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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 display 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.

Keywords

Foxd3; Nodal; Forkhead; Mesoderm; Transcription; Zebrafish

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

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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 later 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 TGFβ 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., 2000; 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 know 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 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* (pregastrula 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

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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 penentrance 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.

Material 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*$^{\mathrm{sym1}}$ 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).

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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): *foxd3*MO1 (5'-TGCTGCTGGAGCAACCCAAGGTAAG-3') (a gift of David Raible; Lister et al., 2006) is complementary to nucleotides 160–184 of the 5' UTR and *foxd3*MO2 (5'-TGGTGCCTCCAGA CAGGGTCATCAC-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 either individual oligonucleotide at higher dosage (30–40ng) yielded similar results, but with some associated toxicity. As specificity controls, a mismatch oligonucleotide was injected at equal dosage (5'-TGGTcCCTaCAGAgAGGcTCATaA C-3'), and RNAs encoding *Xenopus foxd3* (30pg) (Steiner et al., 2006) or zebrafish *cyclops* (20pg) (Feldman et al., 1998) were injected to rescue. For Foxd3 knockdown in *sym1* embryos a mixture of FoxD2MO1 and FoxD3MO2 was injected at a total dosage of 2–4ng. Due to the slightly delayed development of morphants, embryos were stage matched for phenotypic and gene expression analyses.

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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 from 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 5dpf. For each phenotypic class (wild-type, reduced jaw, 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'-GCGAATTCCTTCGTC AAGATCCCACG-3'; reverse 5'-CATATGGAATTCACCCGGCGAA TTCAG-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 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 "reduced jaw" embryos and 8 "short axis with reduced 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.

Results

Foxd3 induces dorsal mesoderm and dorsalization of the body axis

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 gastrula and 24hpf stages (Fig. 1). Wild-type embryos were injected at the one-cell stage with 25pg of *foxd3* mRNA, and 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=556), as compared to uninjected embryos (Fig. 1A,B). Expansion of dorsal or panmesodermal gene expression was observed in a majority of injected embryos, along with a reduction in ventral gene expression (Fig. 1C–L). For *cyclops*, *chordin*, and *goosecoid* (88%, n=637; 93%, n=473; 86%, n=641), genes normally restricted to the shield domain (Stachel et al., 1993;Miller-Bertoglio et al., 1997;Rebagliati et al., 1998), Foxd3 induced lateral expansion (Fig. 1D,F,H), as well as animal expansion for *chordin* (Fig. 1F) and ectopic lateral expression for *goosecoid* (Fig. 1H). The panmesodermal gene *no tail* is expressed throughout the margin at the shield stage (Schulte-Merker et al., 1992), and Foxd3 induced an expansion of *no tail* towards the animal pole (81%, n=634), resulting in a broader marginal domain of *no tail* expression (Fig. 1J). Expansion of *squint* expression was also observed in response to *foxd3* overexpression (86%, n= 652) (data not shown), similar to the response of *cyclops*. Consistent with the expansion of dorsal gene expression, the ventrolateral domain of *bmp7* expression (Schmid et al., 2000) was reduced and limited to the ventralmost margin in response to Foxd3 (95%, n=342) (Fig. 1L).

Foxd3-injected embryos were strongly dorsalized at 24hpf (90%, n=230) (Fig. 1M,N), consistent with the expansion of dorsal mesodermal genes at the shield stage. To assess the formation of axial mesoderm at 24hpf, *no tail* and *sonic hedgehog* expression was examined

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(Fig. 1O–R). *no tail* expression was not perturbed throughout much of the body axis, but was disorganized in the tailbud (Fig. 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 Foxd3 induced expanded (83%, n=64) or ectopic (17%, n=13) expression (Fig. 1R), consistent with axial dorsalization and, in a minority of embryos, axial duplication. The morphological 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 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 (Symes and Smith, 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 (see Fig. S1 in supplementary materials). Therefore, zebrafish Foxd3 has potent dorsal mesoderm-inducing activity, identical to that previously described for *Xenopus* Foxd3 (Steiner et al., 2006).

