Dusp6* promoter was not observed (lanes 18-19).

To determine if PEA3 ETS factors can directly bind to the mouse *Dusp6* promoter *in vivo*, Chromatin Immunoprecipitation (ChIP) assays were performed as part of a collaboration with Dr. Anne Moon's laboratory from the University of Utah. In this assay, pharyngeal tissue was isolated from E9.5 mouse embryos, a region with active FGF signaling that expresses high

levels of *Etv4* (an ortholog of *Pea3* in zebrafish), *Etv5*, and *Dusp6* (Dickinson et al., 2002; Ilagan et al., 2006; Park et al., 2006). By ChIP and PCR, it was found that the Pea3B binding site was enriched with specific ETV4 antibodies, suggesting that ETV4 directly binds to the *Dusp6* promoter *in vivo* **(Figure 37 A-C)**. qPCR quantitation revealed a 7-fold enrichment of this element in the ETV4 ChIPed sample compared to the negative control **(Figure 3 7D)**. Taken together, this shows that *Dusp6* is directly regulated by PEA3 ETS factors during zebrafish and mouse embryonic development.

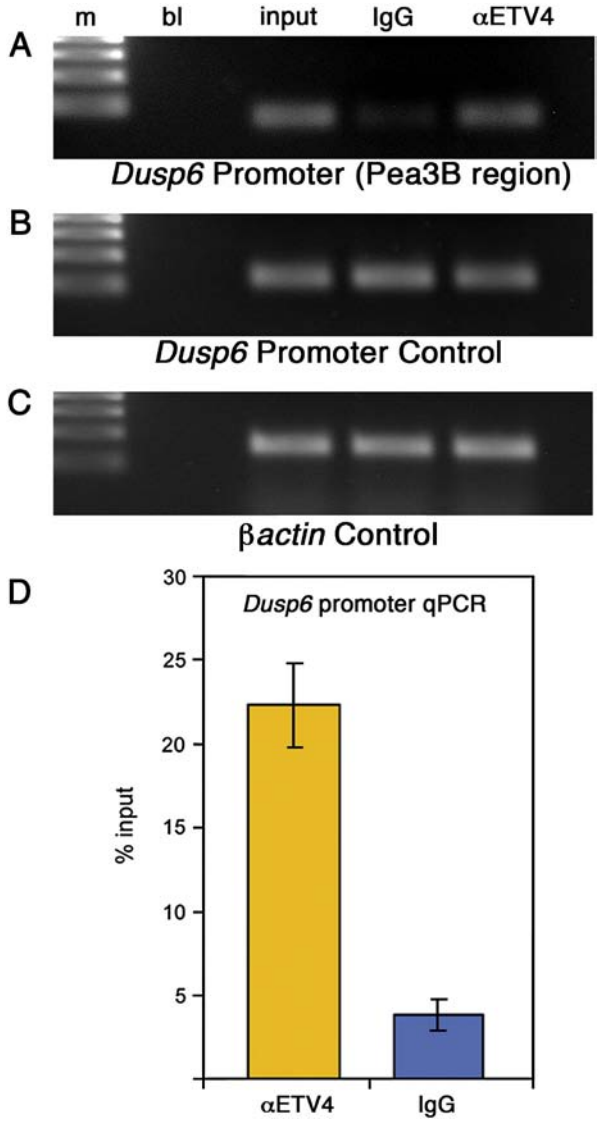

Figure 37: ETV4 Directly Binds to the *Dusp6* Promoter *in vivo* in Mouse.

