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TRANSCRIPTIONAL AND TRANSLATIONAL CONTROL OF ZEBRAFISH MESODERMAL DEVELOPMENT

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Abstract
Establishment of the mesodermal germ layer is a process dependent on the integration of multiple transcriptional and signaling inputs. Here I investigate the role of the transcription factor FoxD3 in zebrafish mesodermal development. FoxD3 gain-of-function results in dorsal mesoderm expansion and body axis dorsalization. FoxD3 knockdown results in axial defects similar to Nodal loss-of-function, and was rescued by Nodal pathway activation. In Nodal mutants, FoxD3 did not rescue mesodermal or axial defects. Therefore, FoxD3 functions through the Nodal pathway and is essential for dorsal mesoderm formation. The FoxD3 mutant, sym1, previously described as a null mutation with neural crest defects, was reported to have no mesodermal or axial phenotypes. We find that Sym1 protein retains activity and induces mesodermal expansion and axial dorsalization. A subset of sym1 homozygotes display axial and mesodermal defects, and penetrance of these phenotypes is enhanced by FoxD3 knockdown in mutant embryos. Therefore, sym1 is a hypomorphic allele, and reduced FoxD3 function results in reduced cyclops expression and subsequent mesodermal and axial defects. The sym1 molecular lesion, a point deletion leading to a seven codon frameshift and premature termination, is predicted to be a truncated protein lacking part of the DNA-binding domain and an essential Groucho corepressor interaction domain (GEH). Strongly predicted to be functionally inactive, the hypomorphic character of sym1 suggested that a -1 translational frameshift may correct the reading frame and produce functional protein. Consistent with this hypothesis, mutation of the distal GEH ablated sym1 cDNA activity. Within the frameshift region are three rarely used codons predicted to cause ribosomal pausing and promote translational frameshifting. Conversion of these codons to highly used codons encoding the same residues rendered the sym1 cDNA inactive. Biochemical analyses confirm that a full length FoxD3 protein is produced from sym1 cDNA, but not when the rare codons are replaced. These results indicate that the null character of the sym1 mutation is suppressed by a novel translational frameshifting mechanism, and support the conclusion that FoxD3 is a Nodal-dependent regulator of zebrafish mesodermal development.

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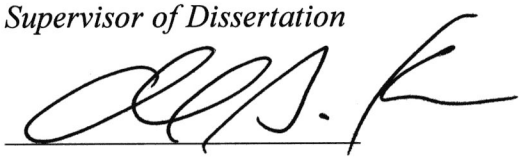

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Dedication

To my loving parents.

ABSTRACT

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Establishment of the mesodermal germ layer is a process dependent on the integration of multiple transcriptional and signaling inputs. Here I investigate the role of the transcription factor FoxD3 in zebrafish mesodermal development. FoxD3 gain-of-function results in dorsal mesoderm expansion and body axis dorsalization. FoxD3 knockdown results in axial defects similar to Nodal loss-of-function, and was rescued by Nodal pathway activation. In Nodal mutants, FoxD3 did not rescue mesodermal or axial defects. Therefore, FoxD3 functions through the Nodal pathway and is essential for dorsal mesoderm formation. The FoxD3 mutant, *sym1*, previously described as a null mutation with neural crest defects, was reported to have no mesodermal or axial phenotypes. We find that Sym1 protein retains activity and induces mesodermal expansion and axial dorsalization. A subset of *sym1* homozygotes display axial and mesodermal defects, and penetrance of these phenotypes is enhanced by FoxD3 knockdown in mutant embryos. Therefore, *sym1* is a hypomorphic allele, and reduced FoxD3 function results in reduced *cyclops* expression and subsequent mesodermal and

axial defects. The *sym1* molecular lesion, a point deletion leading to a seven codon frameshift and premature termination, is predicted to be a truncated protein lacking part of the DNA-binding domain and an essential Groucho corepressor interaction domain (GEH). Strongly predicted to be functionally inactive, the hypomorphic character of *sym1* suggested that a -1 translational frameshift may correct the reading frame and produce functional protein. Consistent with this hypothesis, mutation of the distal GEH ablated *sym1* cDNA activity. Within the frameshift region are three rarely used codons predicted to cause ribosomal pausing and promote translational frameshifting. Conversion of these codons to highly used codons encoding the same residues rendered the *sym1* cDNA inactive. Biochemical analyses confirm that a full length FoxD3 protein is produced from *sym1* cDNA, but not when the rare codons are replaced. These results indicate that the null character of the *sym1* mutation is suppressed by a novel translational frameshifting mechanism, and support the conclusion that FoxD3 is a Nodal-dependent regulator of zebrafish mesodermal development.

Table of Contents

List of Figures

1. Chapter 1 – Introduction

The three primary germ layers—the ectoderm, the mesoderm, and the endoderm—are established from the epiblast during the process of gastrulation. The embryonic body plan arises from these major cell lineages, which are progressively patterned and specialized to give rise to the many tissues and organs in the body. The lineages derived from the mesodermal germ layer give rise to a broad range of tissues and organs, including the embryonic tissues prechordal plate, notochord, somites, heart, pronephros, and hematopoietic precursors. The developmental mechanisms that control the induction and patterning of the mesodermal germ layer have been studied in a variety of embryonic model systems for decades. These studies have revealed cellular and molecular mechanisms that underlie the induction, patterning, differentiation, and morphogenesis of mesodermal lineages. Beginning during gastrulation and continuing at later stages, the instructive signals received by mesodermal progenitors are interpreted as transcriptional programs that initiate positive and negative regulatory feedbacks, conferring the stability of cell-fate choices and leading ultimately to determination and differentiation. Conserved mechanistic similarities have been identified when defining the cell movements and the inductive signals that control mesodermal development in vertebrates and invertebrates, as well as interesting and unexpected differences in developmental mechanism.

In *Xenopus* and zebrafish, mesoderm-inducing signals arise largely from the vegetal pole or yolk cell, whereas the major mesodermal patterning center is the Spemann organizer or embryonic shield. The distinction between mesoderm induction and patterning is somewhat arbitrary at the molecular level, where in vitro, an individual

pathway is capable of inducing mesoderm de novo while differing levels of that same signal can induce distinct types of mesoderm. In the embryo, however, the formation and patterning of mesoderm is dependent on the integration of multiple inductive signals that arise in a spatially and temporally dynamic manner; a number of pathways are required but no individual pathway is sufficient for complete mesodermal development (Heasman, 1997, 2006). Much has been learned about the formation of the mesodermal germ layer, including the identity of major mesoderm-inducing pathways and the complex regulatory networks that modulate pathway activity, however, despite the advances in this research area, many questions are yet to be answered. For example, given that the major mesoderm-inducing pathways regulate distinct lineages throughout development, how is cellular response controlled to ensure an appropriate mesodermal response during early development? Mesoderm formation is a critical embryonic event and its study has, and will continue to provide profound insight into fundamental developmental mechanisms that could aid in developing successful molecular therapeutics for human benefit.

1.1. Embryonic induction and formation of the Zebrafish shield

During the first hours of zebrafish embryonic development the zygote is composed of a small blastoderm disc over a vast yolk cell. The yolk syncytial layer (YSL) forms by fusion of the marginal blastomeres with the yolk cell at the mid-blastula stage (1000 cells) and will then separate the blastoderm from the yolk cell, while the enveloping layer (EVL) covers the outer surface of the blastoderm as a single cell layer. The onset of gastrulation in zebrafish begins with the process of epiboly, where cells from the blastoderm start to spread out and surround the yolk cell. Next, when the leading

edge of the epibolyzing blastoderm reaches the equator cells move inward and coalesce to form the hypoblast, while the outer cells form the epiblast. At the same time clonal groups of blastoderm cells become fate restricted, and the shield forms as a thickening along the blastoderm margin on the future dorsal side. Cells in the blastoderm disc form a single homogeneous population where the margin of the blastoderm contains mesoderm and endoderm precursors. Cells initially located near the blastoderm margin involute first and form the deepest layer of the newly forming hypoblast and give rise to mesodermal and endodermal derivatives, cells that are more distant from the margin involute later and form only mesoderm while non-involuting cells farther from the margin form ectoderm (Kimmel et al., 1990; Warga and Kimmel, 1990). After involution, mesoderm is positioned as a ring of cells that includes the dorsal embryonic shield domain. Further, transcription factors required for the formation of the shield have been localized to the YSL at early gastrula stage and importantly, shown to act in a non-cell-autonomous manner (Koos and Ho, 1998; Yamanaka et al., 1998). The shield will ultimately contribute cells to the hatching gland, head mesoderm, notochord, somatic mesoderm, endoderm and part of the neuraxis (Shih and Fraser, 1996). (Fig. 1.1)

In *Xenopus*, localization of dorsal determinants to the vegetal blastomeres on the future dorsal side of the embryo and rearrangement of the egg cytoplasm result from a process of cortical rotation which occurs prior to the first cleavage (Gerhart et al., 1989; Gerhart et al., 1981). However, in zebrafish, no cortical rotation occurs, presumably because of the large dense yolk cell, therefore the dorsoventral axis of the embryo is not set during or before the first cleavage. Alternatively, it is thought that the dorsal

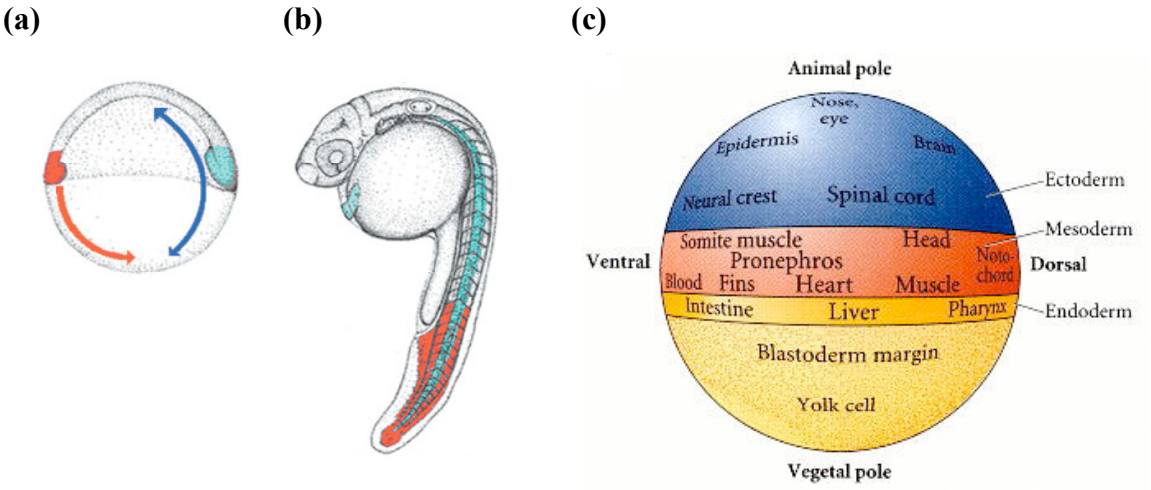

Figure 1.1 The Zebrafish gastrula and 24 h fate map. Position and movement of ventral (red) and dorsal (blue) marginal cells at the **(a)** shield stage, and at **(b)** 24 h after fertilization. The dorsal margin contributes to axial structures like the hatching gland, head mesoderm, notochord, somatic mesoderm, endoderm and part of the neuraxis (blue); ventral margin to non-axial tissues (red). (Adapted from Agathon et al., 2003) **(c)** Zebrafish fate map after cell mixing has stopped. (Adapted from Langeland and Kimmel, 1997)

determinants present in the vegetal yolk are transported to the future dorsal side later, during early cleavage stages (1-8 cell stage), through microtubule-mediated transport (Jesuthasan and Strahle, 1997). These dorsal determinants are derived and transported from the yolk cell to the dorsal blastomeres through the YSL (Jesuthasan and Stahle, 1997; Jesuthasan and Strahle, 1997; Mizuno et al., 1996), which will later act as the equivalent of the *Xenopus* Nieuwkoop center. The Nieuwkoop center induces the dorsally localized Spemann organizer in *Xenopus*, and in a similar manner the YSL induces formation of the dorsal shield in zebrafish. In addition, formation of the shield on the dorsal side of the blastoderm is one of the first signs of dorsoventral polarity and will proceed to pattern the mesoderm to establish the proper organization of the zebrafish body axis. The dorsal shield forms in response to Nodal and Wnt signals and serves as a source for multiple signaling antagonists. The removal of the shield from a developing embryo prevents formation of dorsal structures, and conversely, when transplanted into a host embryo, is able to induce a secondary axis (Saude et al., 2000).

1.2. Mesoderm induction in Zebrafish

Mesoderm is initially induced at the equator of the embryo by signal emanating from the YSL, and this is followed by activation of inducing signals expressed in the mesendodermal blastomeres localized dorsally and closest to the YSL. Mesodermal development therefore is a progressive process in which the exposure of competent cells to inducing signals during the blastula and early gastrula stages results in the specification of mesodermal fates. In zebrafish, extensive cell rearrangements occur during early cleavage divisions, so cell fates cannot be assigned until the onset of

gastrulation (Kimmel et al., 1990). In contrast, in *Xenopus* the initial cleavage divisions define the dorsal–ventral and left–right axes. Therefore, the mesodermal fate map reflects the distribution of distinct maternal factors that establish animal–vegetal and dorsal–ventral patterns and that initiate regional gene expression during the mid-blastula stage (Dale and Slack, 1987; Moody, 1987). Mesoderm is further patterned by zygotic factors produced by cells of the organizer and by competing factors expressed in non-dorsal mesoderm (lateral and ventral mesoderm) (Heasman, 2006). Beginning during gastrulation and continuing at later stages, the instructive signals received are interpreted as transcriptional programs that initiate positive and negative regulatory feedbacks, conferring the stability of cell-fate choices and leading ultimately to determination and differentiation. Although embryonic induction involving communication between cell populations via extracellular factors is an inherently cell-nonautonomous process, cell-autonomous factors functioning within mesodermal precursors are required to bias cell fate decisions during induction and to stabilize mesodermal identity during determination and differentiation. In zebrafish, the mesodermal fate map is set at the gastrula stage and reflects a precise dorsal–ventral organization, with notochord and anterior somite precursors in the dorsal domain, pronephros and trunk somites in lateral domains, and tail somites and blood in the ventral domain. Although there is much conservation in developmental mechanisms between organisms, the coordination of cell division, cell adhesion, cell rearrangements, and the timing of embryonic induction differ among embryos of different organisms. These differences determine the stage at which meaningful fate maps can be established.

An essential aspect of embryonic development is the ability to limit the responsiveness of cells both temporally and spatially. The organizer is a source of secreted antagonists of multiple signaling pathways rather than a source of positive-acting instructional signals. Rather than producing signals that directly confer dorsal identity of adjacent tissue, it produces inhibitors of pathways that promote ventral identity, thus permitting the dorsal development of adjacent tissues. The function of these signaling antagonists in developmental patterning of the mesoderm is to exclude pathway activity from the organizer domain and to produce an activity gradient in the non-organizer mesoderm, with the highest levels at the ventral marginal zone (Harland and Gerhart, 1997).

1.3. Molecular mediators of mesoderm induction

A number of major signaling pathways, including Nodal, Bmp, Wnt, and Fgf, are required for multiple aspects of vertebrate development. Fgfs and Activin-like members of the transforming growth factor (TGF)-β superfamily have the ability to induce mesoderm formation in *Xenopus* animal pole explants. Furthermore, when these signaling pathways are inhibited in vivo, mesodermal development is disrupted. Although the stimulation of individual pathways is sufficient to influence mesoderm induction and patterning, cellular integration of multiple signaling inputs and cross-talk between the components of these signaling pathways are essential for the correct development and differentiation of the germ layers (Candia et al., 1997; Kretzschmar et al., 1997; Nishita et al., 2000). In addition, given the roles of these pathways in many distinct embryonic

processes, mechanisms must exist that confer the spatial and temporal specificity of cellular response.

The TGF-β ligands that influence mesodermal development are: activins, Vg1, Nodals, Gdfs and Bmps. TGF-β ligands stimulate signaling by binding to heterotetrameric receptor complexes with intrinsic serine/threonine kinase activity, and this results in the phosphorylation and activation of Smad proteins that mediate the cellular response (Massague, 1998). Nodals are a subfamily of the TGF-β superfamily of signaling factors. Nodal ligands bind to the extracellular domain of a type II receptor, a type I receptor is recruited into a signaling complex and phosphorylated to activate the serine/threonine kinase activity of the type I receptor. The type I receptor will then phosphorylate and activate Smad 2 or Smad 3 proteins, which then bind to the co-smad, Smad 4, and as a complex are the intracellular signaling mediators of TGF-β signaling. Active Smad proteins translocate to the nucleus, complex with specific DNA-binding proteins, and function as coactivators for transcriptional target genes. Three classes of Smad proteins have been identified in vertebrates, including the receptor-activated Smads (R-Smads), Smads 1, 2, 3, 5, and 8, which each contain an SSXS motif that is phosphorylated by active receptor. This R-Smads class is subdivided into two groups based on the types of TGF-β signals transduced, with Activin-like signals mediated by Smads 2 and 3 and BMP-like signals mediated by Smads 1, 5, and 8. A second class includes Smad 4, a collaborating Smad (co-Smad). The third class includes the inhibitory Smads (I-Smads) 6 and 7, which bind to type I receptors and limit the access of R-Smads. Smad 7 negatively regulates a broad range of TGF-β signaling pathways, including Activin-like and BMP-like pathways, whereas Smad 6 specifically regulates only the

BMP-like pathways. Signaling through a subset of TGF-β ligands including Nodal, Vg1, Gdf1 and Gdf3 requires a coreceptor protein of the EGF-CFC family (FRL1 in Xenopus, Cryptic in chick, Cripto and Cryptic in mouse and human, and one-eyed pinhead in zebrafish) (Shen and Schier, 2000).

Activin was identified early in the search for mesoderm-inducing factors as a protein that was present in the supernatants of *Xenopus* tissue culture cells and macrophage cell lines. Activin induces dorsal mesoderm at high doses, muscle at intermediate doses and ventral–posterior mesoderm at low doses. However, despite the presence of maternal activin protein, a series of inhibitor studies suggested that activin was not an endogenous inducer of mesoderm (Kessler, 2004), but is required to maintain maximal levels of mesodermal gene expression at the gastrula stage (Piepenburg et al., 2004).

A maternal mRNA that is localized to the vegetal cortex of the Xenopus oocyte encodes Vg1. After fertilization, cleavage divisions trap Vg1 protein in vegetal blastomeres, and, therefore, Vg1 has been viewed as a strong candidate for endogenous mesoderm inducer. However, the native form of Vg1 is not processed efficiently, and, although the mature domain can strongly induce mesoderm like activin, native Vg1 was not found to have significant mesoderm-inducing activity (Kessler, 2004). In *Xenopus*, a second Vg1-related gene, Derriere, is efficiently processed, and it is required for the development of posterior mesoderm, but not for the initiation of mesoderm formation (Sun et al., 1999). The knockdown of Vg1, like activin knockdown, results in the reduction of mesodermal gene expression during the gastrula stage, but it does not prevent the initial induction of mesoderm (Birsoy et al., 2006). Vg1 orthologs and Vg1-

like factors have been identified in the zebrafish (DVR1), the chick (cVg1), and the mouse (Gdf1 and Gdf3), and, although processed proteins can each strongly induce mesoderm, loss-of-function studies indicate that Vg1-like proteins is not essential for the initiation of mesodermal development in these systems. Given that Vg1-related proteins signal via the same signaling complex as Nodal proteins, it may be that some degree of functional redundancy obscures the early developmental requirement for a subset of Vg1-like proteins (Kessler, 2004).

BMPs function in the early vertebrate embryo to promote ventral mesoderm formation and to limit the domain of dorsal mesoderm formation. BMPs 2, 4, and 7 are expressed in ventral–lateral regions of the early embryo, and they play essential roles in the dorsal–ventral patterning of mesoderm in *Xenopus* and zebrafish (De Robertis and Kuroda, 2004). BMP gain-of -function suppresses dorsal mesodermal development, whereas the knockdown of BMP function or the overexpression of BMP inhibitors results in an expansion of the dorsal mesodermal domain and the induction of ectopic axial structures (Reversade et al., 2005). Although it is essential for dorsal–ventral patterning of the mesoderm, BMP function is not required for the initiation of mesodermal development. Zebrafish mutants with null alleles of BMP ligands or BMP signaling components are strongly dorsalized, and they show an expansion of dorsal mesoderm (Hammerschmidt and Mullins, 2002). In the mouse, BMP signaling in the epiblast is essential for the proper recruitment of epiblast cells into the primitive streak, and embryos that are null for the BMP receptor BMPR1A fail to gastrulate or form mesoderm normally (Beppu et al., 2000; Mishina et al., 1995; Winnier et al., 1995).