Foxd3 is essential for mesodermal 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), were injected at the one-cell stage (total dosage 20ng), and embryos were analyzed at the shield and 24hpf stages (Fig. 2). At the shield stage, Foxd3 knockdown embryos had reduced or absent shield structures (93%, n=593) (Fig. 2A,B), and a significant reduction of dorsal mesodermal gene expression (Fig. 2C–H). *cyclops* and *goosecoid* expression was detectable, but substantially reduced in a majority of knockdown embryos (86%, n=438 and 73%, n=554, respectively) (Fig. 2D,F), and *no tail* expression was reduced to a thin marginal expression domain (70%, n=523) (Fig. 2H). In addition, *chordin* and *squint* expression in the shield was strongly reduced (79%, n=431 and 81%, n=633, respectively) (data not shown).

At 24hpf, Foxd3 knockdown embryos display reduced head structures, notochord defects, loss of trunk somites, and retention of tail somites (78%, n=330) (Fig. 2I,J). Analysis of *sonic hedgehog* expression indicated disruption of notochord and floor plate development (87%, n=138) (Fig. 2K,L). Moreover, the mesodermal and axial defects observed in Foxd3 knockdown embryos are similar to the single *cyclops* or *squint* mutants, as well as the zygotic *oep* mutant (Feldman et al., 1998; Gritsman et al., 1999), consistent with the reduction of *cyclops* and *squint* expression in knockdown embryos.

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 perfect match oligonucleotides, a *foxd3* oligonucleotide with multiple mismatches did not produce any mesodermal or axial phenotypes (data not shown). In addition, injection of knockdown embryos with *Xenopus foxd3* (30pg) rescued normal development in most embryos (81%, n=126) (see Fig. S2 in supplementary materials). Taken together, the observations indicate that a specific knockdown of endogenous *foxd3* results in severe

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mesodermal and axial defects, strongly supporting an essential and conserved role for Foxd3 in mesodermal development. Furthermore, the similarity of phenotypes for Foxd3 knockdown and Nodal pathway partial loss-of-function is consistent with the predicted role of Foxd3 in promoting Nodal expression and signaling (Steiner et al., 2006). The regulatory relation of Foxd3 and the Nodal pathway is further supported by the ability of injected *cyclops* mRNA (20pg) to fully rescue normal development in Foxd3 knockdown embryos (73%, n=202) (see Fig. S2 in supplementary materials).

It is important to note that previous Foxd3 knockdown attempts in the zebrafish 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. In our studies, a mixture of two *foxd3*-specific oligonucleotides was used, while the previously studies made use of only a single oligonucleotide. If our approach results in a more complete knockdown of Foxd3 protein, it would suggest that neural crest development is more sensitive to Foxd3 dosage than is mesodermal development, which could account for the differences in results obtained. Consistent with this idea, injection of lower doses of either single or combined morpholinos can recapitulate the neural crest defects previously reported (Kelsh et al., 2000; Lister et al., 2006; Montero-Balaguer et al., 2006; Stewart et al., 2006), in the absence of axial phenotypes (data not shown). However, a difference in knockdown efficiency cannot 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).

Foxd3 function is dependent on an active Nodal signaling pathway

The Foxd3 gain-of-function and knockdown results support a role for Foxd3 in maintaining Nodal expression and activity in the organizer, where *foxd3* and *nodal* genes are coexpressed during gastrulation (Odenthal and Nusslein-Volhard, 1998). To further examine the regulatory relation of Foxd3 and the Nodal pathway, we made use of *MZoep* mutant zebrafish that lack an essential Nodal coreceptor (Gritsman et al., 1999). In *MZoep* embryos, Nodal cannot bind its functional receptor complex and the elimination of Nodal signaling output results in a loss of dorsal mesodermal gene expression (Fig. 3H,N,T,Z). 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* (25pg) and mesodermal gene expression was examined at the shield stage (Fig. 3). As predicted, Foxd3 did not rescue or induce the expression of *cyclops* (100%, n=132), *goosecoid* (100%, n=120), *chordin* (100%, n=114), or *no tail* (100%, n=102) in any of the injected embryos (Fig. 3I,O,U,A'). As a control for rescue, *MZoep* embryos were injected with *activin* (25pg), a TGFβ ligand that activates the Nodal pathway independent of the *oep* coreceptor requirement (Gritsman et al., 1999), and strong rescue of mesodermal gene expression was observed (Fig. 3J,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. 3K,L,Q,R,W,X,C',D'). Injected *MZoep* embryos were also examined at later stages for axial and midline rescue (Fig. 4) and Foxd3 failed to rescue head, trunk or notochord development (Fig. 4E,F), while Activin partially rescued head and trunk, and fully rescued notochord (Fig. 4H,I) (Gritsman et al., 1999).

