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Foxd3 is an essential Nodal-dependent regulator of zebrafish dorsal mesoderm development

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ABSTRACT

Establishment of the embryonic mesoderm is dependent on integration of multiple signaling and transcriptional inputs. We report that the transcriptional regulator Foxd3 is essential for dorsal mesoderm formation in zebrafish, and that this function is dependent on the Nodal pathway. Foxd3 gain-of-function results in expanded dorsal mesodermal gene expression, including the Nodal-related gene *cyclops*, and body axis dorsalization. Foxd3 knockdown embryos displayed reduced expression of *cyclops* and mesodermal genes, axial defects similar to Nodal pathway loss-of-function, and Nodal pathway activation rescued these phenotypes. In *MZoep* mutants inactive for Nodal signaling, Foxd3 did not rescue mesoderm formation or axial development, indicating that the mesodermal function of Foxd3 is dependent on an active downstream Nodal pathway. A previously identified *foxd3* mutant, *sym1*, was described as a predicted null mutation with neural crest defects, but no mesodermal or axial phenotypes. We find that Sym1 protein retains activity and can induce strong mesodermal expansion and axial dorsalization. A subset of *sym1* homozygotes displays axial defects and reduced *cyclops* and mesodermal gene expression, and penetrance of the mesodermal phenotypes is enhanced by Foxd3 knockdown. Therefore, *sym1* is a hypomorphic allele, and reduced Foxd3 function results in a reduction of *cyclops* expression, and subsequent mesodermal and axial defects. These results demonstrate that Foxd3 is an essential upstream regulator of the Nodal pathway in zebrafish dorsal mesoderm development and establish a broadly conserved role for Foxd3 in vertebrate mesodermal development.

Introduction

The vertebrate body plan forms in response to a network of signaling cascades that are integrated in time and space to induce and pattern the primary germ layers. These major signaling systems, including the Nodal, BMP, Wnt, FGF and other pathways, are subject to precise feedback and feedforward mechanisms that reinforce or inhibit signaling output (Kimelman, 2006). The modulation of signaling required for proper germ layer patterning is under the control of multiple extracellular signaling inhibitors, and a primary source of these inhibitors is the organizer, a major signaling center responsible for germ layer patterning in the gastrula (De Robertis and Kuroda, 2004). The transcriptional networks initiated in response to these signaling pathways establish a spatial framework in the gastrula for further elaboration of the body plan. Defining the interplay between lineage-specific transcriptional networks and embryonic signaling inputs is essential for a mechanistic understanding of germ layer formation.

Nodal ligands, members of the TGFß superfamily, are essential inducers of mesendoderm in the vertebrate embryo (Schier, 2003). In mouse, Nodal loss-of-function results in incomplete gastrulation, a failure of mesoderm formation, and developmental arrest (Conlon et al., 1994). Inhibition of Nodal signaling in *Xenopus* causes developmental arrest at gastrulation and a failure to form mesodermal and endodermal lineages (Osada and Wright, 1999). A zebrafish double mutant in two *nodal* genes (*cyclops* and *squint*) or a maternal zygotic mutant in the Nodal co-receptor *one eyed pinhead* (*MZoep*) fails to gastrulate, and lacks all head mesoderm, trunk mesoderm, and endoderm (Feldman et al., 1998, 2000; Gritsman et al., 1999; Whitman, 2001). Single mutants for *cyclops* or *squint* have a less severe phenotype, as do maternal or zygotic *oep* mutants (Dougan et al., 2003; Zhang et al., 1998). Spatial and temporal control of the Nodal pathway is dynamic and subject to multiple positive and negative inputs that reinforce Nodal activity in the mesodermal and endodermal domains and silence pathway activity in the adjacent ectodermal domain. While much is known about the inhibitory control of Nodal signaling, less is understood regarding the transcriptional mechanisms that restrict or silence the expression of *nodal* genes.

Foxd3, a member of the forkhead class of transcriptional regulators, has multiple roles in vertebrate embryogenesis, including

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maintenance of stem cell and progenitor cell populations, control of dorsal mesoderm formation in the gastrula, and regulation of neural crest development. *foxd3* is expressed in mouse and human embryonic stem cells, in mouse trophoblast stem cells, and in the epiblast cells of the preimplantation mouse embryo (Hanna et al., 2002; Sutton et al., 1996; Tompers et al., 2005). Neither embryonic stem cell lines nor trophoblast stem cell lines can be established from *foxd3* null embryos, indicating an essential role for Foxd3 in controlling maintenance, survival, and differentiation of these stem cell populations (Hanna et al., 2002; Tompers et al., 2005). At the gastrula stage in *Xenopus* and zebrafish, *foxd3* is expressed in the organizer (Odenthal and Nusslein-Volhard, 1998; Pohl and Knochel, 2001; Sasai et al., 2001) where it is coexpressed with multiple *nodal*-related genes. We have demonstrated in *Xenopus* that Foxd3 is necessary and sufficient for dorsal mesodermal development, and that Foxd3 functions as a repressor to maintain *nodal* expression and signaling activity in the Spemann organizer (Steiner et al., 2006; Yaklichkin et al., 2007). In the neural crest, studies in mouse, chick, zebrafish and *Xenopus* indicate that Foxd3 is required for the determination, migration, survival and/or differentiation of multiple neural crest lineages (Dottori et al., 2001; Kos et al., 2001; Sasai et al., 2001; Cheung et al., 2005; Whitlock et al., 2005; Lister et al., 2006; Montero-Balaguer et al., 2006; Stewart et al., 2006; Teng et al., 2008). Therefore, Foxd3 is an essential transcriptional regulator of diverse cell lineages at distinct stages of vertebrate development.