The Wnt genes constitute a large family of secreted, cysteine-rich, lipid-modified glycoproteins that are involved in many critical processes of early embryonic development (Nusse, 2005). Wnt signaling can stimulate a number of distinct signaling outputs, including the canonical β-catenin–dependent activation of transcription, the planar cell polarity, a calcium-dependent response, and others. In mesodermal development, the canonical pathway plays several important roles. In *Xenopus*, cortical rotation during the first cell cycle results in the displacement of dorsal determinants to the future dorsal side of the embryo. The resulting activation of the canonical Wnt pathway stabilizes β-catenin, which accumulates in the nuclei of dorsal blastomeres during the blastula stage and activates the transcription of dorsal gene expression, including Nodal-related genes. Recent evidence suggests that Wnt 11 is the maternal ligand that is responsible for the early activation of the Wnt pathway (Tao et al., 2005). During the gastrula stage, zygotic activation of the canonical Wnt pathway results in the stabilization of β-catenin in ventrolateral regions, which promotes the developmental of ventral–posterior mesoderm and antagonizes dorsal–anterior mesoderm. Therefore, the canonical Wnt pathway is first used during the maternal phase of development to promote dorsal fates and then to promote ventrolateral fates in response to zygotic signals.

The fibroblast growth factors comprise a large family of signaling factors that play essential roles in mesoderm induction and maintenance. Purified Fgf protein was one of the first proteins identified as a mesoderm inducer in the *Xenopus* animal explant assay. Fgf signaling is crucial as a competence factor in mesoderm induction, and FGF activity is required for the response of animal explants to Activin-like signals. Fgfs also regulate the T-box transcription factors that are necessary for the specification and

maintenance of mesoderm. Dominant-negative, inhibitor, and knockdown studies indicate that Fgf signaling is essential during the gastrula stage for the development of trunk and tail structures, but not for the initial induction of mesoderm. In addition, studies in zebrafish suggest an early role for Fgf signaling in repressing BMP transcription during the late blastula stages to promote dorsal development (Furthauer et al., 2004; Koshida et al., 2002; Londin et al., 2005).

1.4. Nodal signaling and regulation

Nodal-related genes are conserved in vertebrates and are essential for mesodermal development (Shen, 2007). Many Nodal-related genes are expressed during early embryogenesis in the prospective mesoderm and in the organizer domain, however, Nodals are also essential in additional developmental processes, therefore mechanisms must exist that confer spatial and temporal specificity to cellular response. In contrast with Activin and Vg1, Nodal-related proteins appear to be the critical signaling factors for mesoderm induction in all vertebrates. Nodals require the co-receptor EGF-CFC protein (cripto/one-eyed pinhead, Oep in zebrafish) extracellularly for Nodal ligands to bind to and activate their receptors (Gritsman et al., 1999), in contrast to activin which does not require a co-receptor to initiate signaling. Therefore, Oep could function as a spatial and temporal regulator of Nodal signaling. Humans, mice, and chicks have a single Nodal gene, while zebrafish have three, *squint*, *cyclops*, and *southpaw*, and *Xenopus* has five Nodal-related genes, *Xnr-1, -2, -4, -5,* and *-6*, with mesoderm-inducing activity.

In *Xenopus*, Nodal expression is regulated by the maternal factors VegT and β-catenin, and this results in a dynamic expression pattern first in vegetal blastomeres during the late blastula stage, then in the organizer domain during the early gastrula stage, and finally at lower levels throughout the marginal zone during the midgastrula stage. Nodal expression in the marginal zone establishes a dorsal to ventral gradient of Nodal signaling activity that contributes to mesodermal patterning. The inhibition of Nodal signals with the specific inhibitor Cerberus-short results in a complete block of mesoderm induction; this supports the idea that Nodal proteins are the essential initiators of mesoderm formation (Piccolo et al., 1999). Similarly, *cyc* and *sqt* are expressed in partially overlapping domains before gastrulation, with *sqt* expressed earlier and found in both the YSL and future mesendoderm, while *cyc* is only expressed in mesendoderm (Feldman et al., 2000; Feldman et al., 1998; Gritsman et al., 2000; Schier and Talbot, 2001)**.** Both signals activate the same downstream targets and in a concentration dependent manner. Further, *sqt* has been shown to activate target genes in distant cells while *cyc* acts more locally (Chen and Schier, 2001). Nodal gain- and loss-of-function in the zebrafish results in the dramatic perturbation of mesodermal development (Schier and Talbot, 2005). As in *Xenopus*, Nodal gain-of-function in zebrafish induces ectopic dorsal mesoderm and axial duplication (Feldman et al., 1998). Single mutants in *sqt* or *cyc* loss of function results in mild phenotypes with minor defects in mesoderm induction (Hatta et al., 1991). Zebrafish embryos that are null for both *cyc* and *sqt*, or have a maternal-zygotic loss-of-function for the Nodal coreceptor (*mzOep*), lack all trunk and head mesoderm as well as endoderm, and display defects during the initial induction of mesoderm during the gastrula stage (Feldman et al., 2000; Whitman, 2001). However, in

contrast to *Xenopus*, zebrafish embryos lacking active Nodal signaling form some tail mesoderm. In additional, *nodal* loss-of-function in mouse results in a failure to maintain the primitive streak and a failure to form embryonic mesoderm and extraembryonic ectoderm, and this leads to death during early gastrulation (Conlon et al., 1994). Consistent with an essential role for Nodal signaling in mesoderm formation, loss-of-function for the Nodal antagonists Lefty/Antivin in mouse, *Xenopus* and zebrafish results in an expansion of mesodermal derivatives. Furthermore, overexpression of Lefty/Antivin in zebrafish results in a phenotype strikingly similar to *cyc;sqt* or *mzOep* (Bisgrove et al., 1999). Nodals are autoregulatory, and a target of Nodal signaling Lefty/Antivin acts as a competitive feedback inhibitor by binding Nodal receptors, and restricting Nodal activity (Meno et al., 1999). Consistently, expression of the Nodal inhibitor Lefty/Antivin closely follows the expression of *cyc* and *sqt* in the developing gastrula, and overexpression of Nodal signals induces the widespread expression of Lefty/Antivin. Additional negative regulators of Nodal, signaling include Tomoregulin-1, which binds the Nodal coreceptor to inhibit Nodal signaling, Dapper2 which promotes receptor turnover, and BAMBI, a pseudoreceptor that functions in a dominant-negative manner to inhibit signaling by most type I receptors. Smurf1, Smurf2, and Ectodermin are ubiquitin ligases that target Smads 1 and 5, Smad 2, and Smad 4, respectively, for proteosome-mediated degradation. Protein inhibitor of activated STAT (PIASy) associates with Smad proteins in the nucleus to inhibit transcriptional coactivation function. Therefore, the Nodal pathway is subject to multiple layers of negative regulation to limit its activity during development, and this stringent regulation of Nodal

pathway activity is critical for proper organization of the embryonic mesoderm and other tissues of the vertebrate gastrula (Fig. 1.2).

1.5. The role of FoxD3 in mesoderm and neural crest development

FoxD3, a member of the forkhead class of transcription factors, has multiple roles in the vertebrate embryo during early embryogenesis and later in neural crest development. FoxD3 expression is present in mouse and human embryonic stem cells, in mouse trophoblast stem cells and in the preimplantation mouse embryo (Hanna et al., 2002; Sutton et al., 1996; Tompers et al., 2005). Interestingly, neither embryonic stem cell lines nor trophoblast stem cell lines can be established from *foxd3* null embryos (Hanna et al., 2002; Tompers et al., 2005). During early embryogenesis in *Xenopus*, FoxD3 functions as a transcriptional repressor to induce dorsal mesoderm and axis formation, and the antagonism or knockdown of FoxD3 results in severe axial defects and a loss of dorsal mesodermal gene expression. FoxD3 is also necessary and sufficient for the expression of several Nodal-related genes and is dependent on the active Nodal signaling pathway to regulate mesodermal development (Steiner et al., 2006; Yaklichkin et al., 2007). As in *Xenopus*, *foxd3* in zebrafish also presents a biphasic expression. It is expressed at shield stage, and later in the premigratory neural crest cells (Odenthal and Nusslein-Volhard, 1998). Several groups have studied FoxD3 function in zebrafish neural crest development and have determined that FoxD3 is necessary for differentiation of neural crest derivatives, including craniofacial cartilage, peripheral neurons, glial, and iridophore pigment cells (Kelsh et al., 2000; Lister et al., 2006; Montero-Balaguer et al., 2006; Stewart et al., 2006), however, an early function during zebrafish gastrulation has

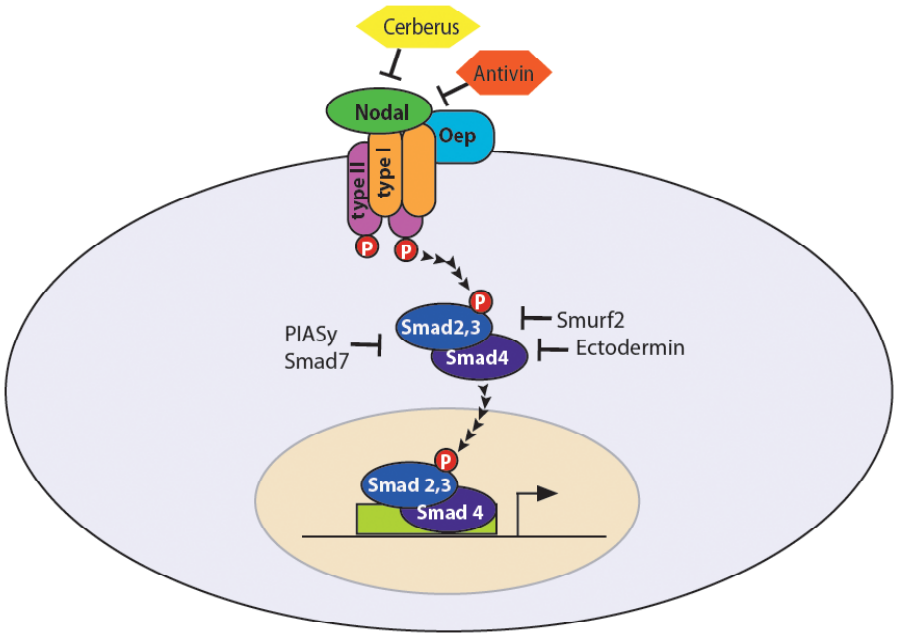

Fig 1.2. The Nodal pathway. Nodal binds to the type II receptor and the co-receptor Oep. The type II receptor phosphorylates the type I receptor. This results in activation of the type I receptor, which phosphorylates Smad2/3. Smad2 and Smad3 are specific mediators of Nodal pathway. Phosphorylated Smad binds Smad4 and translocate to the nucleus where they bind to specific DNA-binding factors to allow transcriptional activation of specific targets.

not been demonstrated.

During vertebrate development the neural crest (NC) is a transient population of cells that delaminates from the neural tube. Early migrating NC cells are specified as neurons and glial cells, while later migrating cells form most pigment cells, melanoblasts. FoxD3 regulates the fate choice between neural/glial and pigment cells in a subset of neural crest cells during the early phase of neural crest migration to repress melanogenesis and inhibit melanoblast differentiation into melanophores or melanocytes. FoxD3 acts by repressing the *mitfa* promoter indirectly in the initial subset of migrating cells. The mechanism of repression involves FoxD3 interaction with the transcriptional activator Pax3 to inhibit Pax3 binding to the mitf promoter (Curran et al., 2009; Thomas and Erickson, 2009). The expression of *foxd3* is controlled by Disc1, which has a role in the transcriptional repression of *foxd3* and *sox10 (Arduini et al., 2009)*. *sox10* is expressed by all neural crest cells and is essential for their differentiation into pigment and glia cells. Disc1 is thought to regulate migration of the cranial NC and sublineage specification (Arduini et al., 2009; Drerup et al., 2009). *kit* and *foxd3* interact to regulate melanophore survival at later stages in the zebrafish. In *kit* mutants, loss-of-function for *foxd3* results in partial rescue of melanophores from apoptosis, while FoxD3 overexpression induces early melanoblast death. Therefore, Kit and FoxD3 may cooperate to establish proper melanophore patterning and number (Cooper et al., 2009). Consistent with a conserved role in neural crest development, *foxd3* has been found to be a strong candidate gene for vitiligo susceptibility in AIS1-linked families with autosomal dominant vitiligo linked to chromosome 1p (Alkhateeb et al., 2005)

FoxD3 has also been reported to function in a variety of other processes. FoxD3 regulates OTK18, a C_2H_2 type zinc finger protein involved in the regulation of HIV-1, by binding its promoter after HIV-1 infection (Buescher et al., 2009). It has also been reported that FoxD3 plays a role in regulating myf5 in somite and adaxial cells but not in presomitic mesoderm (Lee et al., 2006), although we could not replicate or confirm these results (unpublished).

Two FoxD3 mutants have been identified in zebrafish from ENU mutagenesis screens, the *mother superior* (*mosm*^{188}) mutant and the *sympathetic mutation 1* (*sym1*) mutant. *mosm*^{188} was isolated based on its prominent craniofacial phenotype and the mutation likely resides in an distal neural crest-specific regulatory element of the FoxD3 locus, leading to depletion of neural crest derivatives (Montero-Balaguer et al., 2006). The *sym1* mutant was identified in a screen for genes required for normal development of the peripheral sympathetic nervous system. The sym1 molecular lesion is a point deletion that results in the production of a truncated protein predicted to be a null allele based on the absence of essential domains. *sym1* embryos have defects in a subset of neural crest derivatives, including peripheral neurons, glia and craniofacial cartilage (Stewart et al., 2006). However, neither of these *foxd3* mutants have reported defects in mesoderm formation, gastrulation or axial development, as would be expected from the described role of FoxD3 during early embryogenesis of the mouse and *Xenopus*.

1.6. Regulation of gene expression

Gene expression is a highly specific and regulated multilayer process with a plethora of interconnections as well as safeguard and feedback mechanisms. Organisms

contain genomes that vary from thousands to tens of thousands genes and each and every one of these individual genes has to be properly controlled so to have the correct expression of their products at the right time, at the right place and at the right rate. They also have to be able to modulate protein expression in response to new signals and changing environments depending on the needs of the cell or organism and therefore, drive processes of cellular differentiation and morphogenesis. The study of these regulatory mechanisms is not only to understand how the pattern of gene expression is regulated in a specific cell or tissue, but also to understand misregulated gene expression that may lead to disease.

There are several mechanisms for gene expression regulation: structural modification, transcriptional regulation, post-transcriptional modification, RNA processing and transport, mRNA stability, ribosomal translation efficiency, and post-translational modification. A brief explanation of mechanisms of gene expression regulation follows, with a more detailed focus on translational regulation.

One form of regulation at the DNA level is structural, in which regions of DNA are more or less tightly packed so that transcriptional machinery has more or less access to target genes. Histones, around which DNA is packaged, have “tails” which extend outwards and present amino acid sequences that can be modified to alter the packaged state of DNA. Another mechanism to control gene transcription is to alter the availability, quantity, or activity of transcription factors. The activity of transcription factors is regulated in many different ways: they can themselves be produced in higher or lower levels; they may be activated or deactivated by chemical modifications such as phosphorylation; their localization to the nucleus can be regulated, such as by binding to

a ligand; or their activity can be enhanced or inhibited by interaction with other cofactors and transcription factors (Lackner and Bahler, 2008; Munshi et al., 2009; Sonenberg and Hinnebusch, 2009).

At the level of the RNA transcript, RNA molecules are constantly being synthesized and degraded. Every RNA has a different longevity, depending on features such as the length of the polyadenylation tail and the presence of sequence elements in the regulatory region of the transcript. These features influence the interaction of each RNA with the cellular degradation machinery. Messenger RNA was thought of as a mere subcarrier of genetic information and only recently has been recognized as a key player of regulation and control of gene expression. Moreover, the awareness of not only protein, but also mRNA as a modulator of genetic disorders has vastly increased in recent years. MicroRNAs (miRNAs) function at this level to alter target RNA stability. miRNAs are short non-coding RNAs that regulate gene expression post-transcriptionally. They generally bind to the 3′-UTR of their target mRNAs and repress protein production by destabilizing the mRNA and silencing translation. In animals, once dsRNA enters the cell, it is cleaved by an RNase III –like enzyme, Dicer, into double-stranded small interfering RNAs (siRNA) 21-23 nucleotides in length (Nykanen et al., 2001). The miRNA single strands base pairs with their complementary mRNA molecules and inhibit translation or sometimes induce mRNA degradation by Argonaute proteins (Martinez et al., 2002).

Nonsense mediated mRNA decay is a post-transcriptional surveillance mechanism which results in destruction of mRNA encoding incomplete protein products, such as those with premature termination codons. This applies, for example, to

termination codons of upstream ORFs, to termination codons that are followed by splice events in the 3′ untranslated region, or to termination codons that are introduced into an ORF as the result of somatic DNA rearrangements or mutation, alternative splicing, ribosomal frameshifting, or mRNA editing (Neu-Yilik and Kulozik, 2008). During the process of splicing, a dynamic multiprotein complex, the exon junction complex, is deposited 20–24 nucleotides 5′ to the exon junctions and accompanies the mRNA into the cytoplasm and polysomes. If positioned in the 3′ UTR, this complex serves during translation as a spatial reference point for the discrimination between premature and "normal" termination codons. In contrast, if positioned within the ORF, it enhances translation. A translation termination codon is generally seen as "normal" if no exon junction follows more than 50–55 nucleotides downstream (Nagy and Maquat, 1998). In this way, the physiological termination codon would be found in the last exon.

Finally, genes are extensively regulated at the protein level. Proteins can have active or inactive conformations, or can require chemical modifications or cofactors to be functionally active. This allows a cell to control where and when a protein is active and to allow a rapid conversion in a functional or nonfunctional state. In addition, cellular levels of proteins can be downregulated by targeted degradation. A well-known mechanism to selectively degrade proteins in the cell is through the ubiquitin–proteasome pathway, a process in which a small signal molecule is added to designated proteins, marking them for transport to the proteasome where ubiquitinated proteins are degraded into peptides and amino acids.

Viewed together, the activity of genes can be regulated on every level, from DNA to RNA to protein. In many instances, the control of any single gene may occur on

several different levels, with continuous precise adjustments to ensure the levels of gene activity are appropriate.

1.7. Translation and frameshifting

The ribosome translates the genetic information of an mRNA molecule into a sequence of amino acids. Amino acids are transferred to a growing polypeptide chain at the ribosomal site of protein synthesis during translation by transfer RNA (tRNA), a small RNA (74-95 nucleotides in length). tRNA has a 3' terminal site for amino acid attachment and also contains a three base region called the anticodon that can base pair to the corresponding three base codon region on an mRNA. Each type of tRNA molecule can be attached to only one type of amino acid, however because of the genetic code being degenerate, tRNA molecules with different anticodons can carry the same amino acid. tRNA molecules corresponding to specific codons are present in different concentrations, depending on cell or tissue type within an organism or between distinct organisms. In general, there is a ~10-fold difference in concentration between the most and least abundant tRNAs (Ikemura and Ozeki, 1983). Organisms also have a codon usage bias, which refers to differences among organisms in the frequency of occurrence of specific codons in protein-coding DNA sequences. Different factors have been proposed to be related to codon usage bias, including gene expression level, %GC composition, amino acid conservation, transcriptional selection, RNA stability, and optimal growth temperature (Ermolaeva, 2001; Lynn et al., 2002; Paul et al., 2008).

Three tRNA-binding sites are located on the ribosome, termed the A, P and E sites. The A site, containing the decoding centre, binds an aminoacyl-tRNA

corresponding to the codon displayed at this site. The P site binds the peptidyl-tRNA, the tRNA carrying the nascent chain before peptide-bond formation, and the E site binds deacylated tRNAs, tRNAs that have already participated in chain elongation. In the course of two elongation cycles a tRNA moves in succession through the ribosomal A, P and E sites. The first and the third tRNA-binding sites, the A and the E sites, are coupled in a reciprocal fashion. A tRNA bound to the E site induces a low-affinity state for an aminoacyl-tRNA or a ternary complex at the A site and vice versa; occupying the A site promotes a low-affinity state for the E site, thus triggering the release of the deacylated tRNA (Geigenmuller and Nierhaus, 1990). A consequence of this mechanism is that statistically, only two tRNAs are found on the ribosome during protein synthesis, either at the A and P sites (PRE state) or at the P and E sites (POST state). This will be relevant in discussing the process of translational frameshifting, where a mismatch in one site will result in decoding a different amino acid than predicted (Vimaladithan et al., 1995).