Further support for these conclusions was obtained by coexpressing *foxd3* and *antivin*, an atypical TGFβ-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* (100%, n=160), *goosecoid* (100%, n=132), or *no tail* (100%, n=116) (see Fig. S3 in supplementary materials).

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These 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.

Reexamination of the foxd3 sym1 mutant reveals mesodermal and axial defects

The *sympathetic mutation 1* (*sym1*) is a *foxd3* mutant (*foxd3^{zdf10}*) identified in a genetic screen for mutations that disrupt the development of sympathetic neurons (Stewart et al., 2006). Homozygous *sym1* embryos have defects in multiple neural crest lineages, including peripheral neurons, glia and cartilage, resulting in major craniofacial abnormalities. The molecular lesion present in *sym1* is a point deletion (G537) that results in a short frameshift and premature stop, truncating the Foxd3 protein within the C-terminal region of the winged helix DNA-binding domain. This truncation is predicted to disrupt wing 2 of the winged helix, a region required for minor groove contacts, site recognition specificity and transcriptional function (Berry et al., 2005). Based on the predicted truncation and inactivation of the DNA-binding domain, Stewart and colleagues conclude that *sym1* is functional null of *foxd3*. Subsequent to the identification of *sym1*, a conserved C-terminal Groucho corepressor interaction motif was identified in *Xenopus* Foxd3 that is essential for transcriptional and developmental function (Yaklichkin et al., 2007). The truncated *sym1* product, with impaired DNA-binding function and absence of an essential transcriptional effector domain, is strongly predicted to be a functional null allele of *foxd3*.

Given the 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 (n=351) were examined at 24hpf and 5dpf, and assigned to phenotypic classes (Fig. 5). At 24hpf, a subset of cross progeny (10%, n=119) displayed axial phenotypes with reduced head structures, shortened axes, and expanded ventral tail somites (Fig. 5D), consistent with the Foxd3 knockdown phenotype (Fig. 2). Craniofacial defects are not morphologically apparent at this early stage. At 5dpf (n=494), while most embryos fit the previously identified phenotypic classes − 77% wild-type (Fig. 5E,F) and 14% with craniofacial defects (Fig. 5G) – a previously unreported phenotypic class was observed (9%) with both craniofacial defects and axial defects, including curved or shortened axes (Fig. 5H). This novel phenotypic class was not previously reported for the *sym1* mutant (Stewart et al., 2006), but the nature of the axial abnormalities at 24hpf and 5dpf is consistent with Foxd3 function in mesoderm formation. To correlate each of these phenotypic classes with Foxd3 genotype, multiple individual embryos from each class were subjected to genotyping analysis. At 24hpf, the axial defect class consisted exclusively of *sym1* homozygotes, and at 5dpf, the craniofacial defect class and the craniofacial-axial defect class consisted only of *sym1* homozygotes (see Materials and Methods). At 24hpf, the phenotypically wild-type class consisted of wild-type, heterozygous and homozygous embryos, while at 5dpf, the phenotypically wild-type class consisted of wild-type and heterozygous embryos. Importantly, these results show that a genetic loss-of-function in Foxd3 does lead to phenotypic defects consistent with an essential function for Foxd3 in mesoderm and axis formation.