The mouse *Dusp6* promoter region bearing highly conserved PEA3 binding sites is enriched in chromatin immunoprecipitated from mouse embryonic tissues with anti-mouse ETV4 antibody. **(A)** Agarose gel showing PCR products obtained from equal amounts of input, mouse IgG ChIPed, and anti-ETV4 ChIPed DNAs with the *Dusp6* promoter primers that amplify the region containing the putative PEA3 binding sites. **(B)** Agarose gel showing PCR products obtained on the same DNA samples as in A with *Dusp6* control primers, amplifying a region of the *Dusp6* promoter not containing the Pea3B site. **(C)** Agarose gel showing PCR products obtained on the same DNA samples as in A with βactin control primers. Only the *Dusp6* promoter was differentially precipitated by the anti-ETV4 antibody. B and C control primers give identical bands as they detect the non-specific, background precipitated DNA. **(D)** Graphical representation of quantitative PCR results show nearly 7-fold enrichment of the amplicon containing the highly conserved region of the *Dusp6* promoter in ETV4 ChIPed DNA compared to IgG negative control expressed relative to that detected in input. m, marker; bl, blank lane. Error bars indicate standard deviation.

5.7 DISCUSSION

5.7.1 PEA3 ETS Factors Bind to the *Dusp6* Promoter Directly at a Specific Binding Site

The process by which FGF signaling is relayed into a transcriptional response in development is not fully defined. Because *Dusp6* is a known target of FGF activity, I focused on identifying cis-elements within the *Dusp6* promoter to determine if PEA3 ETS factors can directly regulate its transcription (Molina et al., 2007; Tsang et al., 2004). I identified several conserved putative ETS binding sites within 1Kb upstream of the *Dusp6* transcription start site and through reporter and binding assays, indentified one specific site that was critical for reporter gene activity and *in vitro* binding. This site is identical to what has been recently described in the mouse *Dusp6*

promoter (Ekerot et al., 2008). Ekerot et al. (2008) had demonstrated by ChIP studies in NIH3T3 cells that both Ets1 and Ets2 can bind to this particular region. Our studies demonstrate for the first time that Pea3 can bind to the *Dusp6* promoter *in vivo* and provide strong evidence that FGF signaling regulates *Dusp6* transcription by PEA3 ETS factors during development. These differences may reflect varying expression levels of these ETS factors between NIH3T3 cells and mouse pharyngeal tissue. Since *Fgf8*, *Etv4*, *Etv5* and *Dusp6* are strongly expressed in pharyngeal arch, our results provide strong evidence for the direct binding of ETV4 to the *Dusp6* promoter (Dickinson et al., 2002; Ilagan et al., 2006; Park et al., 2006). In contrast, both Ets1 and Ets2 transcripts are predominantly expressed in vascular and lymphatic tissues and not co-expressed with *Dusp6* (Maroulakou et al., 1994). However, in NIH3T3 cells, expression of Ets1 and Ets2 may play a greater role in regulating *Dusp6* expression *in vitro*.

5.7.2 The Direct Binding of PEA3 ETS Factors to the *Dusp6* Promoter is Conserved between Zebrafish and Mouse

The results in this aim suggest the evolutionary conserved binding of PEA3 ETS factors to the *Dusp6* promoter at a specific ETS binding site in zebrafish and mouse. Interestingly, since the conserved ETS domain was used in the EMSA studies, this indicates that all three of the PEA3 ETS factors have the potential of binding to a specific site within the *Dups6* promoter (since the ETS binding domain is ~95% conserved between the three family members)**(Figure 3 6)**. However, luciferase assays performed using the mouse *Dusp6* promoter indicated a larger increase in luciferase activity with the co-transfection of Pea3 compared to the co-transfection of Erm **(Figure 34)** . This may indicated preferential binding of Pea3 at this site, but further analysis, such as competition assays, will need to be performed to address this question. In

addition, the region at which the mutation is generated within the putative Pea3 site on the *Dusp6* promoter appears to be important **(Figure 34)**. Mutations generated within the most extreme 5' end or the central region of the putative Pea3 binding site inhibits both Pea3 and Erm activity, while mutations generated in the extreme 3' end of the Pea3 binding site only inhibit Pea3 binding based upon luciferase activity. This again may be due to preferential binding of Pea3 to this site, or it could be the result of other binding co-factors found near the 3' end of the putative binding site that are necessary to facilitate Erm binding. When the extreme 3' region of the binding site is mutated, this may alter the binding of co-factors. The hypothesis of other co-factors functioning with PEA3 ETS factors will be analyzed more in the Discussion of this thesis.