The correct decoding of genetic information is a crucial step in the process of protein synthesis. As mentioned previously, ribosomal frameshifting is a mechanism that can alter the primary structure of proteins, either by introducing alternate amino acid sequences or a premature termination codon. Therefore, frameshifting can alter the function or production of protein products. Unusual translational events, including frameshifting are well established. Ribosomes are known to misincorporate amino acids at frequencies as high as 10^{-4} to 10^{-3} per codon. Ribosomes have been documented to engage in alternative reading frames during translation, such as frameshifting to −1, +1, to undergo short and long distance ribosomal hopping (+6 or +60), and read through stop codons in both eukaryotic and prokaryotic cells (Atkins et al., 1990; Parker, 1989). For

example, the HIV gag-pol gene is a well-known system in which a highly efficient (12% of products) −1 frameshift takes place. In this case, frameshifting occurs within an overlap region for the open reading frames of the genes encoding the structural (gag) and enzymatic (pol) products involved in the process of reverse transcription. Many retroviruses synthesize gag-pol fusion proteins even though these two genes lie in different translational reading frames with the gag and pol open reading frames overlapping by 230 nucleotides (Jacks et al., 1987). The production of the fusion protein requires a programmed –1 ribosomal frameshifting within the overlap region as ribosomes translate the viral messenger RNA. This frameshift occurs at a slippery sequence followed by an RNA structural motif that stimulates frameshifting. This motif is commonly a stem–loop for HIV-1 and the sequence following the stem–loop can influence the frameshift efficiency in HIV-1 (Dulude et al., 2002). Frameshifting occurs only when the ribosome pauses occurs at a sequence that can induce the shift in reading frames. The mechanisms leading to this pause include blockage of ribosomal movement by a RNA pseudoknot, slow decoding of an in-frame sense codon, and slow recognition of an in-frame termination codon by peptide release factor. Since frameshift efficiency appears to vary directly with the duration of the pause, rare codons and those will low abundance tRNAs are likely targets for physiological frameshifting (Farabaugh, 1996). In our studies of the FoxD3 *sym1* mutant we have found that translational frameshifting suppresses the otherwise null character of this mutation, providing an interesting example of frameshifting control in a developmental context.

1.8. Summary of Results Presented in this Thesis

In my thesis research I have focused on regulation of dorsal mesoderm development in zebrafish, specifically, the role of the transcription factor FoxD3 in this regulatory network. Two zebrafish *foxd3* mutants have been identified: the *mother superior* (*mosm*^{188}) mutant that leads to a depletion of neural crest derivatives (Montero-Balaguer et al., 2006) and the *sympathetic mutation 1* (*sym1*) mutant which shows defects in a subset of neural crest derivatives (Stewart et al., 2006). Both mutants were identified in a mutational analysis screening for neural crest deficiencies. FoxD3 function is necessary and sufficient for dorsal mesoderm formation in *Xenopus*, where it maintains *nodal* expression in the Spemann organizer (Steiner et al., 2006). *foxd3* expression in the *Xenopus* organizer is conserved in the zebrafish shield where *foxd3* is coexpressed with the Nodal-related genes, *cyclops* (*cyc*) (Rebagliati et al., 1998) and *squint* (*sqt*) (Erter et al., 1998). Therefore, we would expect defects in dorsal mesoderm development, in the gastrula and during axis formation in the zebrafish *foxd3* mutants, but this has not been reported. To assess the role of *foxd3* in zebrafish mesodermal development I examine the function of FoxD3 during early gastrulation in wild type zebrafish and in the *sym1* mutant to address these contrasting findings.

I have found that in the zebrafish embryo FoxD3 acts to modulate dorsal mesoderm formation through the Nodal signaling pathway (Chapter 2). These results confirm that FoxD3 is an essential and conserved component of the regulatory pathways that pattern the embryonic mesoderm of vertebrates. Further, studies of FoxD3 mutant, *sym1*, show indeed, these mutants do display defects in mesodermal and axis formation, consistent with a requirement for FoxD3 function in these processes. However, the *sym1* product retains activity, contrary to the predictions based on the molecular lesion, and is a

hypomorphic mutation (Chapter 3). Finally, I present evidence that the retention of activity in the *sym1* mutant is a result of an unexpected translational frameshifting process that suppresses the null character of *sym1* (Chapter 4).

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2. Chapter 2: FoxD3 regulation of mesodermal development in Zebrafish

2.1. Summary

FoxD3, a member of the Fox family of transcription factors, has roles in regulation of neural crest development and maintenance of mammalian stem cell lineages. Mouse FoxD3 null embryos die by 6.5 dpc, and FoxD3 null trophoblast progenitors are defective in self-renewal and differentiation. Further, neither embryonic stem cell lines nor trophoblast stem cell lines can be established from FoxD3 null embryos. Differentiation of embryonic stem cells into distinct tissue lineages has also been shown to be regulated by expression of Nodal. In addition, Nodal ligands, members of the TGFβ superfamily, are required for germ layer induction in vertebrates. During zebrafish gastrulation, the expression domain of the Nodal-related genes, Cyclops (Cyc) and Squint (Sqt), overlaps that of FoxD3 in the shield, suggesting a possible role for FoxD3 in mesodermal development. At shield stage, dorsal mesoderm markers are expanded when FoxD3 is overexpressed, and reduced after FoxD3 knockdown (KD) by morpholinos. KD phenotypes observed at 24hrs are similar to the Nodal loss-of-function phenotype. FoxD3 is dependent on a functional Nodal pathway for dorsal mesoderm induction as FoxD3 does not rescue mesodermal development or induce ectopic mesoderm in MZoep embryos that lack a functional Nodal pathway. The results suggest that FoxD3 regulates dorsal mesoderm induction through the Nodal pathway, at least in part.

2.2. Introduction

The basic vertebrate body plan is initiated by signaling cascades induced by Wnt, BMP, Nodal, and FGF ligands. These signals act in a temporally and spatially coordinated manner (Kimelman, 2006) and are regulated by extra cellular inhibitors of Wnt, BMP and Nodal proteins, that are secreted by the organizer (De Robertis and Kuroda, 2004). These inhibitors shape and refine the growth factor gradients into dynamic, overlapping signaling territories. Although a great deal is known about these signaling pathways, much is to be explored on how the specific germ layer boundaries are formed and maintained.

The primary signaling center responsible for dorso-ventral patterning of the mesoderm during gastrulation is the Spemann organizer in Xenopus, and the dorsal embryonic shield in zebrafish. Nodal ligands, members of the TGF β superfamily, are expressed in the shield and are required for induction and patterning the mesoderm and endoderm in all vertebrates (Schier, 2003). The zebrafish nodal Sqt is expressed in a dorsal region of the blastula that includes the extraembryonic yolk syncytial layer (YSL) while Cyc is expressed only in the dorsal blastula (Feldman et al., 1998). Nodals are conserved and expressed in all vertebrates and require EGF-CFC proteins as cofactors (Ding et al., 1998; Gritsman et al., 1999). In zebrafish, nodals signal by binding type II and type I receptors together with the EGF-CFC coreceptor (Oep). This induces phosphorylation and activation of smads 2/3, which form a complex with smad4. This complex can then enter the nucleus and regulate target gene expression.

Nodal antagonists are also present during early embryogenesis. Antivin proteins negatively regulate mesoderm induction through inhibition of Nodal activity by

competing for binding to their common receptors. Further, overexpression of antivin in zebrafish embryos blocks head and trunk mesoderm formation that results in a phenotype identical to that of Nodal-loss of function (Bisgrove et al., 1999; Meno et al., 1999; Thisse and Thisse, 1999). In a similar level, tomoregulin-1 (TMEFF1) binds to the nodal coreceptor Oep, however does not associate with either nodal or the type I ALK4 receptor (Harms and Chang, 2003). Cerberus, on the other hand, functions by directly binding Nodals to block their activity, and its expression is activated by Nodals during gastrulation therefore acting as a feedback inhibitor of Nodals (Piccolo et al., 1999). Dapper2 (dpr2) is positively regulated by Nodal signals and is expressed in mesoderm precursors during embryogenesis. Dpr2 acts by binding Alk5 and Alk4 receptors to aid in their lysosomal degradation (Zhang et al., 2004). The zinc-finger transcription factor Churchill (ChCh) represses transcriptional response to Nodal signaling and can be induced by FGFs. It also regulates cell fate by regulating cell movements, therefore, ChCh shows a dynamic role in regulating cell movement and fate during early development (Londin et al., 2007a; Londin et al., 2007b).

FoxD3, a member of the forkhead class of transcription factors, is essential for neural crest development and maintenance of mammalian stem cell lineages. FoxD3 expression is present in mouse and human embryonic stem cells, in mouse trophoblast stem cells and in the preimplantation mouse embryo. FoxD3−/− embryos die after implantation; they present a reduced epiblast and lack a primitive streak. FoxD3 is also required for maintenance of embryonic cells of the early mouse embryo and ES cell lines cannot be established from FoxD3−/− embryos. Taken together, FoxD3 is a factor required for the maintenance of progenitor cells in the mammalian embryo (Hanna et al.,

2002; Sutton et al., 1996). Interestingly, trophoblast progenitors in FoxD3−/− embryos do not self-renew, and are not multipotent, but instead give rise to an excess of trophoblast giant cells, therefore trophoblast stem cell lines can not be established from FoxD3 null embryos (Tompers et al., 2005). During Xenopus early embryogenesis FoxD3 is essential for Nodal expression in the Spemann organizer, dorsal mesodermal development and axis formation. FoxD3 function in mesoderm induction is dependent on the recruitment of transcriptional corepressors of the TLE/Groucho, and blocking FoxD3 activity results in axial defects and loss of dorsal mesodermal gene expression. FoxD3 also requires the Nodal signaling pathway for expression of several Nodal-related genes (Steiner et al., 2006; Yaklichkin et al., 2007). In zebrafish, FoxD3 is expressed at shield stage in the dorsal cells and in paraxial mesoderm, and later in the premigratory neural crest cells (Odenthal and Nusslein-Volhard, 1998). FoxD3 function in the neural crest has been determined to be necessary for differentiation of neural crest derivatives, including cranio-facial cartilage, peripheral neurons, glia, and iridophore pigment cells (Kelsh et al., 2000; Lister et al., 2006; Montero-Balaguer et al., 2006; Stewart et al., 2006), however, its early function during gastrulation has not been nearly as well documented.

Here we show that FoxD3 gain-of-function is able to induce dorsal mesoderm markers and dorsalized embryos and that it is required for dorsal mesoderm development as FoxD3 knockdown results in reduced dorsal marker expression. Further, FoxD3 knockdown phenotypes are similar to those for nodal loss-of-function, further implying a role for FoxD3 is dorsal mesoderm development. We make use of MZoep fish, which are deficient in the EGF-CFC co-receptor essential for nodal signaling, to examine the regulatory relation of FoxD3 and the Nodal pathway. FoxD3 overexpression does not

rescue the MZoep fish. This result places FoxD3 upstream of *Oep* and shows the requirement of a functional nodal pathway for FoxD3 to regulate dorsal mesoderm gene expression.

2.3. Materials and methods

2.3.1. Zebrafish methods and microinjection

Zebrafish (Tubingen strain) were raised under standard laboratory conditions as previously described (Mullins et al., 1994), and developmental stage was determined according to (Kimmel et al. 1995). Microinjection of wild-type embryos was performed at the one-cell stage using standard methods (Westerfield, 1993). WT and injected embryos were stage matched for all experiments.

2.3.2. FoxD3 expression constructs

A pCS2-myc-FoxD3 plasmid (Lister et al., 2006) was used for expression of wild-type zebrafish FoxD3. The pCS2-myc-FoxD3 construct (Lister et al., 2006) was used for expression of FoxD3 protein. For microinjection in vitro transcribed mRNA was generated from NotI linearized plasmid templates using the Ambion SP6 mMessage mMachine system (Austin, TX). Microinjections were done at the one cell stage.

2.3.3. Morpholino oligonucleotides

Morpholino antisense oligonucleotides were obtained from Gene Tools (Philomath, OR). Lyophilized oligonucleotides were resuspended in water, then diluted into 1X Danieau buffer (Nasevicius and Ekker, 2000) and 1nl was injected into one-cell

stage embryos. Two morpholino antisense oligonucleotides were designed to *Danio rerio* FoxD3 (BC095603): FoxD3MO1 (5'-TGCTGCTGGAGCAACCCAAGGTAAG-3') is complementary to nucleotides 160-184 of the 5' UTR and FoxD3MO2 (5'-TGGTGCCTCCAGACAGGGTCATCAC-3') is complementary to nucleotides 194-218 and overlaps the start codon. A mixture of the two oligonucleotides (total dosage 20ng per embryo) was used for knockdown experiments in wild-type embryos. Injection of the individual oligonucleotides at higher dosage (30-40ng) yielded similar results but with some associated toxicity. As specificity controls, a mismatch oligonucleotide was injected at equal dosage, and Cyc or *Xenopus* FoxD3 (Steiner et al., 2006) were injected to rescue.

2.3.4. In situ hybridization

Whole-mount in situ hybridization was performed as previously described (Schulte-Merker et al., 1992), using the following digoxigenin-labeled antisense RNA probes: *bmp7* (Schmid et al., 2000), *chordin* (Miller-Bertoglio et al., 1997), *cyclops* (Rebagliati et al., 1998), *goosecoid* (Stachel et al., 1993), *no tail (Schulte-Merker et al., 1992)*, and *sonic hedgehog* (Krauss et al., 1993). All images were taken from an MZFLIII12.5 stereomicroscope (Leica) with a Retiga 1300 camera (Q-imaging) and processed using Adobe Photoshop.

2.4. Results

2.4.1. Dorsal mesoderm induction by FoxD3

To determine the activity of FoxD3 in mesodermal development, gastrulation and axis formation, gain-of-function analysis was performed by mRNA injection, and embryos were examined at the gastrula and 24hpf stages (Fig. 2.1). Wild-type embryos were injected at the one-cell stage with 25pg of FoxD3 mRNA, and at the shield stage morphology was assessed in live embryos and mesodermal gene expression was evaluated by whole-mount in situ hybridization. In response to FoxD3, excessive convergence to the midline was observed as a thickening of the dorsolateral blastoderm and shield (94%, n=500), as compared to uninjected embryos (Fig. 2.1A,B). In a majority of injected embryos, expansion of dorsal or panmesodermal gene expression into lateral or animal domains was observed (92%, n= 556) (Fig. 2.1C-J). For *cyclops*, *chordin*, and *goosecoid*, genes normally restricted to the shield domain (Miller-Bertoglio et al., 1997; Rebagliati et al., 1998; Stachel et al., 1993), FoxD3 induced lateral expansion (Fig. 2.1D,F,H), as well as animal expansion for *chordin* (Fig. 2.1F) and ectopic expression for *goosecoid* (Fig. 2.1H). The panmesodermal gene *no tail* is expressed by mesodermal cells throughout the margin at the shield stage (Schulte-Merker et al., 1992), and an expansion of *no tail* towards the animal pole was observed in response to FoxD3, resulting in a broader marginal domain of *no tail* expression (Fig. 2.1J). Consistent with the expansion of dorsal markers, the ventrolateral domain of *bmp7* expression (Schmid et al., 2000) was reduced and limited to the ventralmost margin in response to FoxD3 (Fig. 2.1L).

FoxD3-injected embryos were strongly dorsalized at 24hpf (90%, n=230) (Fig. 2.1M,N), consistent with the expansion of dorsal mesodermal genes at the shield stage. To assess the organization of the axial mesoderm at 24hpf, *no tail* and *sonic hedgehog* expression was examined (Fig. 2.1O-R). *no tail* expression was not perturbed throughout

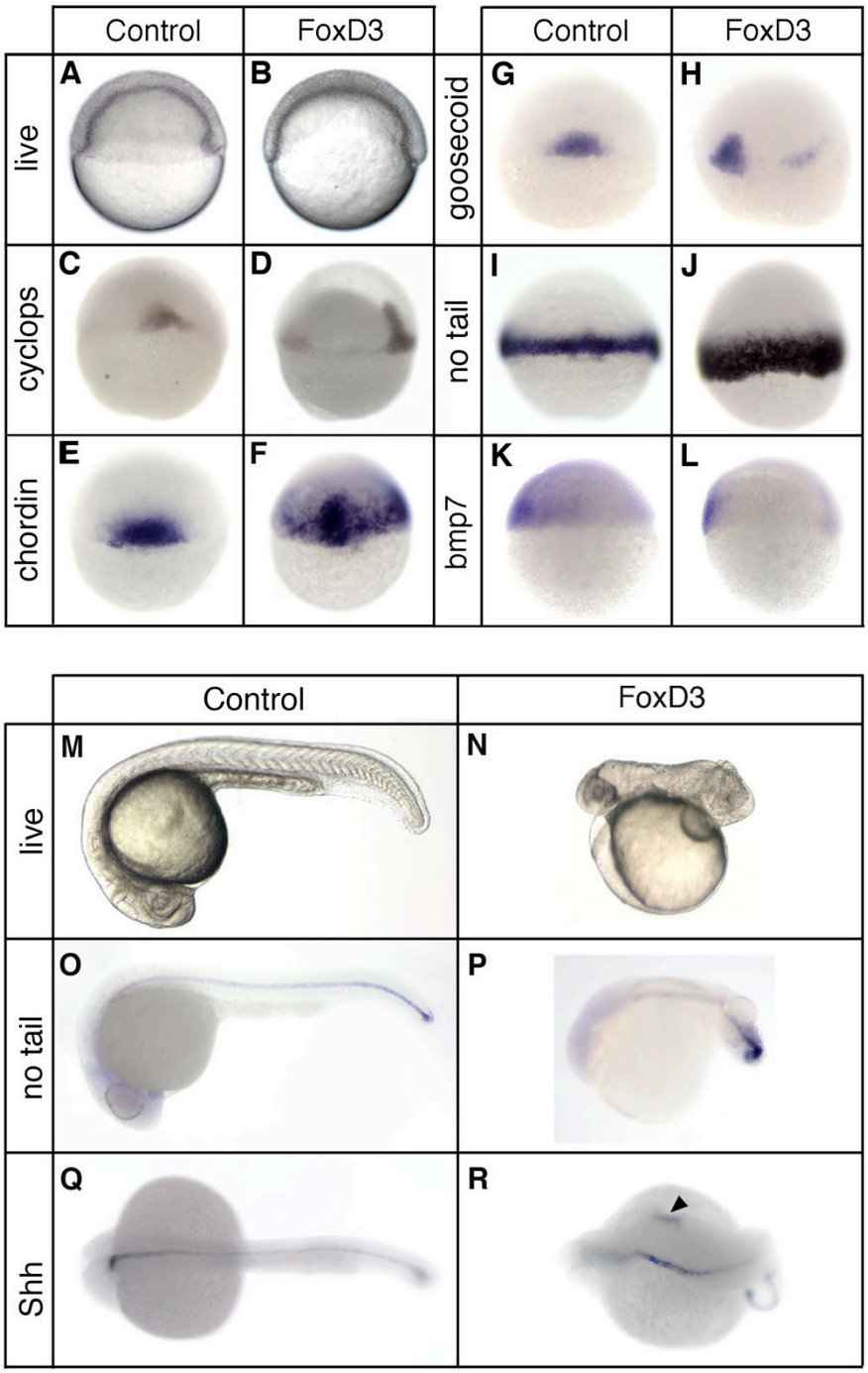

Figure 2.1 FoxD3 expression results in dorsalized embryos. (A-L) whole mount view of shield stage embryos (6hpf). (A,B) live embryos showing (A) WT shield structure and blastoderm and (B) expanded shield and blastoderm after FoxD3 overexpression. In situ hybridization of WT control embryos showing expression of (C) cyc in the shield, (E) chd in the shield and paraxial mesoderm, (G) gsc in the shield (I) ntl throughout

mesoderm, and (K) bmp7 in the ventral region. In situ hybridization of FoxD3 overexpressing embryos showing expansion of dorsal markers (D) expansion of cyc laterally, (F) expansion of chd laterally and towards the animal pole, (H) misexpression and expansion of gsc, (J) expansion of ntl towards the animal pole and (L) reduction of ventral marker bmp7 from ventral regions. (M-R) 24hpf embryos (M) control embryo showing normal phenotype, (N) FoxD3 overexpressing embryos showing a dorsalized phenotype. (O) In situ hybridization of control embryo for ntl expression seen in the notochord, (P) ntl expression in the FoxD3 overexpressing embryo, (Q) Shh expression in the ventral neural tube along the dorsal axis of a control embryo (dorsal view), (R) ectopic expression of Shh in a FoxD3 overexpressing embryo (dorsal view) (arrow head).

much of the body axis, but was disorganized in the tailbud (Fig. 2.1P), consistent with the morphogenetic disruption of posterior structures in dorsalized embryos (Holley, 2006). At 24hpf *sonic hedgehog* is expressed in the notochord, floor plate, and part of the diencephalon (Krauss et al., 1993), and expanded (83%, n=64) or ectopic (17%, n=13) expression was observed in response to FoxD3 (Fig. 2.1R), consistent with axial dorsalization and, in a minority of embryos, axial duplication. The phenotypic and gene expression changes observed at the shield and 24hpf stages demonstrate that FoxD3 can strongly induce the expansion of the dorsal mesoderm, resulting in a predicted dorsalization of the body axis. Furthermore, the embryonic response to FoxD3 is similar to that observed for Nodal pathway gain-of-function in the zebrafish (Feldman et al., 1998).