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. 6). While ~80% of cross progeny had normal mesodermal gene expression, ~20% of the embryos displayed a substantial reduction of *cyclops* (n=321), *chordin* (n=299), *goosecoid* (n=317), and *no tail* (n=253) expression (Fig. 6B,D,F,H). Following in situ hybridization, embryos from each class were genotyped, and while embryos with normal gene expression were either wild-type or *sym1* heterozygotes, embryos with reduced mesodermal gene

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expression were homozygous for *sym1* (data not shown). Importantly, these results confirm the requirement for Foxd3 in dorsal mesodermal development at the gastrula stage. It is interesting to note that while most of the predicted 25% homozygous embryos show mesodermal gene expression deficits at the shield stage, the phenotypic severity appears to diminish during development such that by 24hpf only 9% of embryos display axial defects. This difference in penetrance of gene expression and axial phenotypes may reflect a developmental threshold with reduced levels of mesodermal gene expression being sufficient to support normal axial development in some embryos. Alternatively, compensation or regulation, during gastrulation or thereafter, may moderate the consequence of Foxd3 loss-of-function in some, but not all, homozygous embryos.

sym1 is a hypomorphic allele of Foxd3 with partial penetrance of mesodermal defects

Despite the strong prediction that *sym1* is a functional null allele of *foxd3*, the partial penetrance of 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 (150pg) was injected into wild-type embryos at the one-cell stage. Injected embryos were examined for mesodermal gene expression at the shield stage and for axis formation at 24hpf (Fig. 7). Surprisingly, *sym1* injection resulted in expanded expression of *cyclops* (75%, n=310), *goosecoid* (73%, n=316), *chordin* (76%, n=328), and *no tail* (66%, n=334) in most embryos (Fig. 7B,E,H,K). Similarly, *sym1* induced strong dorsalization of the body axis at 24hpf (76%, n=330) (Fig. 7N). When *sym1* mRNA was injected at doses 6-fold higher than wild-type *foxd3*, the embryonic response was indistinguishable (Fig. 7C,F,I,L,O). This retention of activity indicates that *sym1* is a hypomorphic allele, not a null, despite the strong prediction otherwise.

To confirm the retention of a low level of Foxd3 function in *sym1* mutants, 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 (2.5ng) into the one-cell stage progeny of *sym1* het crosses. At this low dosage no phenotypic response was observed in wild-type embryos (Fig. 8). In contrast, injection of this low dosage of oligonucleotides into *sym1* progeny resulted in a dramatic increase in both the craniofacial only and craniofacial-axial phenotypic classes. In these experiments, the phenotypic distribution of uninjected *sym1* progeny was 76% wild-type, 18% craniofacial only, and 5% craniofacial-axial (n=167) (Fig. 8). Foxd3 knockdown in cross progeny resulted in 24% wild-type, 45% craniofacial only, and 29% craniofacial-axial (n=107) (Fig. 8). Genotyping confirmed that the expanded craniofacial only and craniofacial-axial phenotypic classes consisted entirely of *sym1* heterozygotes and homozygotes (data not shown). Taken together, these results indicate that functional Foxd3 protein is retained in *sym1* homozygotes, and that knockdown of the remaining Foxd3 function results in increased penetrance of axial phenotypes, confirming that Foxd3 function is essential for zebrafish mesodermal development.

Discussion

The control of mesodermal development is an area of intense study, and these efforts have provided broad mechanistic insight to the signaling and transcriptional processes that establish and pattern the major lineages of the vertebrate embryo (Kimelman, 2006; Chang and Kessler, 2007). Here we show that the transcriptional protein, Foxd3, is an essential regulator of mesodermal and axial development in the zebrafish. Foxd3 function is required for maintenance of *cyclops* expression in the shield, formation of dorsal mesodermal lineages, and

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development of normal axial structure. The mesodermal activity of Foxd3 is fully dependent on a functional Nodal signaling pathway, indicating that Foxd3 acts as an upstream positive regulator of Nodal pathway activity. Furthermore, we demonstrate that *sym1*, a *foxd3* mutant previously reported to be a null allele with no mesodermal or axial defects (Stewart et al., 2006), does indeed exhibit reduction of *cyclops* expression, loss of dorsal mesodermal lineages, and disruption of axis formation, albeit at reduced penetrance. Despite predictions that *sym1* is a functional null, we find that knockdown of residual Foxd3 activity in *sym1* embryos strongly enhances the mesodermal and axial phenotypes, indicating that *sym1* is a hypomorphic allele of *foxd3*. These studies define the developmental requirement for Foxd3 in the zebrafish gastrula and demonstrate that Foxd3 is an upstream regulator of the Nodal pathway that is essential for dorsal mesoderm development.