Early and late functions for Foxd3 have been described in both mouse and *Xenopus* (pre-gastrula or gastrula function early and neural crest function later). Surprisingly, despite conservation of *foxd3* expression in the organizer domain, only a neural crest function has been described for zebrafish Foxd3. Knockdown and mutant studies in zebrafish have demonstrated a requirement for Foxd3 in the differentiation of neural crest derivatives, including craniofacial 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, there have been no reports of defects in mesoderm formation, gastrulation or axial development for zebrafish Foxd3 knockdown or loss-of-function analyses. This apparent lack of gastrula function is especially striking in the *foxd3* mutant *sympathetic mutation 1* (*sym1*), a predicted null mutation (Stewart et al., 2006). These results suggest that, unlike mouse and *Xenopus*, Foxd3 function in the gastrula is not essential in the zebrafish, indicating an unexpected lack of developmental conservation. Another possible explanation for these results would be the presence of a second compensating *foxd3* gene, but no second zebrafish *foxd3* locus has yet been identified. These observations suggest either that Foxd3 is not essential in the zebrafish gastrula, or that the *sym1* mutation is not a functional null for Foxd3.

Here we report gain-of-function, knockdown and mutant analyses that demonstrate an essential function for Foxd3 in zebrafish mesodermal development and axis formation, as well as the dependence of Foxd3 on an active, downstream Nodal signaling pathway. We show that the *sym1 foxd3* mutation, previously predicted to be a functional null, is a hypomorphic allele with reduced function, resulting in partial penetrance of mesodermal defects. These studies define an early developmental requirement for Foxd3 in the zebrafish and confirm an essential conserved function of Foxd3 as a Nodal pathway regulator in the vertebrate gastrula.

Materials and methods

Zebrafish methods and microinjection

Zebrafish 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 and *sym1* (*foxd3*^{zdf10}) embryos (a gift of Thomas Look; Stewart et al., 2006) was performed at the one-cell stage using standard methods (Westerfield, 1993).

FoxD3 expression plasmids and mutagenesis

A pCS2-myc-*foxd3* plasmid (Lister et al., 2006) was used for expression of wild-type zebrafish Foxd3. 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′-CGACCCCCAGTCGGAAGATATTTCGACAACGGTAGCTTTCTG-3′ and reverse 5′-CAGAAAGCTACCGTTGTCGAAATATCTTCCGACTGGGGGTCG-3′. For microinjection, in vitro transcribed mRNA was generated from linearized plasmid templates using the Ambion SP6 mMessage mMachine system (Austin, TX).

Morpholino oligonucleotides

Morpholino antisense oligonucleotides were obtained from Gene Tools (Philomath, OR). Lyophilized oligonucleotides were resuspended in water, then diluted into 1× Danieau buffer (Nasevicius and Ekker, 2000) and 1 nl was injected into one-cell stage embryos. Two morpholino antisense oligonucleotides were designed to *Danio rerio foxd3* (BC095603): *foxd3*MO1 (5′-TGCTGCTGGAGCAACCCAAGG-TAAG-3′) (a gift of David Raible; Lister et al., 2006) is complementary to nucleotides 160–184 of the 5′ UTR and *foxd3*MO2 (5′-TGGTGCCTCCAGACAGGGTCATCAC-3′) is complementary to nucleotides 194–218 and overlaps the start codon. A mixture of the two oligonucleotides (total dosage 20 ng per embryo) was used for knockdown experiments in wild-type embryos. Injection of either individual oligonucleotide at higher dosage (30–40 ng) yielded similar results, but with some associated toxicity. As specificity controls, a mismatch oligonucleotide was injected at equal dosage (5′-TGGTcCCTaCAGAgAGGcTCATaAC-3′), or RNAs encoding *Xenopus foxd3* (30 pg) (Steiner et al., 2006) or zebrafish *cyclops* (20 pg) (Feldman et al., 1998) were injected to rescue. For Foxd3 knockdown in *sym1* embryos a mixture of *foxd3*MO1 and *foxd3*MO2 was injected at a total dosage of 2–4 ng. Due to the slightly delayed development of morphants, embryos were stage matched for phenotypic and gene expression analyses.

Whole mount 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 with an MZFLIII12.5 stereomicroscope (Leica) with a Retiga 1300 camera (Q-imaging) and processed using Adobe Photoshop.

Genotyping

Heterozygous *sym1* adults were crossed and individual progeny were harvested for genotyping at 5 dpf. For each phenotypic class (wild-type, reduced jaw, and short axis with reduced jaw) 7–14 individual embryos were analyzed. Genomic DNA was isolated as previously described (Westerfield, 1993) with the modification of incubating embryo lysates 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′-GCGAATTCCTTCGTCAAGATCCCACG-3′; reverse 5′-CATATGGAATTCACCCGGCGAATTCAG-3′) and products were subcloned into the pCR4-TOPO vector (Invitrogen). For each individual embryo 6–17 subclones were sequenced, and individual fish were assigned to genotypic categories based on the sequence