The gain-of-function studies described here show that zebrafish FoxD3 can influence mesodermal development within the intact embryo, but do not demonstrate an ability of FoxD3 to induce dorsal mesoderm de novo from competent tissue. To assess this function zebrafish FoxD3 was expressed in *Xenopus* animal explants, which normally differentiate as atypical epidermis, but are competent to form mesoderm in response to appropriate inducers (Smith et al., 1987). At the one-cell stage, FoxD3 mRNA (100pg) was injected into the animal pole, explants were isolated at the late blastula stage, cultured to the tailbud stage, and mesodermal gene expression was examined by RT-PCR. Zebrafish FoxD3 strongly induced the expression of *muscle actin* and *collagen II*, markers of somitic muscle and notochord, respectively (Fig. 2.2). Therefore, zebrafish FoxD3 has potent dorsal mesoderm-inducing activity, identical to that previously described for *Xenopus* FoxD3 (Steiner et al., 2006).

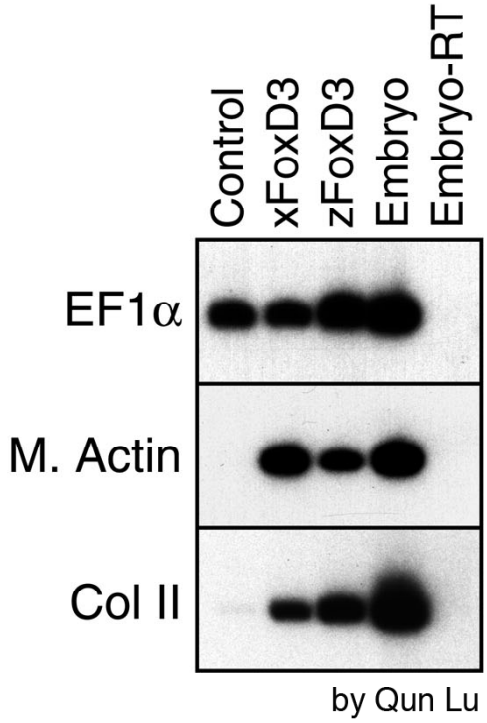

Figure 2.2 FoxD3 can induce dorsal mesoderm de novo from competent tissue. Zebrafish FoxD3 was expressed in *Xenopus* animal explants at the one-cell stage and mesodermal gene expression was examined by RT-PCR at tailbud stage. Similar to the induction of Xenopus FoxD3, zebrafish FoxD3 can induce mesoderm in *Xenopus* animal caps. Zebrafish FoxD3 strongly induced the expression of *muscle actin* and *collagen II*, markers of somitic muscle and notochord, respectively, in the same way Xenopus FoxD3 does. EF1α is used as an expression control. (The *Xenopus* animal explant studies and RT-PCR experiments were performed by Qun Lu).

2.4.2. FoxD3 is essential for dorsal mesoderm and axial development

To determine the requirement for FoxD3 in zebrafish mesodermal development, knockdown studies were performed using morpholino antisense oligonucleotides. A mixture of two oligonucleotides, one targeting the FoxD3 5'UTR and one overlapping the initiator codon (see materials and methods), was injected at the one-cell stage (total dosage 20ng), and embryos were analyzed at the shield and 24hpf stages (Fig. 2.3). At the shield stage, FoxD3 knockdown embryos had reduced or absent shield structures (Fig. 2.3B), and a severe reduction of dorsal mesodermal gene expression (Fig. 2.3C-H). *cyclops* and *goosecoid* expression was detectable, but substantially reduced in a majority of knockdown embryos (86%, n=438 and 73%, n=554) (Fig. 2.3D,F), and *no tail* expression was reduced to a thin marginal expression domain (70%, n=523) (Fig. 2.3H). In addition, *chordin* expression in the shield was strongly reduced (79%, n=431) (data not shown).

FoxD3 knockdown embryos display reduced head structures, notochord defects, loss of trunk somites, and retention of tail somites (78%, n=330) (Fig. 2.3I,J). Analysis of *sonic hedgehog* expression indicated disruption of notochord and floor plate development (Fig. 2.3K,L). Moreover, the spectrum of mesodermal and axial defects observed in FoxD3 knockdown embryos are similar to *cyclops/squint* mutants and *MZoep* mutants (Dougan et al., 2003; Feldman et al., 1998; Gritsman et al., 1999). Specificity controls for the knockdown studies included injection of a morpholino mismatch oligonucleotide, as well as knockdown rescue experiments. At a dosage (20-40ng) equal to or greater than the two perfect match oligonucleotides, a FoxD3 oligonucleotide with multiple

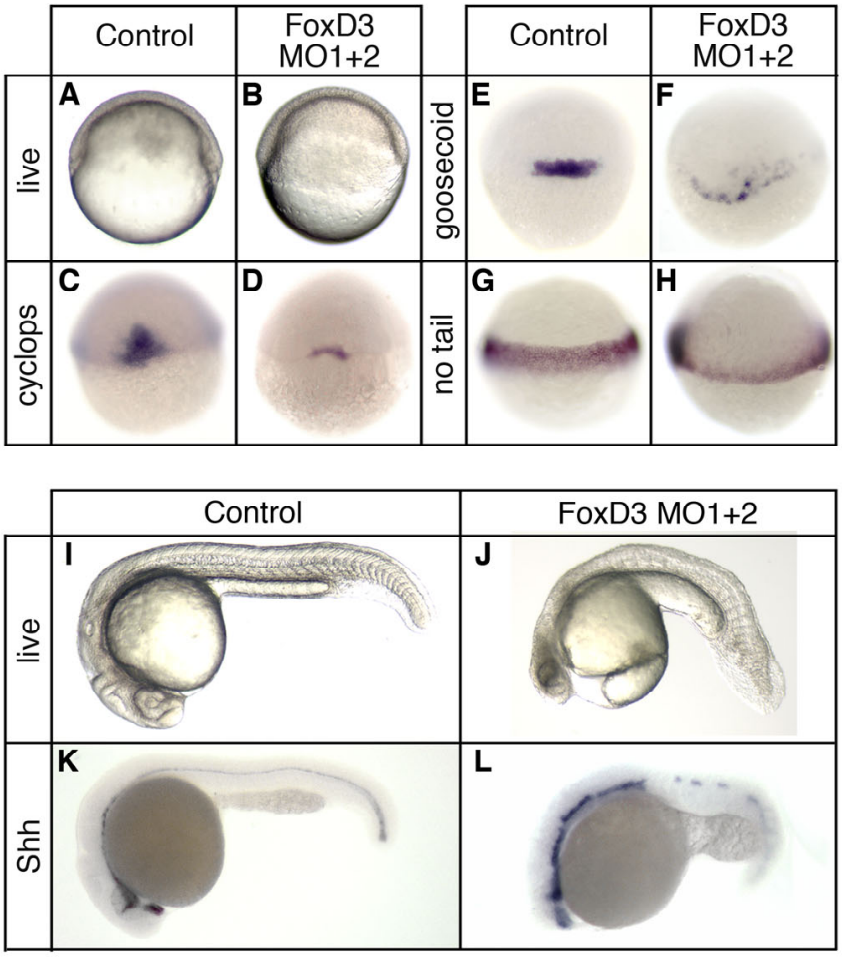

Figure 2.3 Reduced dorsal markers after FoxD3 knock down. Lateral view of, (A-G) shield stage embryos (6hpf), (H-K) 24hpf. (A,B) live embryos showing (A) WT shield structure and blastoderm and (B) FoxD3 morphants with no obvious shield structure and reduced blastoderm. In situ hybridization for (C) cyc in the shield, (E) gsc in the shield, (G) ntl throughout mesoderm. FoxD3 morphants show reduced dorsal marker expression (D) reduced cyc within the shield, (F) reduced and thinning expression of gsc (H) reduced expression of ntl towards the vegetal pole in the shield. At 24hpf (I) control embryo, (J) morphant embryos show nodal loss of function phenotype. (K) Shh expression along the ventral neural tube in a control embryo, (L) Shh expression presents gaps in FoxD3 morphants, particularly in the more posterior tail area.

mismatches did not produce any mesodermal or axial phenotypes (data not shown). In addition, injection of knockdown embryos with *Xenopus* FoxD3 rescued normal development in most embryos (82%, n=126) (fig.2.4 C,E). Taken together, results indicated that a specific knockdown of endogenous FoxD3 results in severe mesodermal and axial defects, strongly supporting an essential conserved role for FoxD3 in mesodermal development. Furthermore, the similarity of phenotype for FoxD3 knockdown and Nodal pathway loss-of-function is consistent with the predicted role of FoxD3 in promoting Nodal expression and signaling (Steiner et al., 2006). This regulatory relation of FoxD3 and the Nodal pathway is further supported by the ability of injected *cyclops* mRNA to fully rescue normal development in FoxD3 knockdown embryos (fig.2.4 D,E).

It is important to note that previous FoxD3 knockdown attempts resulted only in neural crest defects, and not in the mesodermal phenotypes we report (Kelsh et al., 2000; Lister et al., 2006; Montero-Balaguer et al., 2006; Stewart et al., 2006). This difference may simply reflect the efficacy of FoxD3 knockdown. We have used a mixture of two FoxD3-specific oligonucleotides in our studies, while the previously studies made use of only single oligonucleotides. If in fact our approach results in a more complete knockdown of FoxD3 protein, this would suggest that neural crest development is more sensitive to FoxD3 dosage than is mesodermal development, and this would account for the differences in results obtained. However, this explanation would not account for the absence of mesodermal phenotypes in the *sym1* mutant, which has been reported to be a FoxD3 null mutation (Stewart et al., 2006).

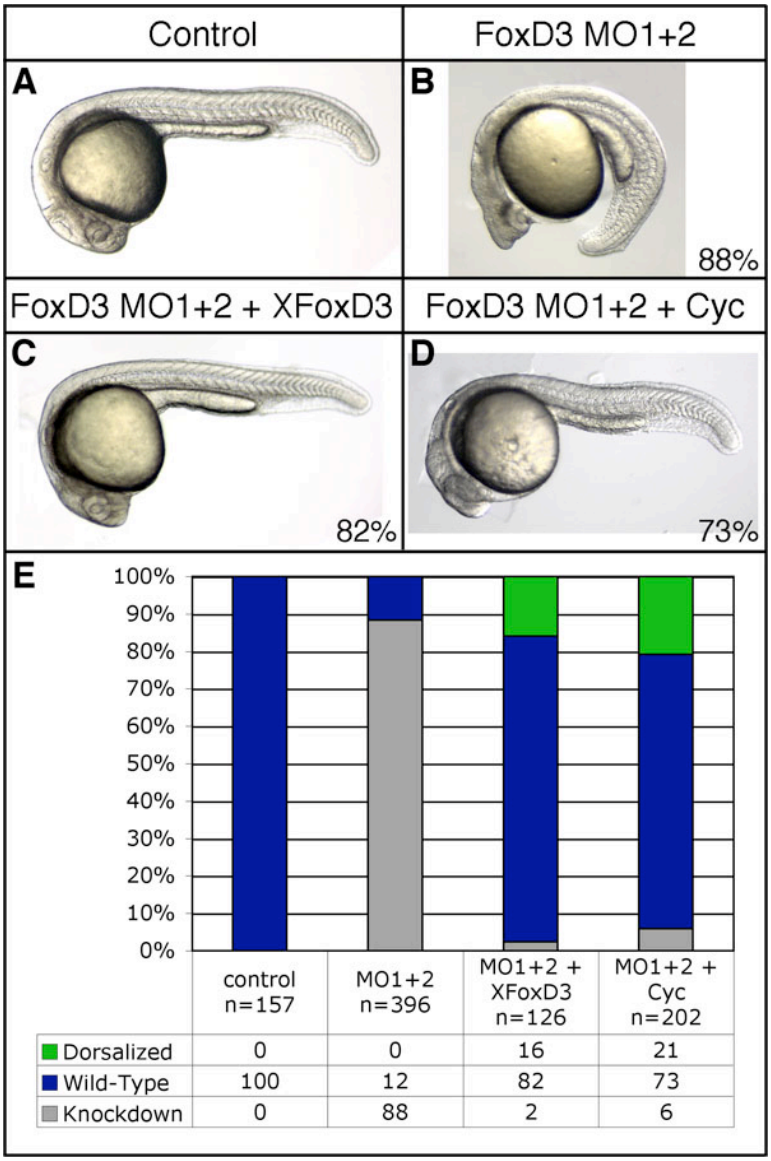

Figure 2.4 Specificity controls for the FoxD3 MO1+2 knockdown. Knockdown rescue experiments were done with *Xenopus* FoxD3 or zebrafish *cyclops*. MO1+2 morpholino combination results in (B) embryos with phenotypes similar to nodal losss-of-function at 24 hpf. Embryos show reduced head and trunk mesoderm as well as expanded tail somites (88%, n=396) compared to (A) uninjected control embryos. Co-injection of knockdown embryos at the one cell stage with (C) *Xenopus* FoxD3 rescued normal development in most embryos (82%, n=126), in a similar manner, co-injection of (D)

cyclops, a zebrafish Nodal protein, results in rescue of the mesodermal and axial tissues (73%, n=202). These results indicate that FoxD3 MO1+2 specifically knocks-down endogenous FoxD3. (E) shows the ability of *Xenopus* FoxD3 and zebrafish *cyclops* to rescue the morphant phenotype. Blue bars show the wild-type phenotype in the control embryos as well as in the rescued embryos, grey bars show the knockdown phenotype after morpholino oligo injection and green bars show dorsalized phenotypes as a result of excess dorsalizing activity of the rescuing *Xenopus* FoxD3 or zebrafish *cyclops*. FoxD3 MO1+2 results in morphant phenotypes in 88% of the embryos. Rescue injections with *Xenopus* FoxD3 result in close to wild-type phenotypes for 82% of the morphant embryos and zebrafish *cyclops* injections results in 73% of morphant embryos showing a close to wild-type phenotype.

2.4.3. FoxD3 function is dependent on an active Nodal signaling pathway

The FoxD3 gain-of-function and knockdown results are consistent with a role for FoxD3 in maintaining Nodal expression and activity in the organizer, where FoxD3 and Nodal genes are coexpressed during gastrulation. To further examine the regulatory relation of FoxD3 and the Nodal pathway, we made use of *MZoep* mutant zebrafish that lack an essential Nodal co-receptor (Gritsman et al., 1999). In *MZoep* embryos, Nodal cannot bind its functional receptor complex and Nodal signaling output is eliminated. If FoxD3 acts solely as an upstream positive regulator of the Nodal pathway in the gastrula, it is predicted that the mesodermal activity of FoxD3 would be fully suppressed in *MZoep* embryos. At the one-cell stage, *MZoep* embryos were injected with FoxD3 and mesodermal gene expression was examined at the shield stage (Fig. 2.5). As predicted, FoxD3 did not rescue or induce the expression of *cyclops*, *goosecoid*, *chordin*, or *no tail* in any embryos (100%, n=97) (Fig. 2.5I,O,U,A'). As a control for rescue, *MZoep* embryos were injected with Activin, a TGFß ligand that activates the Nodal pathway independent of the coreceptor requirement (Gritsman et al., 1999), and strong rescue of mesodermal gene expression was observed (Fig. 2.5J,P,V,B'). As positive controls for FoxD3 and Activin function, wild-type embryos injected with either FoxD3 or Activin showed strong induction of mesodermal gene expression (Fig. 2.5K,L,Q,R,W,X,C',D'). Injected *MZoep* embryos were also examined at 24hpf for axial and midline rescue, and similar results were obtained (Fig. 2.6). FoxD3 failed to rescue head, trunk or notochord development (Fig. 2.6E,F), while Activin partially rescued head and trunk, and fully rescued notochord (Fig. 2.6H,I).

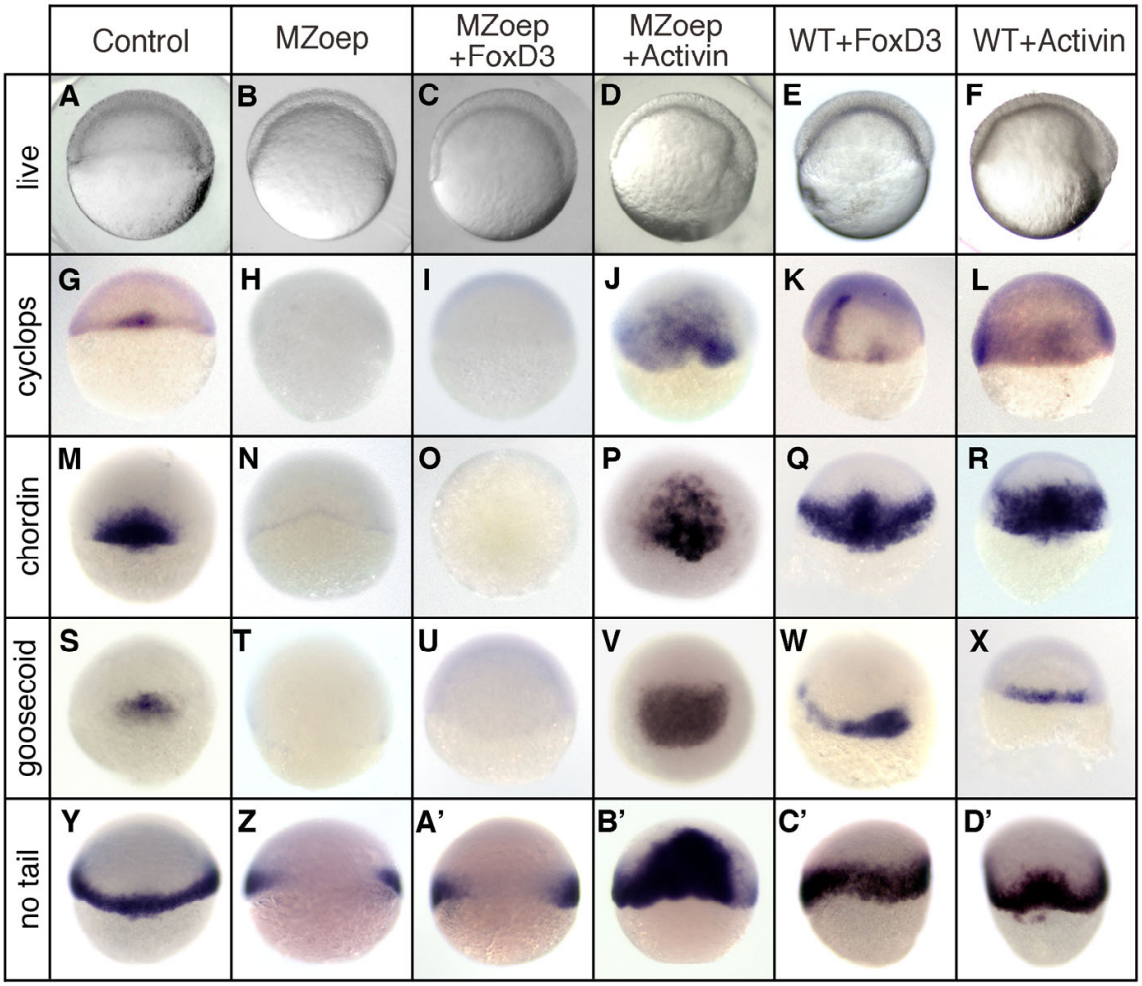

Figure 2.5 FoxD3 acts through the Nodal pathway. (A-F) lateral view of live embryos at shield stage, (A) shield structure in a wt control embryo, (B) no shield structure is evident in a MZoep mutant embryo, (C) no shield structure is obvious in a FoxD3 overexpressing MZoep mutant embryo, (D) expanded shield structure observed on the dorsal side of an MZoep mutant embryo overexpressing activin, (E) control embryo overexpressing FoxD3, the blastoderm and shield are thickened, (F) a control WT embryo overexpressing activin showing an expanded shield. (G-D') Whole-mount in situ hybridization at shield stage, dorsal view. (G,M,S,Y) control embryos showing wt

expression of cyc, chd, and gsc in the dorsal shield, (Y) showing expression of ntl throughout mesodermal tissue. (H-Z) no dorsal markers expressed in the MZoep dorsal shield, (I-A') no dorsal markers expressed after overexpression of FoxD3 in MZoep embryos. (J-B') expansion of dorsal markers after activin overexpression in MZoep embryos. (J) cyc, (P) chd, (V) gsc, (B') ntl. (K-C') control showing expansion of dorsal shield markers after FoxD3 overexpression in WT control embryos (K) cyc is misexpressed and expanded towards the animal pole, (Q) chd is expanded laterally, (W) gsc is expanded laterally, (C') ntl is expanded towards the animal pole. (L-D') control showing expansion of dorsal shield markers after activin overexpression in WT control embryos, (L) cyc is expanded towards the animal pole, (R) chd is expanded towards the animal pole and laterally, (X) gsc is expanded laterally, (D') ntl is expanded towards the animal pole.