Foxd3 regulation of the Nodal pathway

With the onset of gastrulaton in the zebrafish, *foxd3* expression is initiated in the shield domain (Odenthal and Nusslein-Volhard, 1998) where it is coexpressed with the Nodal ligands, *cyclops* and *squint*. Our developmental analyses demonstrate that Foxd3 function is necessary and sufficient for Nodal expression in the gastrula. Foxd3 gain-of-function resulted in ectopic and/or expanded *cyclops* expression, while in knockdown and *sym1* embryos, expression of *cyclops* was substantially reduced (Figs. 1,2,6). A similar Foxd3-dependence was observed for *squint* expression in the shield (data not shown). Given that the onset of Nodal expression in the dorsal domain precedes the initiation of FoxD3 expression in the shield, the results suggest that Foxd3 is not required for the initiation of Nodal expression, but rather for the maintenance of Nodal expression during gastrulation.

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The developmental importance of the Foxd3-Nodal interaction is apparent from the mesodermal and axial defects resulting from Foxd3 loss-of-function, and the similarity of these phenotypes to Nodal pathway loss-of-function. Foxd3 loss-of-function, either by knockdown or *sym1* mutation, results in a reduction of dorsal mesodermal gene expression and axial defects that include reduction of head structures, reduction of trunk mesoderm, disruption of midline structures, and expansion of tail somites (Figs. 2,5,6). This phenotypic spectrum corresponds to those reported for Nodal pathway loss-of-function in either the single *cyclops* or *squint* mutants or the zygotic *oep* mutant (Feldman et al., 1998;Zhang et al., 1998), consistent with the reduction of *cyclops* and *squint* expression in Foxd3 morphants and *sym1* mutants. This phenotypic similarity suggests that the developmental consequences of Foxd3 loss-of-function are largely due to a reduction of downstream Nodal pathway activity. Consistent with this idea, injection of *cyclops* mRNA fully rescued axial development in both Foxd3 knockdown and *sym1* mutant embryos (Fig. S2 and data not shown).

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Strong evidence of the dependence of Foxd3 function on Nodal pathway activity comes from the analysis of Foxd3 activity in the *MZoep* mutant, which is inactive for Nodal signaling. In the *MZoep* background, Foxd3 had no detectable activity, even when expressed at levels sufficient to strongly dorsalize wild-type embryos (Figs. 3,4). Foxd3 could rescue neither dorsal mesodermal gene expression nor axis formation in *MZoep* embryos, indicating that in the zebrafish gastrula, the mesodermal activity of Foxd3 was completely dependent on a functional downstream Nodal pathway. These results provide functional and genetic support for the essential role of Foxd3 as an upstream positive regulator of Nodal pathway activity in the zebrafish gastrula. Furthermore, these studies strongly confirm and extend our model of Foxd3-Nodal interaction in the *Xenopus* gastrula (Steiner et al., 2006), suggesting that this regulatory pathway may play an essential conserved role in vertebrate mesodermal development. It will be of great interest to explore the interaction of Foxd3 and the Nodal pathway in the mouse, where the gastrula-specific function of Foxd3 has yet to be examined.

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sym1 is a hypomorphic allele of foxd3

In striking contrast to our findings, prior knockdown and mutant studies of Foxd3 in the zebrafish revealed no mesodermal or axial phenotypes. Using translation-blocking oligonucleotides, several groups described neural crest lineage defects resulting from Foxd3 knockdown, each reporting largely similar phenotypes, but no mesodermal or axial defects were observed (Whitlock et al., 2005; Lister et al., 2006; Montero-Balaguer et al., 2006; Stewart et al., 2006; Ignatius et al., 2008; Curran et al., 2009). In these studies, it is likely that the Foxd3 knockdown conditions were optimized for neural crest-related phenotypes, and that these conditions were not sufficient for generating mesodermal defects at significant frequency. It should be noted, however, that one group does remark on an apparent dorsalizing activity of overexpressed Foxd3 (Lister et al., 2006), consistent with our results. We observed mesodermal defects at high frequency by injection of a mixture of two *foxd3*-specific oligonucleotides, suggesting that a more complete knockdown of Foxd3 may be necessary to disrupt mesodermal development. When injected at lower doses, the single or combined oligonucleotides did produce the previously described neural crest defects in the absence of mesodermal or axial phenotypes. Therefore, the absence of mesodermal phenotypes in previous knockdown studies may merely reflect differences in experimental design that result in more or less complete knockdown of Foxd3.