6.0 CONCLUSIONS AND FUTURE PROSPECTUS

In this thesis work, the importance of the PEA3 ETS transcription factor family in FGF signaling has been described. Specific residues were isolated on each family member that are phosphorylated via ERKs and have an effect on expression of downstream FGF targets. Mutating these residues to a constitutive active form will increase expression levels of FGF targets. Furthermore, I have also determined that PEA3 ETS factors play a role in FGF-mediated developmental processes, such as MHB formation, early and late heart development, and L/R patterning. Finally, a PEA3 ETS binding motif was identified on the *Dusp6* promoter, and the binding of Etv5 to this motif will increase *dusp6* expression both in zebrafish and in mouse. Thus, within this dissertation, I have determined how these factors are activated, the roles of these family members during development, and how these factors regulate expression of downstream targets.

6.1 PEA3 ETS FACTORS ARE PHOSPHORYLATED AT SPECIFIC RESIDUES TO REGULATE TARGET GENE EXPRESSION

A protein alignment using zebrafish, mouse, and human Etv5 and Erm identified conserved sequences outside of the DNA binding domain within PEA3 ETS family members. Using ScanSite and Netphos2.0 software, specific sites of phosphorylation via ERKs was predicted.

Two threonines (T135 and T139) and one serine (S142) were identified that are conserved among human, mouse, and zebrafish, and are predicted to be phosphorylated by ERKs within Etv5 and Erm. In addition, similar sites have been shown to be direct targets of MEK/ERK signaling in *ets1*, another ETS factor in a different subfamily, within sea urchins (Rottinger et al., 2004). To analyze the importance of these conserved residues in ERK phosphorylation events, I performed site-directed mutagenesis on Etv5 to generate constructs that mimic the charged nature ERK phosphorylation events at these sites. These constructs, when injected into zebrafish embryos, showed a large increase of expression of FGF downstream target genes *dusp6* and *sef* at shield stage, supporting the charge hypothesis for activation. More importantly, these results were verified upon injection of a constitutively active construct (*VP16:Etv5*), also showing an increase of *dusp6* and *sef* at shield stage.

Interestingly, another member of the PEA3 ETS subfamily, Pea3, has only one conserved serine (S100) that was verified to be important in ERK phosphorylation events. Several explanations may indicate why multiple ERK phosphorylation sites exist in some family members (Etv5/Erm), while only one putative ERK phosphorylation site was determined in another family member, Pea3. Since Etv5 contains multiple sites for ERK phosphorylation, it is not unlikely that other types of post-translational modifications are used in combination to temporally and spatially control activities of PEA3 ETS transcription factors. It was found that in the case of Pea3, SUMO modification takes place both *in vivo* and *in vitro* on multiple SUMO attachment sites within the N-terminal region. Importantly, sumoylation of Pea3 was promoted by the activation of the ERK/MAPK pathway, thus the same pathway may control two types of post-translational modifications, phosphorylation and sumoylation. (Guo and Sharrocks, 2009). Due to the powerful response induced from ERK phosphorylation in Etv5 it is likely that other

post-translational modifications, such as sumoylation, are required to regulate the activities of Etv5 throughout development.

6.1.1 Verification of Multiple ERK Phosphorylation Sites on Etv5

Although multiple putative ERK phosphorylation sites elicit an FGF response, all of these putative sites may not be phosphorylated during FGF signaling. In addition, preferential phosphorylation may occur at one site compared to others. Since no zebrafish-specific antibodies exist for Etv5, I have been attempting to generate specific antibodies for unphosphorylated and phosphorylated forms of Etv5 (BioSynthesis, Inc). Column purified rabbit bleeds for the peptide KPLTPPSTPVSPC, an unphosphorylated version of Etv5, in addition to two phosphorylated peptide versions, KPL(pT)PPS(pT)PVSPC and TPPS(pT)PV(pS)PCVPS, have been generated. To test the functionality of these antibodies, Western blot analysis will be performed blotting with these bleeds when running a purified form of Etv5 on an SDS-PAGE gel. When examining for specificity of the phosphorylated versus unphosphorylated versions of these bleeds, the product of phosphorylation assays with Etv5 can be examined on a Western blot. If these antibodies are specific for phosphorylation of Etv5, immunohistochemistry will be performed on whole zebrafish embryos during different stages in development when FGF signaling is critical. These experiments can thus determine which sites on Etv5 are phosphorylated during different developmental processes to relay FGF signals. Interestingly, the sites of ERK phosphorylation on Etv5 may vary depending on which developmental processes are occurring at specific timepoints. These antibodies can also be critical in providing other insights into PEA3 ETS transcription factors, which will be discussed later within the section.