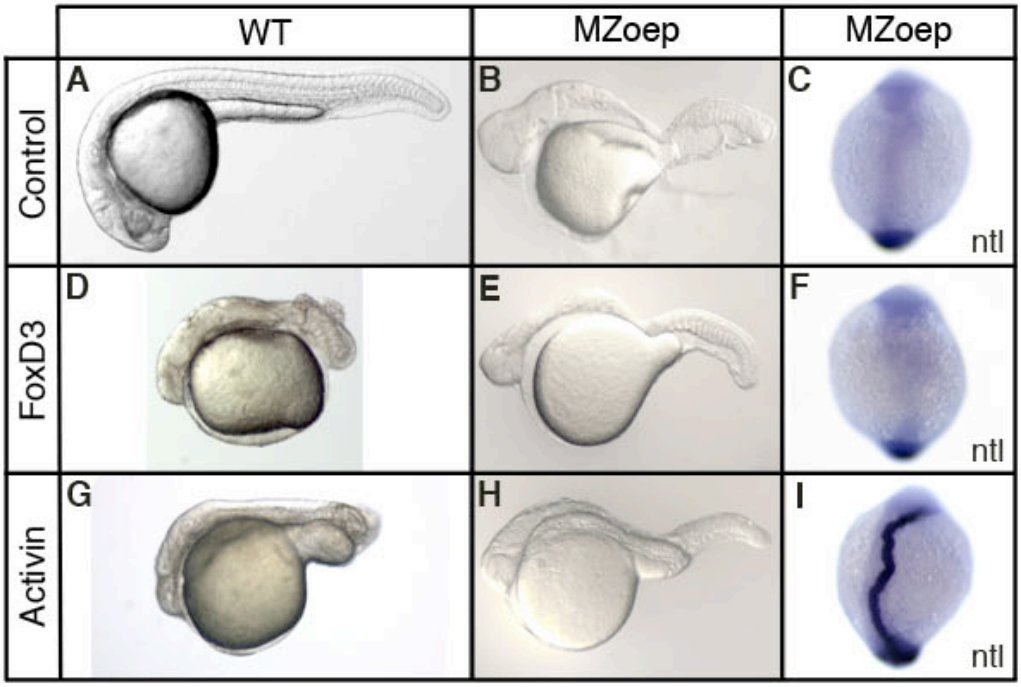

Figure 2.6 FoxD3 acts through the Nodal pathway. (A) 24hpf WT control embryo, (B) 24hpf MZoep embryo, (C) ISH for ntl at early somite stage, no notochord present. (D) 24hpf dorsalized embryo overexpressing FoxD3, (E) 24hpf MZoep embryo overexpressing FoxD3, (F) ISH for ntl at early somite stage MZoep embryo overexpressing FoxD3, no notochord present. (G) 24hpf dorsalized embryo overexpressing activin, (H) 24hpf MZoep embryo partially rescued with activin. Notice some dorsal somites and eye structures. (I) ISH for ntl at early somitogenesis (11hpf), notice formation of the notochord.

Further support for these conclusions was obtained by coexpressing FoxD3 and Antivin, an atypical TGFß-related protein that inhibits Nodal signaling by sequestration of the Oep coreceptor (Thisse and Thisse, 1999). At the shield stage, Antivin fully suppressed mesodermal gene expression and coinjection of FoxD3 did not rescue expression of *cyclops, goosecoid*, or *no tail* (100%, n=102) (Fig. 2.7). The results demonstrate that the mesodermal function of FoxD3 in the gastrula is completely dependent on a functional Nodal signaling pathway, consistent with a model in which FoxD3 acts upstream of Nodal in the organizer domain to promote mesodermal development.

2.5. Discussion

2.5.1. FoxD3 maintains dorsal mesoderm in the zebrafish shield

In this study we show several lines of evidence to support a function for zebrafish FoxD3 is the gastrula in regulating dorsal mesoderm development. These include the timing and location of FoxD3 expression, the dorsalizing activity resulting from ectopic expression of the zebrafish gene in *Xenopus* and zebrafish embryos, and the embryonic phenotypes and reduced dorsal marker expression produced by knockdown of FoxD3.

We have found that FoxD3 protein plays an important role during early dorsal mesodermal gene expression and development in the zebrafish, and that FoxD3 requires a functional Nodal pathway to regulate dorsal mesoderm.

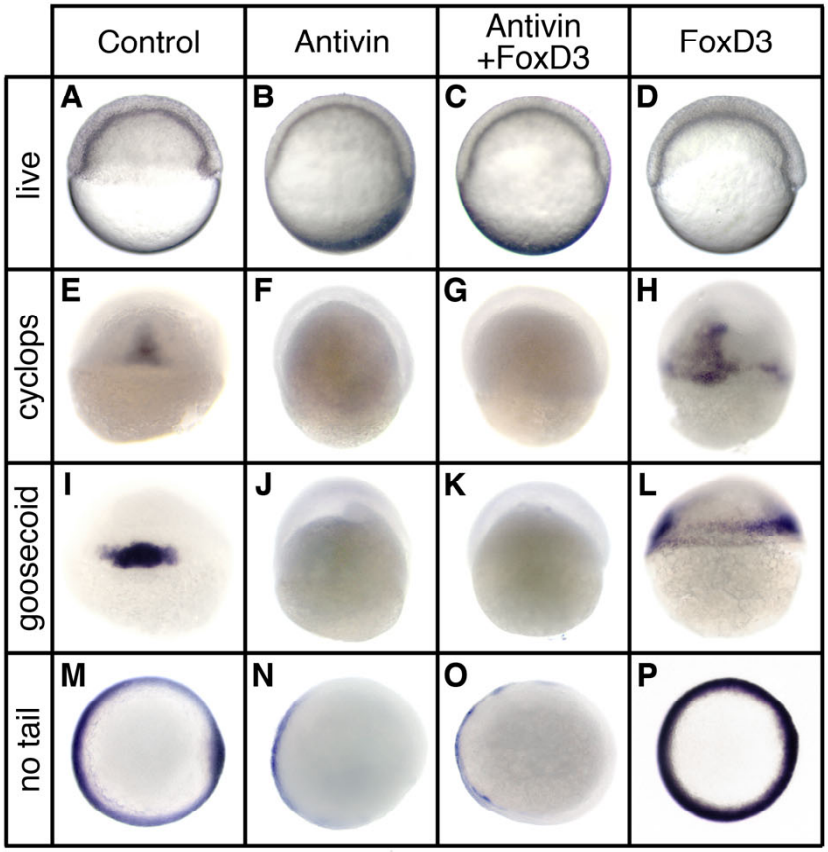

Figure 2.7 Antivin blocks FoxD3s ability to induce mesoderm. (A-D) lateral view of live embryos at shield stage showing (A) wt control shield, (B) antivin treated embryos with no shield structure being formed, (C) embryo showing co-expression of antivin and FoxD3, no shield structure is formed, (D) embryos showing a thickened blastoderm and expanded shield after FoxD3 expression. (E-P) ISH at shield stage (E,I,M) ISH of wt control embryos showing (E) cyc expression in the shield, (I) gsc expression in the shield, (M) Ntl expression through out the mesoderm (animal pole view). (F,J,N) ISH of antivin treated embryos (F) cyc is not expressed in the shield, (J) gsc is not expressed in the

shield, (N) ntl is not expressed in the shield (animal pole view). (G, K, O) ISH of embryos co-expressing antivin and FoxD3 showing (G) no cyclops expression in the shield, (K) no gsc expression in the shield, (O) no expression of ntl in the shield (animal pole view). Note how FoxD3 does not rescue the expression of any of the dorsal genes. (H, L, P) ISH of embryos overexpression FoxD3, (H) cyclops is expanded towards the animal pole and laterally, (L) gsc is expanded laterally and (P) ntl expression is expanded towards the animal pole (animal pole view).

2.5.2. FoxD3 in mesoderm induction

Nodal proteins are endogenous mesoderm inducers in vertebrates (Agius et al., 2000). The precise expression and regulation of Nodal signaling is essential for normal embryo development. Dorsal mesoderm induction results from a balance of inducing and repressing molecules, being expressed from the ventral mesoderm and the dorsal mesoderm (embryonic shield).

Results after overexpression of FoxD3 show that it is able to expand dorsal mesoderm marker expression and so would be regulating expression of target genes required during early dorsal mesoderm development. The Xenopus FoxD3 WH-DNA binding domain was cloned into constructs with VP16 or Eng as activator or repressor domains (Steiner et al., 2006). FoxD3 was found to act as a transcriptional repressor as the Eng-FoxD3 repressor fusion protein showed dorsalizing activity similar to WT FoxD3. In the same way, Eng-FoxD3 induced dorsal mesoderm marker expression and dorsalized zebrafish embryos, acting as a transcriptional repressor. We propose that FoxD3 acts as a repressor to maintain expression of dorsal mesoderm genes in the shield. Outside the shield, FoxD3 would be repressed and Nodal gene expression not maintained.

Morpholino knockdown of FoxD3 interferes with the required maintenance of dorsal mesoderm gene expression, and the result is phenotypes with reduced shield structures and reduced dorsally derived structures at 24hpf as expected. The floor plate, a specialized group of cells in the ventral midline of the neural tube of vertebrates, plays crucial roles in patterning the central nervous system, and is induced by nodals. A group of the MO oligo treated embryos analyzed for *sonic hedgehog* expression at 24hpf show reduced expression in the ventral floor plate, this may be a result of failure to specify

axial mesoderm adequately. These observations are consistent with what is seen in *oep* mutant embryos, where a small minority show defects in formation of the notochord, the more posterior axial mesoderm (Schier et al., 1997). Results are also consistent with the requirement of *cyclops* signaling during gastrulation for induction of the floor plate and ventral brain (Sampath et al., 1998). FoxD3 overexpression results in the expansion of *sonic hedgehog* expression in the floor plate as a result of *cyclops* upregulation. Zebrafish *cyclops* regulates the expression of *sonic hedgehog* in the ventral neural tube (Tian et al., 2003) and the floor plate cells that express *sonic hedgehog* at 24hpf originate from the embryonic shield (Krauss et al., 1993).

Nodal gain- and loss-of-function in the zebrafish results in the dramatic perturbation of mesodermal development (Schier and Talbot, 2005). Embryos that are null for two Nodal genes (*cyclops* and *squint*) or the Nodal coreceptor *one-eyed-pinhead* (mzOep) lack all trunk and head mesoderm as well as endoderm, and display defects during the initial induction of mesoderm at gastrula stages (Feldman et al., 2000; Whitman, 2001). We found that phenotypes observed after FoxD3 knockdown at 24hpf and later show similar characteristics to nodal loss-of-function phenotypes. This further establishes a role of FoxD3 in regulating the nodal signaling pathway and so, in regulating dorsal mesoderm development.

Our model predicts that FoxD3 function is upstream of Oep in the Nodal pathway. Consistent with our model, overexpression of FoxD3 has no effect on dorsal gene expression in MZoep fish or in Antivin-treated embryos, demonstrating that FoxD3 requires a functional nodal pathway to regulate dorsal mesoderm gene expression.

2.5.3. Differences in Nodal requirements between vertebrate species

In mouse, nodal loss-of-function results in drastically reduced mesodermal gene expression and the primitive streak does not form (Conlon et al., 1994), blocking Nodal signaling in Xenopus results in anterior truncations, together with delayed and suppressed induction of mesendodermal markers (Osada and Wright, 1999). In zebrafish, embryos that are null for the two Nodal genes (*cyclops* and *squint*) or the Nodal co-receptor *one-eyed-pinhead* (*mzOep*) do not gastrulate and lack all trunk and head mesoderm as well as endoderm and present expanded tail somites which form more ventral mesoderm (Feldman et al., 2000; Feldman et al., 1998; Gritsman et al., 1999; Whitman, 2001). *Squint;cyclops* double mutants lack mesendodermal derivatives, including notochord, somites, heart, pronephros, blood and gut, and lack head and trunk tissues (Feldman et al., 1998). Single mutants for *cyclops* and *squint* give milder phenotypes, as do single maternal or zygotic oep mutants (Dougan et al., 2003; Hatta et al., 1991). Single mutants display cyclopia and defects in prechordal plate and ventral nervous system. Given the severity of the double mutants, it is thought that these molecules have significant and overlapping activities. Further, because signaling pathways and molecules are conserved between organisms, these inconsistent nodal loss-of-function phenotypes between organisms remain to be understood.

2.5.4. Previous FoxD3 observations

Several FoxD3 loss of function approaches using antisense morpholino oligonucleotides in zebrafish show NC defects, albeit no early gastrulation defects (Cooper et al., 2009; Ignatius et al., 2008; Lister et al., 2006; Montero-Balaguer et al.,

2006; Stewart et al., 2006). A plausible explanation is that affected embryos might have been overlooked since the experiments were designed to study the activity of FoxD3 during neural crest development, a process that occurs several hours (~10h) after gastrulation and mesoderm induction. Further, this could be due to incomplete knockdown with the dose of morpholino used. In our studies we make use of two morpholino oligonucleotides at slightly higher doses, described by Lister et al. (2006) and a second one targeted to the FoxD3 ATG start codon (designed by genetools, see methods). The combination of these two oligonucleotides is effective in knocking down FoxD3 activity at shield stage. Embryos injected with both oligos that survive and develop to 3dpf have craniofacial defects and iridophore as well as melanocyte reduction, as reported previously (Lister et al., 2006). An alternate explanation might be the presence of a FoxD3 ortholog or perhaps the presence of collaborating factors, however, we have not been able to come across either. These results also highlight the difference in requirement of FoxD3 at diverse stages and the importance of understanding FoxD3 function and regulation at different stages of development.

In chapter 3, I reexamine *sym1*, a previously described FoxD3 mutant (Stewart et al., 2006). In conflict with our gain-of-function and knockdown studies, Stewart and colleague conclude that *sym1* is a null allele, but do not report any defects in mesodermal or axial development. We use genetic, phenotypic, and gene expression analyses to reassess mesodermal development in the *sym1* mutant.

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3. Chapter 3: Reexamination of *sym1,* a Zebrafish FoxD3 mutant

3.1 Summary

The FoxD3 mutant, *sym1*, has been reported to encode a truncated protein predicted to be a null allele. Phenotypic deficiencies include craniofacial defects and delayed development of chromatophores, consistent with the role of FoxD3 in neural crest formation. Based on our earlier results, we would predict early gastrulation deficiencies and defects in axial tissues derived from dorsal mesoderm. We assess the function of the Sym1 protein product by expressing *sym1* mRNA in zebrafish embryos. Phenotypic and gene expression analysis demonstrates that *sym1* retains partial function as dorsal markers are induced at the shield stage and embryos are dorsalized at later stages, similar to the activity of wild-type FoxD3. Furthermore, gene expression analysis of *sym1* heterozygous cross progeny showed that 20% of the embryos present reduced dorsal gene expression, while a considerable proportion (9%) of the swimming larva analyzed have axial defects, a phenotypic class not previously reported. This phenotypic class is enhanced by FoxD3 knockdown in mutant embryos. The results suggest that *sym1* is a hypomorphic allele of FoxD3 with partial penetrance of mesodermal defects, consistent with our earlier analysis of FoxD3 function in mesodermal development.

3.2 Introduction

FoxD3 is a member of the Forkhead transcription factor family that contain a conserved 100 amino acid DNA-interaction motif called the forkhead domain (FHD), which is a variant of the helix–turn–helix motif (Lai et al., 1990; Sutton et al., 1996). Forkhead proteins present highly conserved amino acid sequences in the DNA

recognition helix, but maintain particular DNA-binding specificity which is likely conferred by the sequences outside the recognition helix (Overdier et al., 1994). FoxD3 acts as a transcriptional repressor by binding DNA through its conserved winged helix (WH) DNA binding domain. Downstream of this WH domain, FoxD3 contains a Groucho repressor (GEH) motif which is necessary for its repressor activity (Yaklichkin et al., 2007).

FoxD3 in the early Xenopus gastrula is expressed in the Spemann organizer, and this expression domain is conserved in the zebrafish shield, in the chick node and the mouse gastrula. FoxD3 is also expressed at an earlier stage of mouse development in embryonic stem cells where it regulates stem cell maintenance (Pan et al., 2006) and later in pre-migratory and migrating neural crest (NC) cells (Barembaum and Bronner-Fraser, 2005), and motor-neuron progenitors of the developing spinal cord (Hromas et al., 1999; Labosky and Kaestner, 1998).

Two zebrafish FoxD3 mutants have been reported, both mutants arose form a mutational analysis screening for neural crest deficiencies. One is the *mother superior* (*mosm*^{188}) mutant, which was isolated based on its prominent craniofacial phenotype. *mosm*^{188} mutants have no FoxD3 expression in NC progenitors and show defects in early stages of specification and differentiation of NC derivatives, including the craniofacial cartilage, peripheral nervous system, and pigment cells. The authors suggest that the *mosm*^{188} mutation lies in a distal NC-specific regulatory element within the FoxD3 locus (Montero-Balaguer et al., 2006). The second FoxD3 mutant is *sympathetic mutation 1* (*sym1*). The *sym1* embryos have similar defects in a subset of neural crest derivatives, including peripheral neurons, glia and craniofacial cartilage, but retain normal numbers

of melanocytes (Stewart et al., 2006). The only phenotypic descriptions reported are craniofacial defects observed as a reduced jaw, and delayed development of chromatophores after 3dpf, consistent with the role of FoxD3 in neural crest formation. However, there is no mention of defects in gastrulation or dorsal mesoderm derivatives, as would be expected from our earlier results on FoxD3 regulation of dorsal mesodermal development. The *sym1* mutation is a point deletion (G537), which results in a shift of the reading frame and introduction of an early termination codon seven codons downstream of the point deletion. The point deletion in the *sym1* mutant truncates the second wing (W2) of the winged helix domain, which binds the minor groove and confers DNA recognition specificity for binding and transactivation (Berry et al., 2005).

We hypothesize that *sym1* mutants do display previously unappreciated defects in mesodermal and axial development. To assess the early developmental consequences of the *sym1* mutation we pursued genetic, phenotypic, and gene expression analyses. We find that *sym1* is a hypomorphic allele of FoxD3 with reduced penetrance of mesodermal phenotypes.

3.3 Material and methods

3.3.1 Heterozygous zebrafish mating and maintenance

Embryos and adult fish were raised and maintained under standard laboratory conditions (Westerfield, 1993). Two female and one male sym1 +/- fish were obtained from the Look lab (Stewart et al., 2006). Fish were mated and heterozygous individuals were maintained in the fish facility. Embryos were genotyped and phenotyped for experimental use.

3.3.2 FoxD3 and sym1 expression constructs

For expression of Sym1, pCS2-myc-FoxD3^{sym1} was generated by site-directed mutagenesis using pCS2-myc-FoxD3 as template and the following mutagenic primers: Forward 5'-CGACCCCCAGTCGGAAGATATTTCGACAACGGTAGCTT TCTG-3' and reverse 5'-CAGAAAGCTACCGTTGTCGAAATATCTTCCGACTGGGGG TCG-3'. For microinjection in vitro transcribed mRNA was generated from linearized plasmid templates using the Ambion SP6 mMessage mMachine system (Austin, TX). 25pg of FoxD3 mRNA and 25-125pg of sym1 mRNA were injected into one cell stage embryos.

3.3.3 Genotyping

Heterozygous *sym1* adults were crossed and individual progeny were harvested for genotyping at 5dpf. For each phenotypic class (wild-type, no jaw, short axis with no jaw) 7-14 individual embryos were analyzed. Genomic DNA was isolated as previously described (Westerfield, 1993) with the modification of incubating the embryos at 50°C overnight after the addition of extraction buffer. Primers flanking the position of the *sym1* point deletion were used to PCR amplify this region of FoxD3 from genomic DNA (forward 5'-GCGAATTCCTTCGTC AAGATCCCACG-3'; reverse 5'-CATATGGAATTCACCCGGCGAATTCAG-3') and products were subcloned into the pCR4-TOPO vector (Invitrogen). For each individual embryo 6 to 17 subclones were sequenced, and individual fish were assigned to genotypic categories based on the sequence of 6 or more subclones with matching top and bottom strands. For the phenotypically wild-type class, 14 individual embryos were analyzed and of these 7 were wild-type and 7 were *sym1* heterozygotes. For the mutant classes, 7 "no jaw" embryos

and 8 “short axis with no jaw” embryos were analyzed, and in every case were confirmed as *sym1* homozygotes. The genotype of wild-type and *sym1* heterozygous parents was also confirmed using this strategy.

3.3.4 Morpholino oligonucleotides

Morpholino antisense oligonucleotides were obtained from Gene Tools (Philomath, OR). Lyophilized oligonucleotides were resuspended in water, then diluted into 1X Danieau buffer (Nasevicius and Ekker, 2000) and 1nl was injected into one-cell stage embryos. Two morpholino antisense oligonucleotides were designed to *Danio rerio* FoxD3 (BC095603): FoxD3MO1 (5'-TGCTGCTGGAGCAACCCAAGGTAAG-3') is complementary to nucleotides 160-184 of the 5’ UTR and FoxD3MO2 (5’-TGGTGCCTCCAGACAGGGTCATCAC-3’) is complementary to nucleotides 194-218 and overlaps the start codon. A mixture of the two oligonucleotides (total dosage 20ng per embryo) was used for knockdown experiments in wild-type embryos. Injection of the individual oligonucleotides at higher dosage (30-40ng) yielded similar results but with some associated toxicity. As specificity controls, a mismatch oligonucleotide was injected at equal dosage, and *Xenopus* FoxD3 (Steiner et al., 2006) was injected to rescue. For FoxD3 knockdown in *sym1* embryos a mixture of FoxD2MO1 and FoxD3MO2 was injected at a total dosage of 2-4ng.