The discrepancy in obtaining mesodermal phenotypes resulting from Foxd3 knockdown prompted a reevaluation of the identified zebrafish *foxd3* mutants. Two *foxd3* mutants, *sym1* and *mos*, were identified in screens for regulators of neural crest development (Montero-Balaguer et al., 2006; Stewart et al., 2006). In both cases, these mutations result in defects in craniofacial structures, the peripheral nervous system, and pigment cells, but not mesodermal or axial phenotypes. The molecular lesion in *sym1* is predicted to truncate the DNA-binding domain and eliminate an essential C-terminal transcriptional effector domain, so it was concluded that *sym1* was a functional null allele of *foxd3* (Stewart et al., 2006). Given that our knockdown studies in *Xenopus* (Steiner et al., 2006) and zebrafish (this study) demonstrate a requirement for Foxd3 in mesodermal development, the absence of mesodermal defects in *sym1* suggested either the presence of redundant and/or compensating activities in the *sym1* mutant or that *sym1* was not a functional null allele.

The reanalysis of *sym1* provides definitive evidence of mesodermal and axial defects in this mutant, with ~39% of *sym1* homozygotes displaying both craniofacial and axial phenotypes (Fig. 5), and ~80% of homozygotes with reduced *cyclops* and dorsal mesodermal gene expression at the shield stage (Fig. 6). The penetrance of the mesodermal defects is enhanced by specific knockdown of Foxd3 in mutant embryos (Fig. 8), indicating that *sym1* is a hypomorphic allele of *foxd3*, and not a null. In gain-of-function studies *sym1* retains dorsalizing activity (Fig. 7), consistent with a hypomorphic character for the *sym1* mutation. Taken together, these observations demonstrate that reduction of Foxd3 activity in the zebrafish results in mesodermal and axial defects, establishing an essential role for Foxd3 in zebrafish mesodermal development.

The *mos* mutation, in contrast to *sym1*, does not reside in the *foxd3* coding region, but is a mutation in a distal regulatory element required for *foxd3* expression in neural crest lineages (Montero-Balaguer et al., 2006). In *mos* embryos, *foxd3* expression is lost in neural crest progenitor cells, resulting in neural crest phenotypes identical to those observed in *sym1* embryos and in Foxd3 knockdown embryos. At the gastrula stage, however, *foxd3* expression in the shield is normal and no defects in mesodermal or axial development are observed in *mos* embryos. Therefore, gastrula expression and function of Foxd3 is maintained in *mos* embryos, resulting in normal development of the mesoderm and body axis.

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In contrast to the reduced penetrance of mesodermal phenotypes in *sym1* embryos, neural crest phenotypes are fully penetrant in *sym1* and equal in severity to that observed in *mos* mutant embryos, which are null for *foxd3* expression in the neural crest. This suggests that *sym1* is a functional null for neural crest development, despite the sufficiency of this hypomorphic allele to support mesodermal developmental in most embryos. This difference in penetrance likely reflects differing dosage sensitivities in the two lineages, with greater Foxd3 activity required for neural crest development. More recent studies in the zebrafish have examined the genetic interaction of Foxd3 with other neural crest regulators, and conclusions drawn from these studies are based on the assumption that *sym1* is a true null allele of *foxd3* (Ignatius et al., 2008; Arduini et al., 2009; Cooper et al., 2009). Our demonstration that *sym1* is a hypomorph that retains biological activity should prompt a careful reevaluation of these genetic interaction studies. Identification of a true null allele for zebrafish *foxd3* is necessary to provide a definitive analysis of Foxd3 function in both mesodermal and neural crest lineages.