6.2 MULTIPLE ROLES OF PEA3 ETS FACTORS DURING DEVELOPMENT

Microinjection of *etv5MO*, *ermMO* or *pea*3MO alone did not significantly affect development of the zebrafish embryo. Since the expression patterns of these genes are identical during development, it is likely ETS factors function redundantly, so that the loss of one gene can be compensated by other ETS factors. This same redundancy was determined when studying ETS factors in mouse (Arber et al., 2000; Chen and Deng, 2005; Livet et al., 2002). To circumvent redundancy, injections of a MO targeting both *erm* and *etv5* together or a combination of MOs targeting all three family members (*3EtsMO*) were performed. The injection of the *3EtsMO* resulted in a decrease in downstream FGF targets, malformations of the MHB, and alterations in the early and late heart development. These morphant phenotypes are very similar to the zebrafish *fgf8* mutant or embryos injected with *fgf8MO*, indicating that the ETS genes are likely to be critical components of the FGF signal transduction pathway.

Functional redundancy within other ETS transcription factors in zebrafish has also been described (Pham et al., 2007). A recent study examined four ETS family members expressed in the vasculature: *fli1*, *fli1b*, *ets1*, and *etsrp* (Pham et al., 2007). Using an antisense MO approach to knock down expression of all four genes, both vascular and hematopoietic development was disrupted, showing the importance of these genes for vessel sprouting and circulation. Interestingly, a hierarchy was observed, whereby the knock-down of *estrp* showed stronger phenotypes than a single knock-down of *fli1*, *fli1b*, or *ets1* (Pham et al., 2007). Similarly, a reduction of *etv5* was shown in our study to have a strong effect on the *scl* expression domain, indicating a hierarchy may also exist between members of the PEA3 ETS factors. In conclusion, we have defined the importance of PEA3 ETS transcription factors in mediating FGF signaling during development.

6.2.1 Further Analysis of ETS Factors in FGF-mediated Developmental Processes

Within this work, PEA3 ETS transcription factors have been implicated in early and late heart development, but further investigation is necessary to determine how these factors affect overall heart development. *Tg(cmlc2:dsRed^{nuc})* embryos express dsRed in the nucleus of cells found only within the heart. This transgenic line will be used to further evaluate the effect of ETS factors on heart development. From my previous results, it was concluded that FGF signaling and ETS factors play a role in heart shape, size, and looping. Utilizing *EtsMO* injections within *Tg(cmlc2:dsred^{nuc})* embryos, the alterations in heart development will be attributed to either a change in the number of cardiac cells, or a change in size of cells that make up the heart. Using this transgenic line in conjunction with chamber-specific heart antibodies, such as S46, the effect of ETS factors on heart development can be further analyzed within each chamber. Preliminary studies examining the heart of *Tg(cmlc2:dsred^{nuc})* embryos indicate a strong reduction in the number of cells making up the embryonic heart upon injection of the *3EtsMO* **(Figure 38)**. In addition, this transgenic line can be valuable for small molecule screening. To determine the effects of small molecules on FGF signaling or specifically on heart development, *Tg(cmlc2:dsred^{nuc})* embryos can be used as a quick and easy assay to find molecules that alter heart development, just as *Tg(dusp6:EGFP)^{pt6}* has been used previously in small molecule screens for modulators of FGF signaling (Molina et al., 2009a).