3.3.5 In situ hybridization

Whole-mount in situ hybridization was performed as previously described (Schulte-Merker et al., 1992), using the following digoxigenin-labeled antisense RNA

probes: *bmp7* (Schmid et al., 2000), *chordin* (Miller-Bertoglio et al., 1997), *cyclops* (Rebagliati et al., 1998), *goosecoid* (Stachel et al., 1993), *no tail* (Schulte-Merker et al., 1994), and *sonic hedgehog* (Krauss et al., 1993). All images were taken from an MZFLIII12.5 stereomicroscope (Leica) with a Retiga 1300 camera (Q-imaging) and processed using Adobe Photoshop.

3.3.6 Phenotypic analysis

Embryos were analyzed based on their phenotypic appearance between 3dpf and 5dpf, and scored for presence of lower jaw structures as well as axial length and curvature of the body and tail. Complete clutches from $Sym1^{+/-}$ parents were separated into three groups: (1) wt fish, (2) fish with no jaw, (3) fish with no jaw and axial defects (short and curved).

3.4 Results

3.4.1 Reexamination of *sym1* embryos reveals defects in axial development

sym1 is a mutation predicted to inactivate FoxD3 due to a point deletion, causing a frameshift that results in a premature stop codon (Stewart et al., 2006). The *sym1* protein is predicted to be truncated in the W2 domain, which is required for DNA binding and confers DNA recognition specificity necessary for transcriptional activity. Mutations in this region of the winged helix domain have been shown to impair DNA binding and transactivation (Berry et al., 2005). In addition, the truncation removes a distal effector domain (GEH) that recruits Groucho corepressors. Given the strong evidence from our zebrafish and *Xenopus* knockdown studies that FoxD3 is an essential regulator of

mesodermal development, the reported absence of mesodermal phenotypes in *sym1* embryos is difficult to accommodate.

In an attempt to resolve this conundrum, we reexamined the phenotypic consequences of the *sym1* mutation. Mating pairs of heterozygous *sym1* adults were obtained and cross progeny were examined and assigned to phenotypic classes (Fig. 3.1). While most embryos fit the predicted phenotypic classes – 75% wild-type (Fig. 3.1A.B) and 16% craniofacial defects (Fig. 3.1C) – an unexpected smaller phenotypic class (9%) was apparent and these embryos displayed both craniofacial defects and axial defects, including curved or short axes (Fig. 3.1D,E). This novel phenotypic class has not been previously reported for the *sym1* mutant, but the nature of the axial abnormalities is consistent with the mesodermal function of FoxD3. To determine the correlation of each of these phenotypic classes with FoxD3 genotype, multiple individual embryos from each class were subjected to genotyping analysis (see Material and Methods). As predicted, the phenotypically wild-type class consisted of wild-type and *sym1* heterozygous embryos, while the craniofacial defect class and the craniofacial and axial defect class consisted only of *sym1* homozygotes (data not shown). Importantly, these results show that a genetic loss-of-function in FoxD3 does lead to phenotypic defects consistent with an essential mesodermal function for FoxD3.

3.4.2 Analysis of early gene expression in Sym1

To determine the underlying developmental origins of the axial defects present in *sym1* homozygotes, mesodermal gene expression was examined in cross progeny at the shield stage (Fig. 3.2). While ~80% of cross progeny had normal mesodermal gene

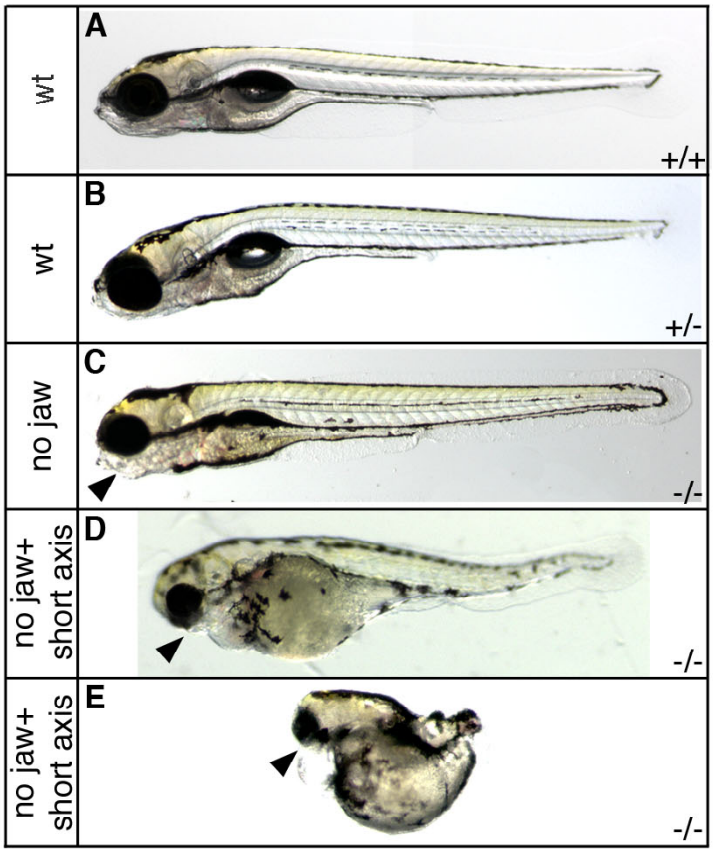


Figure 3.1 Sym1 phenotypes. Phenotypes observed from Sym1 +/- cross. We observed a phenotypic distribution of 77% WT and 23% with no jaw, of which 9% have axial defects (see text). Representative samples where genotyped from each group. (A,B) phenotypically wt larvae, 14 where genotyped, 7 were homozygote wt, 7 where heterozygotes (C) larvae that present no jaw, 7 samples where genotyped and shown to be homozygote mutant. (D, E) Representative samples of larvae that present no jaw as well as a short axis, 8 samples where genotyped and shown to be homozygous mutant.

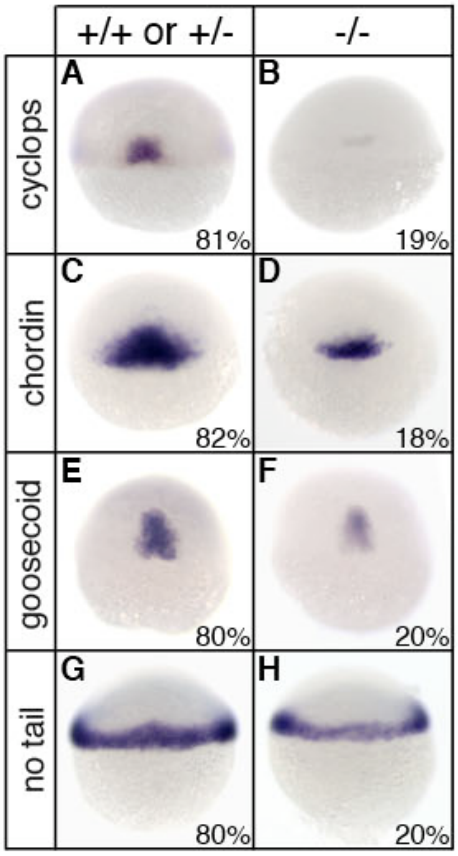


Figure 3.2 Reduced dorsal markers in Sym1. ISH of Sym1 showing wt and reduced dorsal shield marker expression in a Sym1 clutch. ISH was done with a complete clutch per marker. (B,D,F,H) 20% of a single clutch showed reduced shield markers at shield stage for each cyc, chd, gsc and ntl. Representative samples were genotyped and found to be homozygote mutant for sym1. (A,C,E,G) ISH for cyc, chd, gsc and ntl, 80% of each clutch had wt marker expression as shown.

expression, ~20% of the embryos displayed a substantial reduction in of *cyclops*, *chordin*, *goosecoid*, and *no tail* expression (Fig. 3.2B,D,F,H). Following in situ hybridization, embryos from each class were genotyped, and while embryos with normal expression were either wild-type or *sym1* heterozygotes, every embryo with reduced mesodermal gene expression was homozygous for *sym1* (data not shown). Importantly, these results confirm the requirement for FoxD3 for dorsal mesodermal development at the gastrula stage. It is interesting to note that while most of the predicted 25% homozygous embryos show mesodermal deficits at the shield stage, the phenotypic severity appears to diminish during development so that by 24hpf only 9% of embryos display axial defects. This may reflect a process of compensation or regulation, during gastrulation or later, that moderates the consequence of FoxD3 loss-of-function in many, but not all homozygous embryos.

3.4.3 Sym1 protein retains mesoderm inducing activity

Despite the strong prediction that *sym1* is a functional null allele of FoxD3, the reduced penetrance of the mesodermal defects at both the gastrula and 24hpf stages raises the possibility that the *sym1* product may retain some level of activity. To assess the developmental activity of the *sym1* product, the point deletion was introduced into the wild-type FoxD3 cDNA, and *sym1* mRNA was injected into wild-type embryos at the one-cell stage. Injected embryos were examined for mesodermal gene expression at the shield stage and axis formation at 24hpf (Fig. 3.3). Surprisingly, *sym1* injection resulted in expanded expression of *cyclops*, *goosecoid*, *chordin*, and *no tail* in most embryos (84%, n=946) (Fig. 3.3B,E,H,K). Similarly, *sym1* induced strong dorsalization of the

body axis at 24hpf (76%, n=330) (Fig. 3.3N). In fact, when *sym1* mRNA was injected at doses ~5-fold higher than wild-type FoxD3, the embryonic response was indistinguishable (Fig. 3.3C,F,I,L,O). This retention of activity indicates that *sym1* is a hypomorphic allele, not a null, despite the strong prediction otherwise.

3.4.4 Functional FoxD3 protein is produced in *sym1* mutant embryos

The demonstration that *sym1* retains activity, at least in a gain-of-function assay, raises the possibility that a low level of FoxD3 function persists in *sym1* homozygotes. To assess this possibility, we attempted to further knockdown FoxD3 function in *sym1* cross progeny. If *sym1* is indeed a hypomorphic allele, it is predicted that knockdown of the *sym1* product would result in increased penetrance of the axial phenotype. The mixture of two FoxD3-specific oligonucleotides was injected at low dosage (2-4ng) into the one-cell stage progeny of *sym1* het crosses. At this low dosage no phenotypic response was observed in wild-type embryos (Fig. 3.4). In contrast, injection of this low dose into *sym1* progeny resulted in a dramatic increase in both the craniofacial only and craniofacial and axial phenotypic classes. In these experiments, the phenotypic distribution of uninjected *sym1* progeny was 76% wild-type, 19% craniofacial only, and 5% craniofacial and axial (n=167) (Fig. 3.4). FoxD3 knockdown in cross progeny resulted in 24% wild-type, 46% craniofacial only, and 30% craniofacial and axial (n=107) (Fig. 3.4). This distribution, with the two affected classes accounting for 76% of the embryos, likely represents a phenotypic response to FoxD3 knockdown in both homozygous and heterozygous *sym1* embryos. Nevertheless, the results indicate that functional FoxD3 protein is retained in *sym1* homozygotes, and that knockdown of the

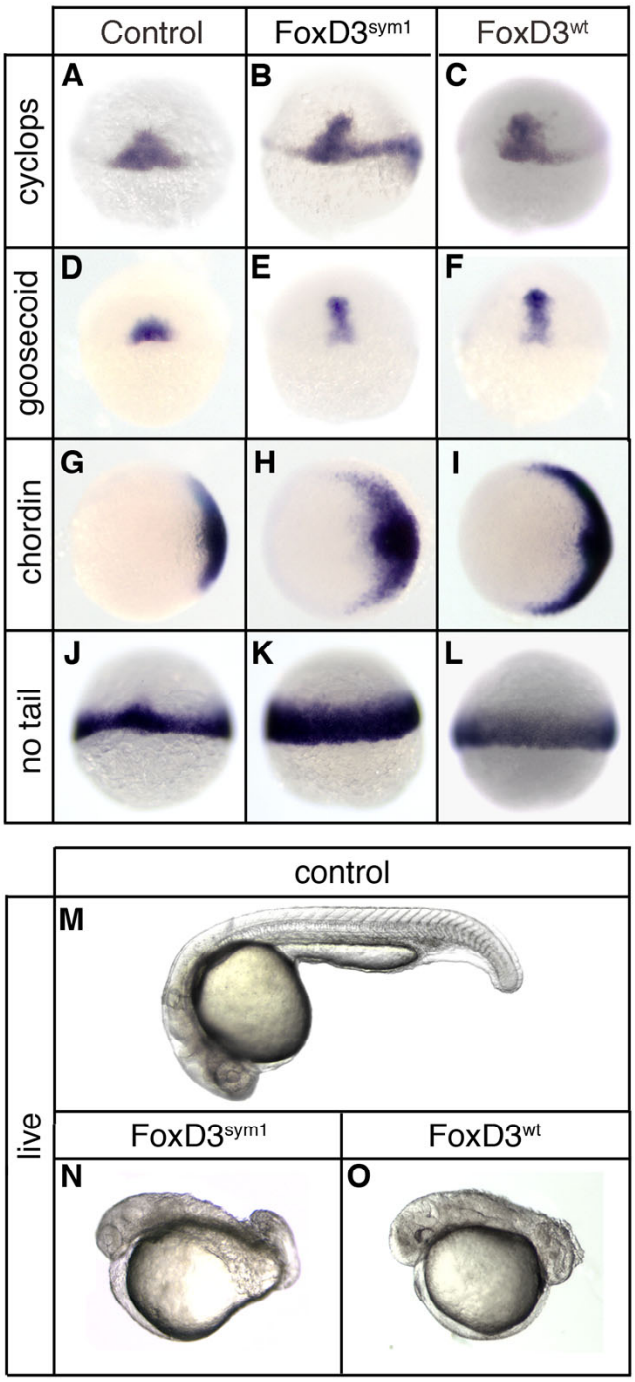
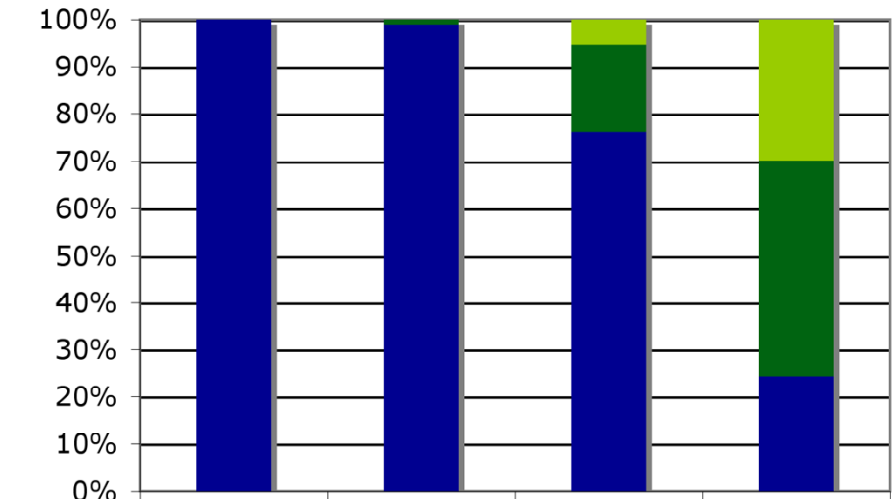


Figure 3.3 Sym1 gain of function. Sym1 mutant retains wt activity (A, D, G,J) ISH for dorsal markers at shield stage in wt control embryos (A) cyc, (D) gsc, (G) chd (animal pole view) expression in the shield (J) ntl expression throughout mesoderm. (B,E,H,K)

ISH for dorsal markers at shield stage after Sym1 overexpression (B) cyc is expanded laterally and towards the animal pole, (E) gsc is expanded towards the animal pole, (H) chd is expanded laterally and towards the animal pole, (K) ntl is expanded towards the animal pole. These results recapitulate what is seen for FoxD3 overexpression, confirming that Sym1 retains dorsalizing activity. (C, F, I, L) ISH showing dorsal markers at shield stage after FoxD3 overexpression (C) cyc is expanded, (F) gsc is expanded towards the animal pole, (I) chd is expanded laterally and towards the animal pole (animal pole view), (L) ntl is expanded towards the animal pole. All dorsal shield view except where noted. (M,N,O) 24hpf live images showing phenotypes (M) wt control, (N) after Sym1 overexpression, (O) after FoxD3 overexpression. Note similar dorsalization in (N) and (O).



	wt n=36	wt + MO n=95	sym1 n=167	sym1+MO n=107
short	0	0	5	30
no jaw	0	1	19	46
wt	100	99	76	24

Figure 3.4 FoxD3MO results in more penetrant Sym1 phenotypes**.** Quantification of sym1 phenotypes with and without FoxD3 MO1+2 treatment. 2.5ng of MO1+2 was used to determine if FoxD3 activity could be further reduced in the no jaw phenotype group. FoxD3 MO1+2 injections result in higher numbers of embryos with jaw and short axis phenotypes when compared to uninjected sym1 phenotypes. The 2.5ng concentration of MO used in sym1 embryos had no activity in WT embryos, implying that the +/+ Sym1 progeny was not affected and does not contribute to the no jaw phenotype group.

remaining FoxD3 function results in increased penetrance of axial phenotypes, confirming that FoxD3 function is essential for zebrafish mesodermal development.

3.5 Discussion

3.5.1 Analysis of a FoxD3 mutant

sym1 is a FoxD3 mutant, in which a point deletion results in an early stop codon that truncates the protein, removing part of the DNA-binding domain and an essential distal transcriptional effector domain. *sym1* has been previously examined for neural crest defects, however no early gastrulation defects had been reported. To assess the role of FoxD3 in zebrafish mesodermal development, we reexamined *sym1* to determine if any early mesodermal and axial defect were present as would be expected from our earlier results. Several FoxD3 studies have been published on the role of FoxD3 in neural crest formation in zebrafish, and these studies also used MO knockdown, however, no early gastrulation defects were reported in these publications. We believe that the early phenotypes may have been overlooked and not taken into consideration as those studies were not focused on early development, but on neural crest development, which occurs some 10 hours later. Also, the levels of FoxD3 activity necessary to mediate FoxD3 function in the neural crest lineage may differ from those in the gastrula. We examined the *sym1* mutant more in depth and found that it does indeed present early mesodermal deficiencies as well as defects in dorsal mesoderm derivatives, which are obvious at later stages. These results are consistent with our pervious results showing that FoxD3 acts through the Nodal pathway to induce dorsal mesoderm (chapter 2 of this thesis).

The second FoxD3 mutant, *mother superior*, has defects only in neural crest development. In contrast to *sym1*, which alters the structure of FoxD3 protein, *mother superior* is a regulatory mutation in a distal transcriptional regulatory element that drives expression in the neural crest. Gastrula expression of FoxD3 in the shield is not perturbed in *mother superior*, and therefore mesodermal defects are not expected in this FoxD3 regulatory mutant.

3.5.2 *sym1* as a hypomorphic allele of FoxD3

From a structural and mechanistic perspective, the *sym1* mutant should not produce functional FoxD3 protein. Instead, it should be a functional null given that it does not contain part of its W2 domain, which has been shown in fox genes to be necessary for DNA binding and transactivation (Berry et al., 2005), as well as the distal Groucho binding motif (Yaklichkin et al., 2007). We have shown that *sym1* retains dorsalizing activity, similar to that of the wild-type FoxD3 protein, as *sym1* can induce and expand dorsal markers at the shield stage. This suggests that *sym1* is a hypomorphic allele, and not a true null. This is further supported by the ability of the FoxD3 morpholino to enhance the penetrance of the short axis phenotypic group from 5% to 30%, indicating that functional FoxD3 protein is present in the *sym1* mutant.

Overall, we propose that the *sym1* mutation does have dorsal mesodermal defects and subsequent defects in axis formation. Importantly, we show that a mutant that was thought to be a null is acting as a hypomorph. However, the retention of FoxD3 activity by the *sym1* mutant is confounding. In Chapter 4, we address one potential hypothesis for

this unexpected retention of activity. We propose that a -1 translational frameshift results in a correction of reading frame and production of a full length FoxD3 protein.

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4. Chapter 4: Translational frameshifting suppresses a potential FoxD3 null mutation in Zebrafish

4.1 Summary

The FoxD3 mutant, *sym1*, has been reported to encode a truncated protein predicted to be a null allele due to a point deletion that results in seven codon frameshift and a premature stop codon. In contrast to this prediction, we have found that *sym1* retains activity, and induces mesoderm and axial dorsalization, as does wild-type FoxD3. To determine if the predicted *sym1* protein product had activity, a FoxD3 cDNA truncated to encode the same product predicted from the *sym1* cDNA was expressed in zebrafish embryos. This product, lacking part of the DNA-binding domain and GEH effector domain, had no developmental activity, in contrast to the intact *sym1* cDNA. This difference in the activity of the intact and truncated cDNAs suggests that 3' sequences, present in the *sym1* cDNA, but not in the truncated FoxD3 cDNA, are required for the activity of the *sym1* product. An inactivating point mutation introduced into the GEH-encoding region of the *sym1* cDNA, downstream of the predicted premature stop, rendered the *sym1* cDNA inactive when overexpressed. This suggests that a corrective frameshift occurs during translation of the *sym1* cDNA to generate a full length FoxD3 protein with an active GEH domain. Within the seven codon frameshift region downstream of the *sym1* point mutation are three codons rarely used in the zebrafish, and rare codons are known to cause ribosomal pausing and promote frameshifting. Conversion of those three residues to highly used codons encoding the same residues rendered the *sym1* cDNA inactive. Biochemical analyses confirm that a full length FoxD3 protein is produced from the *sym1* cDNA, but not when the rare

codons have been replaced. These results indicate that the null character of the *sym1* mutation is suppressed by a novel translational frameshifting mechanism.