Transcriptional function of Foxd3 in development

Foxd3 has multiple roles in the developing vertebrate embryo, including the maintenance of embryonic and trophoblast stem cell lineages in the mouse (Hanna et al., 2002; Sutton et al., 1996; Tompers et al., 2005), regulation of neural crest determination, migration, and/or differentiation in the mouse, chick, *Xenopus*, and zebrafish (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; Arduini et al., 2009; Cooper et al., 2009), and promotion of *nodal* expression and activity during mesodermal development in *Xenopus* (Steiner et al., 2006) and zebrafish (this study). Given the many roles of Foxd3, defining the molecular mechanisms of Foxd3 action, including transcriptional activity and target gene identity, is essential for understanding the common and lineage-specific functions of Foxd3.

The transcriptional function of Foxd3 has been examined in a variety of cell culture and embryonic systems and these developmental and transcriptional studies provide strong evidence that Foxd3 functions as a transcriptional repressor. In *Xenopus* neural crest and mesodermal lineages, a fusion protein containing the Engrailed repressor domain and the Foxd3 winged helix DNA-binding domain mimicked the activity of native Foxd3, while a VP16 activator-Foxd3 fusion antagonized Foxd3 function (Pohl and Knochel, 2001; Sasai et al., 2001; Steiner et al., 2006). Similarly, an Engrailed-Foxd3 fusion protein can mimic the mesodermal and axial activities of native Foxd3 in the zebrafish, and VP16-Foxd3 disrupts zebrafish mesoderm and axis formation (L.L.C. and D.S.K., unpublished). In cell culture, *Xenopus* mesoderm and zebrafish neural crest, transcriptional reporter assays indicate that Foxd3 directly represses transcription of natural and heterologous target genes (Sutton et al., 1996; Freyaldenhoven et al., 1997; Yaklichkin et al., 2007; Ignatius et al., 2008; Curran et al., 2009). Consistent with a function as an obligate transcriptional repressor, all Foxd3 orthologs contain a conserved Groucho corepressor interaction motif, which in *Xenopus* Foxd3 is essential for Groucho recruitment, transcriptional repression activity, and mesoderm induction (Yaklichkin et al., 2007). Taken together, these observations provide compelling evidence that Foxd3 functions as a transcriptional repressor to regulate mesodermal development in the zebrafish gastrula.

Despite the strong evidence that Foxd3 functions as a repressor, transcriptional activation function for mouse FOXD3 has been reported in 293 cells and ES cells (Guo et al., 2002; Pan et al., 2006). Given that mouse FOXD3 can also function as a repressor in 293 cells (Sutton et al., 1996), the transcriptional activity of FOXD3 may be dependent on promoter context and/or transcriptional cofactors that facilitate activation of certain targets and repression of others. These results suggest that FOXD3 may have a dual transcriptional function, with context-

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dependent or lineage-specific activation of a subset of target genes. It should also be noted that the mammalian FOXD3 proteins contain several polyalanine and polyglycine sequences that are not present in other Foxd3 orthologs, and these sequences may confer additional transcriptional functions on the mammalian proteins.

An activation function for Foxd3 has also been reported in zebrafish somitogenesis (Lee et al., 2006). In a yeast one-hybrid screen, zebrafish Foxd3 bound a somite-specific regulatory element of *myf5*, and a transcriptional reporter containing that element was weakly activated by Foxd3 in cell culture studies. Foxd3 is coexpressed with *myf5* in somites, and Foxd3 knockdown resulted in a loss of *myf5* expression in somites, indicating that Foxd3 activates *myf5* expression in newly formed somites. Therefore, the transcriptional function of Foxd3 may differ in distinct lineages of the zebrafish, perhaps due to the lineage-specific expression of coactivators, corepressors, or other interacting factors. However, in conflict with these results, we have found that when introduced into zebrafish embryos by microinjection, the *myf5* reporters were unresponsive to overexpressed Foxd3 at the shield and somite stages, arguing against a direct role for Foxd3 in *myf5* regulation (L.L.C. and D.S.K., unpublished).