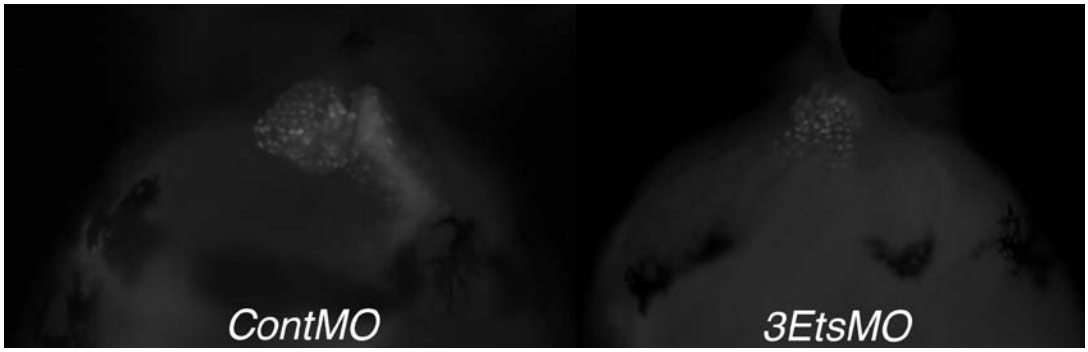

Figure 38: *3Ets* Morphants Exhibit an Overall Reduction in the Number of Cardiomyocytes.

Tg(cmlc2:dsred^{nuc}) embryos at 52hpf, anterior view. When compared to *ContMO*, the overall number of cardiomyocytes are reduced in *3EtsMO*-injected embryos, in addition to having misshaped hearts.

In this work, I have determined a role for PEA3 ETS factors in early and late cardiac formation and looping. However, these effects were seen upon injection of MO at 2-cell stage. Evidence has shown that *EtsMO* also induced early patterning defects that are characteristic of altering early FGF signaling in the embryo. Thus, these heart defects may be secondary effects of disrupting FGF signaling early in development. To circumvent the early polarity defects generated by these MOs, I attempted to employ a strategy that allows for temporal activation of ETS constructs at time points when cardiac progenitors are initially specified, and during migration and differentiation of these cells into a functioning heart. Hormone inducible Etv5 fusion constructs were generated that have been successfully employed with Erm in tissue culture studies (Pelczar et al., 1997). This technique has also proven to be successful with the study of other transcription factors in zebrafish and *Xenopus laevis* embryos (Kolm and Sive, 1995; Picard, 1994). The constitutively active and dominant negative version of *etv5* **(Figure 9)** were fused to the hormone-binding domain of estrogen receptor (ER^{tm}). This mutant estrogen

receptor contains a domain that interacts with the cytoplasmic heat shock proteins, and anchors proteins containing this domain to the cytoplasm. Upon the addition of 4-OH-tamoxifen (10^{-7}M), the binding of the ER domain to Hsp90 will be disrupted, thus allowing transcription factors to enter the nucleus to function (Pelczar et al., 1997). With this approach, *etv5:VP16* and *etv5:EnR* can be activated later in development, when heart formation begins. Thus, the temporal activation of constitutive active and dominant negative forms of an ETS factor can be examined during heart development. Although, in my studies, the activation of the transcription factors via tamoxifen was not easily controlled, and activation was occurring even in the absence of tamoxifen. To get around this problem, BCI was used to hyperactivate FGF signaling **(Figures 22 and 23)**, although this is not analogous to altering PEA3 ETS factors directly. Future studies can take advantage of recent technology where MOs have been synthesized to be photoactivatable. Here, MOs would be generated containing an inhibition linker that is cleavable when light-triggered (Shestopalov et al., 2007). This caged reagent would allow temporal gene regulation *in vivo* and allow a direct analysis of PEA3 ETS transcription factors specifically on heart development.