4.2 Introduction

Translation is the mechanism by which RNA is decoded into a sequence of amino acids. This mechanism requires the ribosomal machinery that will scan the RNA for specific sequences as AUG start sites and required translation initiation factors. Eukaryotes require the Kozak sequence ~6 base pairs before the AUG start codon, while prokaryotes require the Shine-Dalgarno ribosomal binding site (Kozak, 1986; Shine and Dalgarno, 1975). During the translation process, transfer RNAs (tRNAs) move through three binding sites in the ribosome where they encounter specific mRNA codons. These binding sites are the aminoacyl or A acceptor site which binds the tRNA with the amino acid to be added to the growing protein, the peptidyl or P site which carries the tRNA with the growing peptide chain, and the exit or E site which releases the deacylated tRNA. All tRNAs except the initiator tRNA-met have to enter the ribosome at the A acceptor site. The amino acid chain grows when the charged tRNA and its bound amino acid enter the A site, a peptide bond is formed between the A and P sites, and the ribosome translocates to the next codon and the deacylated tRNA leaves the ribosome at the E site.

Proteins are translated from template mRNA by the ribosomal machinery, in which tRNAs recognize the mRNA codons through their anticodon sequence, and add the corresponding amino acid to the growing peptide chain. The 20 amino acids that are the building blocks of proteins are encoded by up to six distinct codon sequences, and for

each codon multiple tRNAs, with differing abundance, contain a matching anticodon sequence. For each individual amino acid, the corresponding codons differ in their frequency of use and protein coding sequence, and this codon usage bias has species-specificity. The pattern of codon usage reflects a complex balance between biases generated by mutation, selection and random genetic drift (Bulmer, 1991; Sharp et al., 1993). The data available in eukaryotes shows that there is a link between codon usage frequency and tRNA abundance, and that strong codon usage bias increases with increasing levels of gene expression (Duret and Mouchiroud, 1999).

There are several instances where the rules for decoding mRNA are altered. While rare, this occurs at specific sites, where signals or structures are present in the mRNA sequence. The specific pausing of ribosomes at such sites is thought to play an essential role in promoting recoding (Gesteland and Atkins, 1996; Kontos et al., 2001). During ribosomal pausing, frameshifts most often occur when one or more ribosome-bound tRNAs slip between cognate or near-cognate codons, but some frameshifts may occur without slippage. Ribosomal pausing can be induced in several ways, including mRNA secondary structures, termination codons, and limiting availability of amino-acyl-tRNAs for low frequency codons, all of which increase the time that ribosomes are held at a recoding site (Farabaugh, 2000). Recoding and frameshift mechanisms are diverse. Most common are -1 frameshifts, +1 frameshifts are rare, and translational hop sites that program the ribosome to bypass a region of several dozen nucleotides are by far the rarest (Gesteland and Atkins, 1996).

It is thought that a minority of genes across species rely on recoding for proper translation. A well studied -1 programmed ribosomal frameshift occurs in the human

immunodeficiency virus type 1 (HIV-1) where frameshifting is used to produce the Gag-Pol polyprotein. Conventional translation of this RNA by the majority of ribosomes produces the Gag polyprotein, the precursor of the structural proteins of the virus, while the -1 frameshift changes the reading frame and extends the translation past the stop codon of the gag gene, producing a product that terminates at a stop codon in the pol gene reading frame (Park and Morrow, 1991). Programmed -1 ribosomal frameshift has also been reported in several other retroviruses, coronaviruses, plant viruses, in a yeast virus, in bacteria and, recently, in humans (Brierley, 1995; Chandler and Fayet, 1993; Shigemoto et al., 2001; Tzeng et al., 1992).

sym1 is a FoxD3 mutant, which, as we show in Chapter 3, retains activity despite a point deletion that results in an early stop codon midway through the protein. FoxD3, a member of the Forkhead (Fox) family, contains a characteristic winged helix DNA-binding domain (Sutton et al., 1996). For Fox family proteins, deletion of the sequence encoding wing 2 (W2) results in a loss of DNA-binding activity (Clark et al., 1993), and this region is downstream of the predicted premature stop in *sym1*. In addition to deletion of W2 domain, *sym1* lacks a distal transcriptional effector domain, a Goosecoid-Engrailed homology motif required in FoxD3 for Groucho corepressor recruitment and mesoderm induction activity (Stewart et al., 2006; Yaklichkin et al., 2007). Both domains, the W2 and the GEH, are necessary for the developmental and transcriptional function of FoxD3, however, in the *sym1* mutant, these domains are predicted to be absent from the protein, yet the *sym1* product retains activity (see Chapter 3).

How is it then possible for *sym1* to retain activity? The protein product of *sym1* is predicted to lack functional domains localized downstream of the early stop codon that

are essential for developmental function of FoxD3 activity. Yet we have found that *sym1* retains dorsalizing activity, albeit at reduced levels. In an attempt to reconcile these results we propose that translational frameshifting occurs immediately downstream of the point deletion to correct the reading frame and allow production for full length functional FoxD3 protein. In this chapter we provide support for this hypothesis, and the results suggest that rare codons present in the *sym1* mRNA cause ribosomal pausing and facilitate a -1 frameshift, correcting the reading frame and suppressing the *sym1* mutation.

4.3 Material and methods

4.3.1 FoxD3 and sym1 expression constructs

The pCS2-myc-FoxD3 construct (a gift of David Raible; Lister et al., 2006) was used for expression of FoxD3 protein. FoxD3 and *sym1* point deletion mutants (F>E, ΔC, ΔN) were generated by using DPNI-mediated PCR mutagenesis. PCR primers: FWD FoxD3/Sym1 F>E 5' - CCGTCCAGTCGACCATCAGAAAGCATAGAAAACATCATC - 3' , REV FoxD3/Sym1 5' - GATGATGTTTTCTATGCTTTCTGATGGTCGACTGGA CGG - 3' . FWD FoxD3/Sym1 Δ, 5'-TCCTCGAGATGACCCTGTCTGGAGGC-3', REV FoxD3ΔC 5'-CCCTCGAGTCAGGTCAGAAAGCTACCGTTG-3', REV Sym1ΔC 5'-ACCCTCGAGTCAGAAAGCTACCGTTGTCGAA-3', FWD FoxD3/Sym1ΔN 5'-ATGATGCAGAGTTTTGGGGCATAC-3', REV FoxD3/Sym1ΔN 5'-GGATCCTGCAAAAAGAACAAGTAGCTT-3'. (Fig. 4.1)

For mRNA injection, plasmids were linearized with NotI, and transcribed with SP6 polymerase (mMessage mMachine, Ambion). Microinjections were done at the one cell stage.

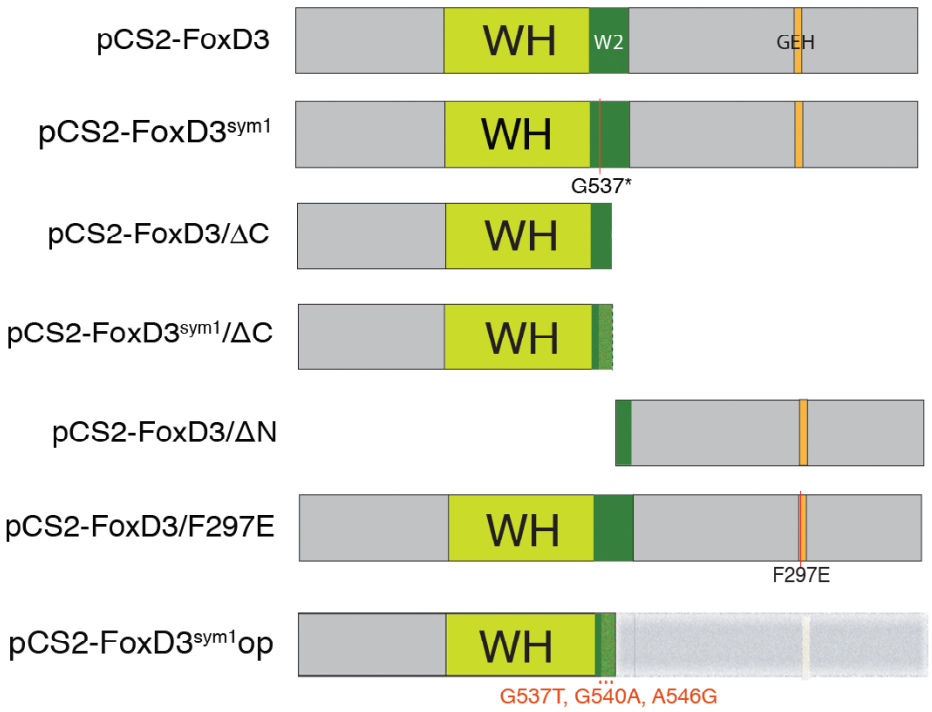


Figure 4.1 FoxD3 and Sym1 constructs. The pCS2-myc-FoxD3 construct was used for expression of FoxD3 protein. FoxD3 and *sym1* point deletion, point mutation and truncated constructs were generated from the pCS2-myc-FoxD3 using DPNI-mediated PCR mutagenesis.

4.3.2 Codon optimization

For the sym1op construct we optimized three low frequency codons found between the point deletion and the premature stop codon. Third base changes were introduced while maintaining the amino acid they encoded. pCS2-myc-Sym1op was obtained by PCR site-directed mutagenesis of pCS2-myc-Sym1 using the following primers FWD 5' - CCAGTCGGAAGATATTTCTACAACAGTGGCTTTCTGAGG - 3' and REV 5'- TTCTCCTCTCAGAAAGCCACTGTTGTAGAAATATCTTC - 3'. For mRNA injection, the plasmids were linearized with SacII or NotI, and transcribed with SP6 polymerase (mMessage mMachine, Ambion). Microinjections were done at the one cell stage with 25-50pg.

4.3.3 Western analysis

Injected embryos and control embryos (50-100 embryos per sample) were harvested at shield stage and manually deyolked by puncturing the yolk cell with forceps. Embryos were collected and lysed in 200ul ice-cold nuclear lysis buffer (50mM Tris pH7.5, 0.5M NaCl, 1% NP-40. 1% deoxycholate, 0.1% SDS, 2mM EDTA, protease inhibitors) by repeated pipetting in a microtube.

For western analysis, 25-100 embryos were lysed, the extracts were cleared by centrifugation and 25ul were loaded per well. An anti-myc (rabbit polyclonal IgG) antibody (Upstate, Lake Placid, NY) was used at 1:5000 dilution and was detected with a 1:5000 dilution of anti-rabbit IgG, horseradish peroxidase linked antibody (GE healthcare, UK) by chemiluminescence with ECL+Plus reagent (GE healthcare, UK).

4.3.4 Phenotypic analysis

Embryos were injected at the one-cell stage with 25-50pg of RNA encoding FoxD3ΔC, Sym1ΔC, FoxD3ΔN, FoxD3F>E, or Sym1F>E and axial phenotypes were analyzed at 1-3 dpf. Embryos were assigned to three phenotypic groups, strongly dorsalized, weakly dorsalized and wild-type.

4.4 Results

Given that *sym1* is predicted to be a null mutation yet retains dorsalizing activity, we were interested in further exploring the mechanism by which *sym1* retains activity. The *sym1* protein is predicted to be approximately half the size (182 aa) of the wild-type protein (371aa), and would lack part of the W2 binding domain, as well as the Groucho repressor interaction motif, both domains necessary for FoxD3 activity. It is important to note that despite the point deletion, the *sym1* mRNA is full length and retains the coding capacity for the C-terminal half of FoxD3 in the -1 reading frame.

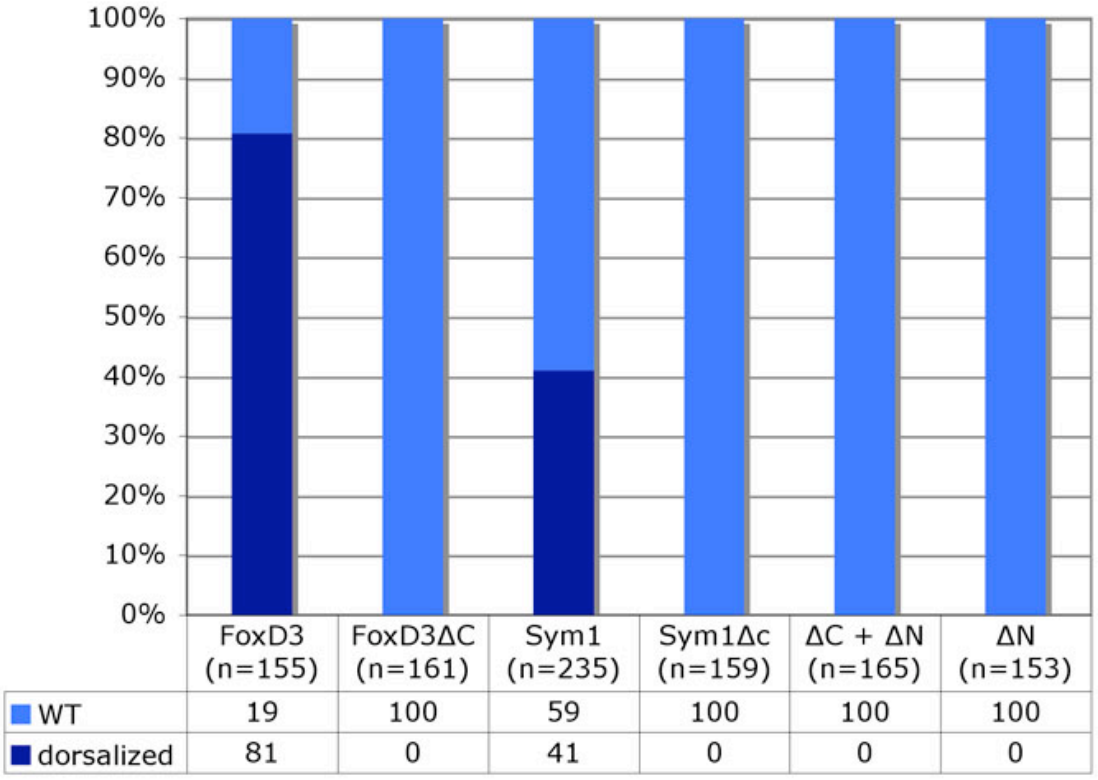
4.4.1 The predicted truncated Sym1 product has no activity

To determine if the predicted *sym1* protein has activity, constructs were generated from the *sym1* and wild-type FoxD3 sequences that lack all coding regions downstream of the premature stop, Sym1ΔC and FoxD3ΔC. Embryos injected with Sym1ΔC or FoxD3ΔC mRNA were not dorsalized (0%, n=159 and 0%, n=161) and were indistinguishable from uninjected wild-type embryos (Figure 4.2). In the same experiments *sym1* (41%, n=235) and *foxd3* (81%, n=155) strongly dorsalized embryos. We also assessed the activity of the C-terminal half of FoxD3 (FoxD3ΔN), which

contains all the sequences downstream of the early stop codon and found that it was inactive (0%, n=153). Finally, we coexpressed the N- and C-terminal halves of FoxD3, to determine if these fragments of FoxD3 could interact in trans to reconstitute FoxD3 activity, and no activity was observed in embryos (0%, n=153). For these experiments, the accumulation of the predicted protein products was verified by western blotting of embryonic extracts (data not shown). The results are consistent with previous structure-function analyses of FoxD3 in *Xenopus* (Yaklichkin et al., 2007), and confirm that the predicted *sym1* product has no biological activity in the zebrafish embryo. This points to a requirement for downstream coding sequence, 3' to the predicted premature stop, for the activity of *sym1*.

4.4.2 The distal GEH domain is required for *sym1* activity

A -1 translational frameshift presents a possible mechanism to return downstream sequences, including the essential GEH motif, back into frame. If the -1 frameshift occurs soon after the G537 point deletion, this frameshift would result in frame correction and translation of a full length protein with minimal amino acid sequence changes. All FoxD3 orthologs contain a conserved GEH sequence (271-FSIENII-303 in zebrafish FoxD3) that is essential for protein function. A single point mutation in the absolutely conserved first residue (F297E) renders the protein inactive (Yaklichkin et al., 2007). Given that sequence 3' to the predicted premature stop is essential for *sym1* activity, we examined the requirement for the distal GEH effector domain in *sym1* activity. A single point mutation was introduced into the GEH encoding region of *sym1* and wild-type FoxD3, substituting phenylalanine for glutamate (F297E). It should be noted that this site is more



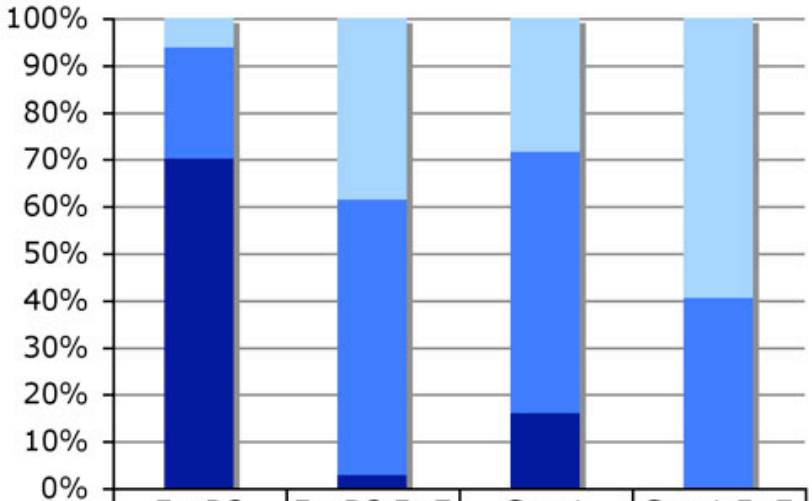
	FoxD3 (n=155)	FoxD3ΔC (n=161)	Sym1 (n=235)	Sym1Δc (n=159)	ΔC + ΔN (n=165)	ΔN (n=153)
WT	19	100	59	100	100	100
dorsalized	81	0	41	0	0	0

Figure 4.2 The expected Sym1 truncated protein has no activity. Deletion of the C terminal domain of Sym1 results in complete loss of activity compared to Sym1, demonstrating that the predicted truncated Sym1 peptide has no activity. Coexpression of ΔC and ΔN has no activity, similar to ΔN and FoxD3ΔC.

than 300 nucleotides 3' to the predicted premature stop of *sym1*. One-cell stage embryos were injected with FoxD3F>E or Sym1F>E, and axial development was examined at 24hpf (Figure 4.3). In both cases a reduction of dorsalizing activity was observed. FoxD3 strongly dorsalized 70% (n=240), while FoxD3F>E strongly dorsalized only 3% (n=300). Similarly, Sym1 strongly dorsalized 16% (n=252) and Sym1F>E did not have strong dorsalizing activity (0%, n=280). Western blotting of embryo extracts confirmed the accumulation of both protein products (data not shown). Therefore, the distal sequence encoding the GEH motif is required for the strong dorsalizing activity of FoxD3 and *sym1*. More important, these results demonstrate that distal sequences, far past the predicted site of the premature stop, encode an essential functional domain of the *sym1* product. This provides strong support for a role for translational frameshifting in the retention of activity in the *sym1* mutant.

4.4.3 Optimization of rare codons suppresses *sym1* activity

Several mechanisms can induce translational frameshifting, including the presence of a rarely used codon (a codon represented at low frequency in the protein coding sequence of a specific organism). Rare codons cause the ribosome to pause and wobble while waiting to engage the cognate tRNA, which are themselves often present at lower abundance. During this waiting period, wobble or shifting of the ribosomal complex can result in engagement of an alternative reading frame containing a more commonly used codon.



	FoxD3 n=240	FoxD3 F>E n=300	Sym1 n=252	Sym1 F>E n=280
WT not dorsalized	6	39	29	60
C1-2 weakly dorsalized	24	59	56	40
C3-C4 strongly dorsalized	70	3	16	0

Figure 4.3 The C-terminal end of Sym1 is necessary for dorsalizing activity. Sym1F>E shows greatly reduced dorsalizing activity compared to Sym1, evidence that Sym1 translates downstream sequences (GEH) previously though to be truncated. As a control, FoxD3 shows high dorsalizing activity, while FoxD3F>E has reduced activity, as expected from the F>E point mutation in the GEH domain. These results support the hypothesis of a translational frameshift to generate a functional full length protein.