6.2.2 FGF Signaling and Cilia Development

Two recent studies indicate a role for FGF signaling during cilia development. However, these two studies have different conclusions on the function of FGFs during ciliogenesis. Hong and Dawid (2008) indicate that the knock-down of Fgf8 via antisense MOs significantly decreased the number of cilia present within the KV. Conversely, Neugabauer et al (2009) demonstrate that knocking down *fgfr1*, the receptor through which Fgf8 signals, does not decrease cilia number, but diminishes cilia length in several ciliated organs within the zebrafish, including the

otic vesicle and KV. Furthermore, it is shown that *ace* fish (mutant for *fgf8*) do not have a decrease in the length of cilia unless these mutants are also injected with *fgf24MO*. Thus, this study concluded that Fgf8 and Fgf24 both signal through FGFR1 to activate ciliary transcription factors that control cilia length.

In the work within this dissertation, I have identified a family of transcription factors that have overlapping expression patterns with *fgf8*, an indication that this ligand is important for relaying the FGF signal. PEA3 ETS factors were shown to play a role in the number of cilia present within KV. However, my work also indicates that *fgf8MO* has minimal affects on cilia length in morphants. Therefore, it is possible that multiple FGF ligands regulate the activation of PEA3 ETS factors in ciliogenesis. Further investigation with Fgf24 is necessary to determine the role of this ligand in ETS factor activation. If so, this would agree with the current model proposed by Neugabauer et al (2009) **(Figure 4)** and define ETS factors as being another family of ciliary transcription factors.

Important to note, although the knock-down of Fgf8 does not alter the formation, size, or number of cilia with KV, it has not been determined if these cilia are functional. Within the zebrafish KV, a complicated network of cilia movements occur, normally in a net counter-clockwise direction when viewed from the apical side, to cause fluid flow in a leftward direction (Kramer-Zucker et al., 2005). As development progresses, the length of cilia within the KV fluctuates until the KV collapses around 18 somite stage (Essner et al., 2005; Okabe et al., 2008). Many genes have been identified to be important for the proper movement of cilia, including motor genes, such as leucine-rich repeat-containing 50, *lrrc50*, an outer-arm dynein subunit (Sullivan-Brown et al., 2008; van Rooijen et al., 2008) and *dnah9* (also known as *lrdr*), a member of the dynein family (Kawakami et al., 2005). Expression of both *lrrc50* and *lrdr* have

been shown to be important for cilia motility. Further studies are necessary to determine the roles, if any, of PEA3 ETS factors, Fgf8, and Fgf24 on the expression of genes involved in cilia motility.

6.3 THE BINDING OF ETS TRANSCRIPTION FACTORS TO DOWNSTREAM TARGETS

Studies using *Dusp6* genomic DNA have indicated that a 10kb fragment upstream and including the first exon contains sequences that recapitulate endogenous *dusp6* expression. Using fragments from this putative promoter to drive luciferase reporter expression, I analyzed transcriptional regulation of *dusp6* by FGFs in *Xenopus* animal caps assays. Data indicated a 2.5-3 fold increase in luciferase expression in the presence of *fgf8*. Consequently, several putative cis-elements within the FGF responsive *dusp6* promoter were identified that are highly conserved among humans, fish, and mouse. These results revealed a requirement of at least one transcription start site proximal specific ETS binding site (Pea3B) for the FGF-mediated induction of luciferase. Microinjection of *etv5:VP16* or *etv5:S142D* increased luciferase activity and this was dependent on the Pea3B ETS binding site, as a mutation of this sequence suppressed luciferase induction. Further analysis using both zebrafish and mouse promoters in EMSA and ChIP assays reiterated the importance of the same Pea3 site within the mouse Dusp6 promoter, indicating the evolutionary conservation of this site. However, this does not negate the potential importance of any potential putative Pea3 sites upstream of the 2Kb promoter region that was analyzed.