In the seven residue frameshift region between the *sym1* point deletion and the premature stop, three rarely used codons in the zebrafish are found, a Serine codon (TCG) representing only 6.8% of serine codons, a Threonine codon (ACG) at 13.4% and a Valine codon (GTA) at 10.5% (Figure 4.4). Given such low frequency of usage, these are potential sites of ribosomal pausing in *sym1*. No codons of such low frequency are found within the sequence of wild-type FoxD3. To determine if these low frequency codons promote frameshifting, we substituted the third position of each codon, converting each into a high frequency codon (20.5-44.3%) encoding the same amino acid (Figure 4.4). We then tested the function of this translationally optimized form of *sym1* (Sym1op). We predict that this optimization will prevent ribosomal pausing, suppress frameshifting, and result in the production of the predicted truncated *sym1* product, which we have shown as non-functional. Zebrafish embryos were injected at the one-cell stage with equal doses of FoxD3, Sym1 and Sym1op and axis formation was evaluated at 24hpf (Figure 4.5). While the strongly dorsalized class represented 82% (n=139) for FoxD3 and 51% for Sym1 (n=156), Sym1op-injected embryos were only weakly dorsalized, 5% (n=146).

These results indicate that low frequency codons present in the *sym1* mRNA are required for developmental activity, strongly supporting a role for ribosomal pausing and translational frameshifting.

FoxD3

DNA	GAT	ATG	TTC	GAC	AAC	GGT	AGC	TTT	CTG
amino acid	D	M	F	D	N	G	S	F	L
% prob	47.15	100	53.33	52.85	59.65	21.96	22.30	46.67	40.52

FoxD3^{SYM1}

DNA	GAT	ATT	TCG	ACA	ACG	GTA	GCT	TTC	TGA
amino acid	D	I	S	T	T	V	A	F	stop
% prob	47.15	34.45	**6.79**	30.85	**13.43**	**10.49**	31.86	53.33	45.16

FoxD3$^{SYM1\ OPTIMIZED}$

DNA	GAT	ATT	TCT	ACA	ACA	GTG	GCT	TTC	TGA
amino acid	D	I	S	T	T	V	A	F	stop
% prob	47.15	34.45	**20.48**	30.85	**30.85**	**44.29**	31.86	53.33	45.16

No pause → efficient translation to early stop

Figure 4.4 Codon frequency in FoxD3, FoxD3^{sym1}, FoxD3^{sym1op}. Three low frequency codons in FoxD3^{sym1}were optimized to higher frequency codons without affecting the coding capacity to create the pCS2-FoxD3^{sym1op} construct. These codons are located within the frameshifted sequence, between the Sym1 point deletion and the early stop codon. We would predict that translation should efficiently read through this sequence and come to a stop at the early termination codon generating a truncated protein with no activity.

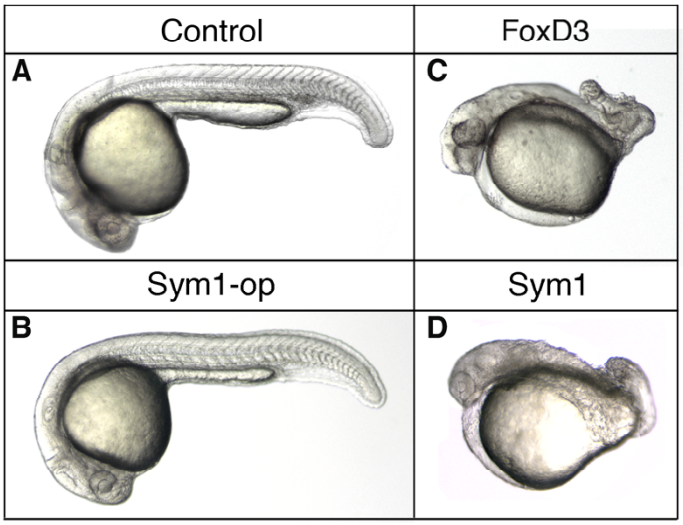


Figure 4.5 Codon optimization in Sym1op results in loss of activity. (A) WT 24hr live embryo. (B) Expression of the Sym1op mRNA weakly dorsalized 5% (n=146) of the embryos. (C) Expression of FoxD3 mRNA dorsalizes the majority of embryos 82% (n=139), while (D) Sym1 mRNA dorsalized 51% (n=156).

4.4.4 Detection of the Sym1 protein product

The translational frameshifting model we propose predicts that a full length FoxD3 protein is produced by the *sym1* mRNA. FoxD3, Sym1 and Sym1op were epitope-tagged, expressed in embryos, and extracts analyzed by western blotting (Figure 4.6). FoxD3 produced the predicted full length protein (~51kD). Two products were detected for Sym1, one with the size of the predicted truncated Sym1 protein and one with the approximate size of full length FoxD3. For Sym1op, only a single product was detected with the size of the predicted truncated protein. These results appear to fulfill expectations based on the translational frameshifting model. In other cases of translational frameshifting, a maximal efficiency of ~10% is observed, so it would be expected that Sym1 would produce a mixture of full length and truncated proteins, with the frameshifted product present at lower abundance. In contrast, Sym1op produced a single product of the size predicted for the truncated product, consistent with a suppression of frameshifting. These results provide biochemical support for the translational frameshifting of *sym1* to produce functional FoxD3 protein. We note, however, that the results shown in Figure 4.6 were only observed in a subset of attempted experiments; so additional biochemical confirmation of this model is required.

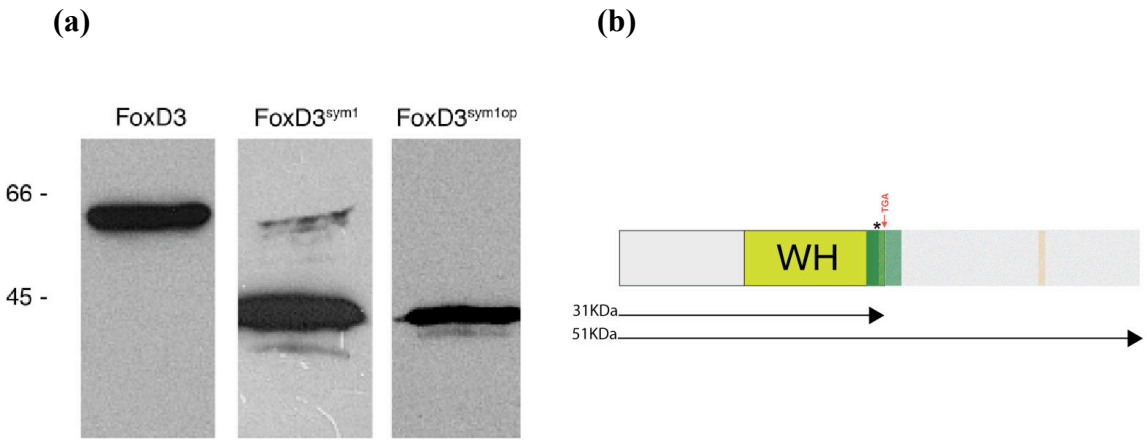


Figure 4.6 Western blots analysis of Sym1-op. **(a)** Western blot showing the FoxD3 protein at 51KDa, the Sym1 protein at its expected 31KD and the full length product at 51KDA and at a much lower concentration, and the Sym1-op protein at the expected 31Kda size with no full length product detectable. **(b)** Cartoon depicting the expected protein sized expected. * site of G537 Sym1 lesion in the W2 domain, TGA early termination codon resulting from the frameshift.

4.5 Discussion

4.5.1 Sym1 structural analysis

The functional analysis of potential Sym1 products demonstrated that the predicted truncated product had no activity. There are several downstream start sites following the point deletion in Sym1, which would produce proteins that could potentially interact with the Sym1 product in trans. We show that there is no activity when co-expressing the N-terminal and C-terminal halves of FoxD3, therefore eliminating the possibility that dorsalizing activity derives from the trans acting protein fragments. In addition, the results obtained after introducing a mutation in the GEH domain further indicates that C-terminal region of FoxD3 is functional in the *sym1* product in zebrafish embryos, and that this protein can actively dorsalize embryos. All together these results further support the idea of a frameshift correction in the *sym1* allele. The argument is strengthened by the observation that the frameshift could be suppressed by substituting the rare codons immediately following the point deletion with frequent codons without altering the amino acid sequence. The contribution of the rare codons to the frameshift can be explained by the low abundance of cognate tRNA, which leads to ribosomal pausing and frameshifting to incorporate a high frequency codon in an alternative reading frame (Gallant et al., 2004). The predicted -1 frameshift then places the ribosomal machinery back into frame with the natural FoxD3 sequence, and therefore results in production of a full length functional protein.

Apart from low frequency codons, the presence of a slippery sequence is a possible mechanism for promoting repositioning of the ribosome. This occurs by the simultaneous slippage of ribosome-bound A- and P-site tRNAs by one base, resulting in

the tRNA binding a non-cognate codon in the -1 position, thus allowing for the next codon to be in frame again (Shigemoto et al., 2001). This slipping of the ribosome has been shown to occur at different specific sequences in prokaryotes and eukaryotes. Interestingly, eukaryotic frameshift signals are not recognized by prokaryotes and the typical prokaryotic slip sequence does not lead to significant frameshifting in eukaryotes (Garcia et al., 1993). However, we have not found any obvious slippery sequences in *sym1*.

4.5.2 Implications of translational frameshifting for protein diversification

Frameshifted products resulting in truncated and out of frame proteins are generally assumed to be loss-of-function changes. However, evolutionarily, frameshift mutations might offer a significant mechanism to create novel functional motifs, which might be essential to the functional diversification of transcription factor families and other gene families. The rate at which frameshifted sequences would adopt new roles and result in novel functional domains is probably very low, but not impossible. Frameshifting has been suggested as the mechanism for floral MADS-box gene evolution (Vandenbussche et al., 2003), but vertebrate examples are yet to be identified. Further insight into sequence conservation and discrepancies between protein families will be important in bringing to light the complexities and consequences of frameshifting in protein diversification over a range of organisms.

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5. Chapter 5: Conclusions and future directions

The results presented in this thesis establish that FoxD3 has an essential role in dorsal mesoderm formation during zebrafish development. We find that FoxD3 function in mesodermal development is dependent on an active Nodal pathway, consistent with its role in maintaining Nodal gene expression in the embryonic shield. This thesis also explores the activity of *sym1*, a zebrafish FoxD3 mutant, demonstrating that *sym1* is a hypomorphic allele that retains activity, correcting a previous conclusion that *sym1* was a functional null allele of FoxD3. Based on the results obtained, we propose that the null character of *sym1* is suppressed by a translational frameshifting mechanism that results in the production of a full-length active protein. This novel mechanism for frame correction of a mutant allele has not been previously reported in vertebrates.

5.1 The role of FoxD3 in the Nodal pathway

Mesoderm induction occurs through activation of the Nodal signaling pathway. Mesoderm is induced in a confined marginal area surrounding the developing embryo, while dorsal mesoderm is induced in only the dorsal side of the embryo. Therefore, signals that instruct dorsal mesodermal development must be restricted to the dorsal domain. FoxD3 expression is limited to the shield and expands laterally towards the paraxial mesoderm when it is first expressed at shield stage. We have shown that FoxD3 knockdown results in strong reduction of Nodal signaling, consistent with the idea that FoxD3 is necessary for Nodal signaling maintenance in the shield as well and that FoxD3 is a component of the autoregulatory loop that maintains Nodal signaling. We propose that FoxD3 regulates dorsal mesoderm development by negatively regulating a repressor

of mesoderm development in the zebrafish, and that it does so through the Nodal signaling pathway. These results are consistent with what has been reported in *Xenopus* for dorsal mesoderm development (Steiner et al., 2006).

Nodal expression is dynamic throughout embryogenesis in a wide array of organisms, implying that multiple developmental processes are regulated by Nodal signaling, and at the same time, meaning that different regulators must be required in a tissue and time dependant manner. FoxD3 is also required at different developmental stages in zebrafish and *Xenopus*. FoxD3 is expressed at shield stage to regulate dorsal mesoderm development, and it is also expressed later in premigratory neural crest cells, where it is necessary for differentiation of neural crest derivatives, including craniofacial cartilage, peripheral neurons, glial, and iridophore pigment cells (Kelsh et al., 2000; Lister et al., 2006; Montero-Balaguer et al., 2006; Stewart et al., 2006). Our lab has carried out a microarray screen in *Xenopus* aimed at identifying transcriptional targets of FoxD3 that negatively regulate Nodal expression. Among the potential FoxD3 targets identified are Blimp1, Xema and Gata4. It will be interesting to further analyze these factors and determine how these and other factors are involved in FoxD3 activity and Nodal regulation. Even though Nodal signaling is essential in all vertebrate species for dorsal mesoderm development, an interesting conundrum arises when the Nodal loss-of-function phenotypes are compared between these species. Nodal loss-of-function phenotypes differ between species in a very broad manner. The basis for these differences has not yet been elucidated, but most certainly it will involve orthologous gene function with conserved as well as differing functions, and perhaps this will include regulatory factors such as FoxD3.

5.2 Differences in the developmental requirements for the Nodal pathway

Two Nodal signaling factors act during dorsal mesoderm development in zebrafish, *cyclops* and *squint*. *Xenopus* presents five Nodal-related genes with mesoderm inducing activity, while mouse has a single *nodal* gene. Single mutants for zebrafish *cyclops* and *squint* give mild phenotypes, as do single maternal or zygotic *oep* mutants (Dougan et al., 2003; Hatta et al., 1991). These single mutants display cyclopia and defects in prechordal plate and ventral nervous system. Further, zebrafish double mutants of *cyclops* and *squint* or maternal-zygotic *oep* (*mzOep*) mutants lack head and trunk mesoderm, but form some tail mesoderm. In contrast, mouse *nodal* mutants lack most embryonic mesoderm and extraembryonic ectoderm, which leads to failure to maintain the primitive streak and death during early gastrulation. Similarly, in *Xenopus* inhibition of Nodal signaling with the antagonist Cerberus-short fully blocks mesodermal development and embryos arrest in gastrulation (Conlon et al., 1994; Feldman et al., 1998; Osada and Wright, 1999; Piccolo et al., 1999; Zhou et al., 1993). These differing Nodal loss-of-function phenotypes between organisms remain to be understood. Importantly, loss-of-function for each individual Nodal gene results in slightly or dramatically differing phenotypes depending on the organism, which points to important differences, either quantitative or qualitative, in the signaling output and/or cellular response to individual Nodal ligands. It will therefore be important to define the precise signaling output of the Nodal pathway in response to specific Nodal ligands, as well as to define the cellular and temporal controls that confer specificity to the developmental roles of the Nodal pathway.

Our findings that FoxD3 regulates Nodal signaling in zebrafish add one small piece to the puzzle. Comparative studies of the molecules involved and discovery of new ones, will aid in resolving many questions. In addition, the early morphological events that take place during the development of these embryos are somewhat distinct. In the early zebrafish embryo, the blastoderm cells form a single homogeneous population on the top half of the yolk, until blastula stages where cell mixing is halted and cells acquire their prospective fates, then the embryonic tissue migrates towards the vegetal pole until it surrounds the yolk cell during epiboly. The *Xenopus* embryo develops as a complete sphere where extensive cell mixing does not occur, this results in a consistent early blastomere fate map. The mouse embryo develops as a cylinder with characteristic extraembryonic tissue, where extraembryonic mesoderm and visceral endoderm will contribute cells to tissues of the developing embryo (Kimmel and Law, 1985; Kimmel et al., 1990; Moody, 1987; Tam and Behringer, 1997). Embryos present these morphological differences in embryonic development even though signaling pathways and transcription factors that regulate early embryo patterning are conserved between species. Further understanding these mechanisms will be of significant interest to understand determinant positioning and embryo patterning.

Finally, because the Nodal pathway is essential in mesodermal development, understanding how its regulated might aid in treating mesoderm defects, which are the cause of many embryonic malformations and abnormalities as well as adult diseases. Furthermore, FoxD3 could be of use in regulating Nodal signaling for mesodermal development from ES cells and for future therapeutics like induction of tissue regeneration.

5.3 When is a null mutation a true null?

In my thesis work I reexamined the developmental defects of the FoxD3 mutant *sym1* zebrafish. This mutation in FoxD3 was strongly predicted to be a functional null allele due to the presence of a point deletion, generating an early termination codon that results in the truncation of the DNA-binding domain and deletion of the C-terminal Groucho corepressor interaction motif. Based on previous structure-function studies of FoxD3, the predicted truncated *sym1* protein products should not have transcriptional or developmental activity. However, I found that *sym1* retains partial function when overexpressed in the zebrafish embryo. Sym1 protein can dorsalize embryos, although higher dosage is required compared to wild-type FoxD3, suggesting that *sym1* is a hypomorphic allele, not a null. These results raise again the importance of functional testing of mutant alleles, as predictions based solely on the nature of the molecular lesion can often mislead.

More important, the hypomorphic nature of *sym1* begs the question, how can this predicted truncated protein retain activity? Given the nature of the *sym1* molecular lesion, the most plausible hypothesis was that a translational frameshift mechanisms corrects the reading frame error caused by the point deletion, bringing the sequence back in frame and allowing a full-length functional protein to be translated. The efficiency of frameshifting, at most 10% in other described cases, could certainly account for the reduced function of *sym1*. We also note that depending on the precise site of the frame correction, a small amino acid substitution (1-5 residues) would be introduced into the C-terminus of the winged helix DNA-binding domain, and this could also contribute to the reduction of

protein function. While we describe an unusual scenario where translational frameshifting suppresses a null mutation, it may be that this mechanism is employed by a subset of "normal" genes for diversification of the vertebrate proteome. In the case of wild-type genes, frameshifting would generate alternative protein sequences, with potential quantitative or qualitative modifications of protein function.

5.4 Larger implications of translational frameshifting

Frameshifting is a mechanism frequently employed by viruses and bacteria to produce two distinct protein products from one mRNA sequence. Frameshifting has also been associated with human disease. For example, a Ubiquitin-B +1 (UBB+1) frameshift results in a protein that cannot tag proteins for degradation, and itself accumulates in neuritic plaques, causing several tauopathies (van Leeuwen et al., 2006). In the case of *sym1*, translational frameshifting plays a role in correcting a point deletion, and likely suppressing the phenotypic consequences of the point deletion, as opposed to causing a loss or reduction of function as seen for UBB+1. This type of correction has not been reported previously in vertebrates and has implications for modifying the functional outcomes of mutation, and for the diversification of the vertebrate proteome. This mechanism may be more prevalent than previously appreciated, perhaps accounting for the disconnect between the predicted and actual activity of specific mutant alleles, as well as the lack of correspondence between the proteome and genome for certain genes. Further investigation into the prevalence and mechanisms of translational frameshifting should be pursued in vertebrate models.

The potential significance of translational frameshifting is especially interesting to consider in the context of proteome diversification. In general, frameshifts within a gene, whether induced by mutation of other conditions, are deleterious and are expected to result in loss-of-function, with altered protein sequence, structure and function. In some cases, the truncated proteins resulting from a frameshift mutation may accumulate and function as a dominant negative that disrupts cellular function. However, seldom is frameshifting considered a mechanism for the generation of novel or alternative protein functions. Point mutations, gene duplication, retroposition, gene fusion and fission, and exon shuffling have been considered likely mechanistic explanations for the generation of novel gene/protein function (Long et al., 2003). Regulated translational frameshifting is an additional viable mechanism for generating novel or altered protein sequences. The region of the open reading frame distal to the site of frameshifting will encode a protein sequence unrelated to the original sequence. Such "new" protein could be viewed as a fusion protein derived from two overlapping reading frames that join at the site of frameshifting. In rare cases the generated fusion protein may gain an important role in development or other biological process. On the other hand, genes that undergo frameshifting must retain structural and functional features of the protein encoded N-terminal to the frameshift, but also accommodate functions conferred by the distal sequences, thus allowing for the expression of novel functions by the frameshifted protein product. The likelihood of these conditions coming together may be low, however, given the complexity of the genome and the evolutionary timescale in which to sample mutations, a role for frameshifting in diversification of the proteome seems plausible (Raes and Van de Peer, 2005). The presence of a second redundant gene copy,

as is the case for many loci in the zebrafish (Meyer and Schartl, 1999), can clearly facilitate the generation of new protein function by frameshifting. The unmodified gene would maintain function, thus allowing the modification of function in a second gene copy. Whether this mechanism, as observed for *sym1*, is exceedingly rare or a mechanism used for a select group of natural genes remains to be determined.

Finally, defining the mechanisms that control frameshifting might be beneficial in identifying therapeutic approaches for viral disease. In the case of coronaviruses, production of RNA polymerase requires a -1 frameshift, and this polymerase is essential for viral replication (Plant and Dinman, 2008). Viral infection by organisms that depend on frameshifting for survival and viral replication could be targeted with small molecules, peptides or oligonucleotides that inhibit frameshifting, thus inhibiting viral multiplication and infectivity, allowing for a potentially effective treatment (Gareiss and Miller, 2009).

5.5 References

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