6.3.1 Binding Partners of ETS Transcription Factors

In addition to multiple putative PEA3 ETS binding sites within a 2Kb region of the *Dusp6* promoter, several other putative sites for transcription factors were also present; among these were IRF2 and HNF-3E sites. The IRF2 site is particularly intriguing due to its proximity to the Pea3B putative site (3 nucleotides downstream). Mutating each of the putative binding sites in *Xenopus* animal cap explant assays indicate that each of these sites are important for activation of Dusp6. This result provides evidence that these sequences may be potential binding sites for protein partners of ETS factors **(Figure 39)**. Since ETS factors have a low binding specificity to targets (all that is required is a core RGAA/T sequence for ETS factors and a core GGAA/T sequence of PEA3 ETS factors), interactions with neighboring proteins appears critical. For example, the Elk subclass of ETS factors must form a complex with Srf to cause activation on the c-fos promoter (Buchwalter et al., 2004). In addition, Escalante et al (2002) have solved the crystal structure of Pu.1, another ETS factor within the Spi subfamily, and IRF4 bound to their respective cis-elements to form a ternary complex to regulate immunoglobulin light chain lambda expression (Escalante et al., 2002a; Escalante et al., 2002b). Therefore, it is tempting to speculate that other ETS factors may interact with IRF proteins to regulated gene expression, and these elements within the *Dusp6* promoter represent another example for ETS and IRF factor cooperation.

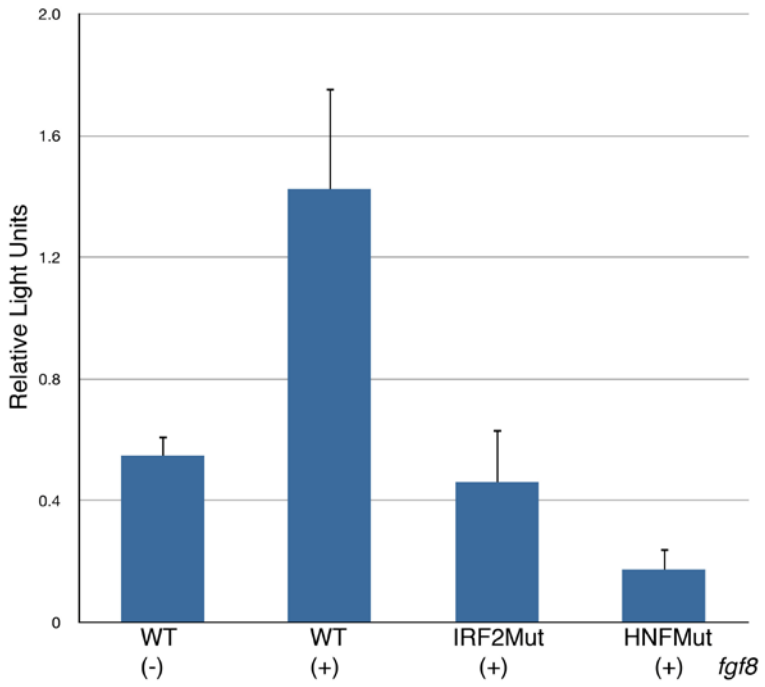

Figure 39: Putative IRF2 and HNF Sites may be Co-Factor Binding Sites with Pea3 to Activate *Dusp6*.

fgf8 could activate the *Dusp6* reporter (WT), and mutation of the IRF2 site or the HNF site diminished this activity. Error bars indicate standard deviation.

6.3.2 Identification of PEA3 ETS Transcription Factor Targets

With the availability of potential zebrafish specific Etv5 antibodies (see 6.1.1), the implications of other possible experiments in the future, including ChIP assays using whole embryo lysates, are possible. Several studies have successfully established protocols to detect transcription factor binding to promoters in whole embryos (Havis et al., 2006; Hirayama et al., 2005; Kim et al., 2004; Park et al., 2005). To determine the importance of PEA3 ETS factors during different times in development, embryos can be lysed at different stages, thus determining the temporal activation of Etv5 by FGF signaling to regulate gene expression, such as *dusp6, in vivo*. A more

global evaluation of the DNA-protein interactions with PEA3 ETS factors can also be examined with the advent of new technologies such as ChIP-chip or ChIP-Seq. ChIP-chip technology has recently been performed using zebrafish embryos (Wardle et al., 2006), where genome-wide DNA-protein interactions could be identified **(Figure 40)**. Importantly, these experiments have the potential to identify direct targets of ETS transcription factors *in vivo* in addition to identifying potential binding partners with these factors, adding a more global context to the importance of PEA3 ETS factors during development, both within the FGF signaling pathway and among other pathways.

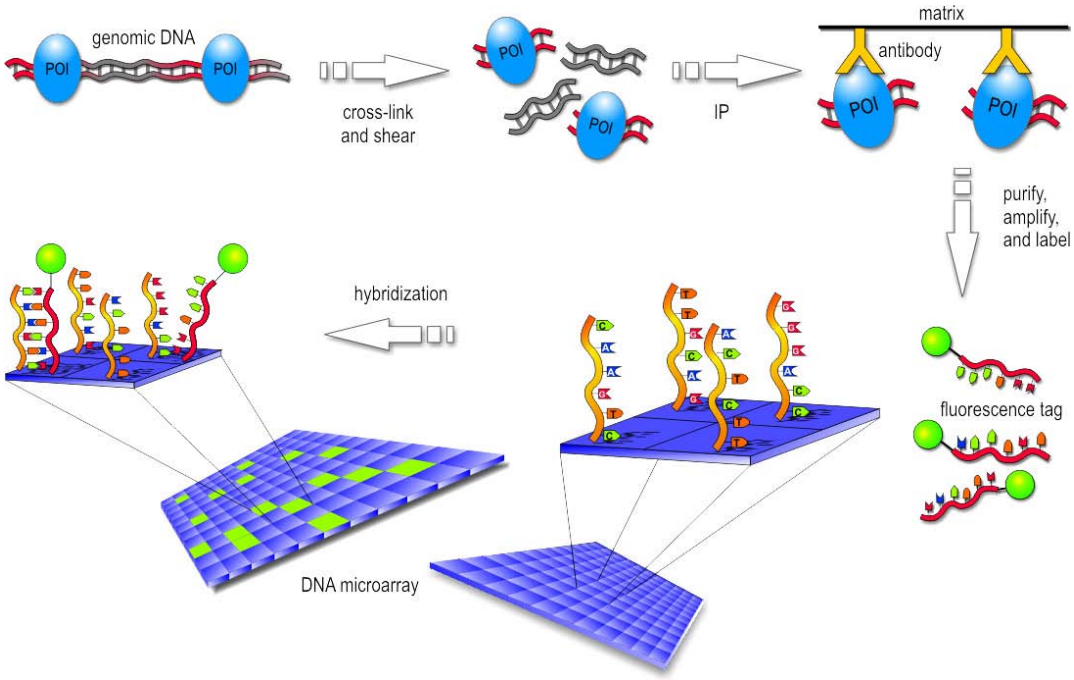

Figure 40: Method of ChIP-chip Technology.

Overview of ChIP-chip methodology to identify genome-wide interactions with PEA3 ETS factors. Using whole embryo lysates, formaldehyde cross-linking is performed followed by shearing of the DNA. Using potential zebrafish Etv5 antibodies, DNA-Etv5 complexes can be purified. Upon reverse cross-linking, DNA fragments can be fluorescently tagged and amplified. These fragments can then be hybridized to a zebrafish DNA microarray (Wardle et al., 2006), and analyzed for protein-Etv5 interactions (Figure from Thomas Hentrich; used with permission).

6.4 SUMMARY

PEA3 ETS factors are a subfamily of transcription factors involved in cell proliferation, growth, and apoptosis. In this thesis, the importance of these factors within the FGF signaling pathway was examined. The way these factors were activated via ERK phosphorylation was described, in addition to the roles ETS factors played during FGF-mediated developmental processes. Futhermore, the way in which ETS factors bind to downstream FGF targets was also identified. It will be interesting to further examine the importance of ETS factors during development, specifically in heart formation and L/R patterning. Importantly, the identification of other genome-wide targets of PEA3 ETS factors using ChIP-chip or ChIP-Seq technology can have far-ranging advances in both FGF signaling and developmental biology.

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