The Nodal pathway is subject to multiple levels of negative regulation that strictly limit signaling activity, both spatially and temporally (Schier, 2003; Shen, 2007). Due to the non-autonomous and autoregulatory function of Nodal signaling, negative regulatory inputs are essential to limit the amplification and spread of Nodal activity, thus ensuring proper embryonic organization. The ability of Foxd3 to promote *nodal* expression as a transcriptional repressor suggests that Foxd3 functions as an indirect activator by repressing a negative regulator(s) of *nodal* expression and/or activity in the shield. Foxd3 may repress antagonists of Nodal ligand-receptor interaction or signal transduction, or repressors of Nodal transcription. If Foxd3 were to repress antagonists of Nodal signaling, it might be expected that the transcriptional response to Foxd3 would be mediated largely by the conserved autoregulatory enhancer within intron 1 of the *nodal* locus (Osada et al., 2000). In preliminary analyses of the *Xenopus nodal-related-1* gene, we identified a distal Foxd3-responsive region upstream of the start site of transcription, while the autoregulatory intron 1 element was unresponsive to Foxd3 (Qun Lu and D.S.K., unpublished). We therefore favor a role for Foxd3 in repressing a transcriptional repressor of *nodal* expression in the shield.

How does sym1 retain biological activity?

The reanalysis of *sym1* demonstrates that this hypomorphic allele of *foxd3* results in mesodermal and axial defects with reduced penetrance, but one confounding question remains unanswered. How does the protein product of *sym1* retain biological activity? The molecular lesion of the *sym1* mutation is a point deletion within the C-terminus of the winged helix DNA-binding domain (Stewart et al., 2006). This results in a short frameshift followed by a premature stop, truncating much of the W2 region of the DNA-binding domain and eliminating a distal Groucho interaction motif (GEH). Disruption of either the W2 or GEH domains has been shown to result in a near complete loss-of-function (Berry et al, 2005; Yaklichkin et al., 2007), and disruption of both is strongly predicted to result in a functionally inactive Foxd3 protein. So how is it that the product of *sym1* retains biological activity, as shown in our studies?

In considering this question we note that the coding capacity for full-length Foxd3 protein is present within the *sym1* mRNA, which is not truncated, so it may be that corrective mechanisms bypass the premature stop to generate a Foxd3 protein containing the essential C-terminal domains. In support of such a corrective mechanism, when truncated at the position of the predicted premature stop, the *sym1* cDNA has no dorsalizing activity (data not shown). Therefore, sequences distal to the C-terminus of the predicted *sym1* product are required for the biological activity of *sym1*, raising the possibility that full-length functional Foxd3 protein may be produced from the *sym1* cDNA. Suppression of the premature stop would not be

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sufficient to restore Foxd3 function as the downstream reading frame has been shifted due to the point deletion. On the other hand, translational frameshifting, prior to the premature stop, would correct the open reading frame and allow production of full-length Foxd3 protein. Ongoing efforts focus on defining the molecular mechanisms responsible for the unexpected retention of biological activity for *sym1*.

Foxd3 is an essential transcriptional regulator for the development of multiple lineages in the vertebrate embryo. A number of important questions remain to be to explored, including the identification of Foxd3 targets that mediate the distinct functions of Foxd3 in specific lineages. It will be especially interesting to determine whether lineage-specific targets and mechanisms mediate Foxd3 function in the mesoderm, neural crest and stem cell populations, or if an underlying common regulatory pathway is utilized in each of these lineages. Ongoing studies of Foxd3 in the organizer, neural crest, and stem cell populations will provide further insight into the developmental and molecular mechanisms of vertebrate embryogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Arduini BL, Bosse KM, Henion PD. Genetic ablation of neural crest cell diversification. Development 2009;136:1987–1994. [PubMed: 19439494]

Berry FB, Tamimi Y, Carle MV, Lehmann OJ, Walter MA. The establishment of a predictive mutational model of the forkhead domain through the analyses of FOXC2 missense mutations identified in patients with hereditary lymphedema with distichiasis. Hum. Mol. Genet 2005;14:2619–2627. [PubMed: 16081467]

Chang, LL.; Kessler, DS. Formation of the embryonic mesoderm. In: Moody, SA., editor. Principles of Developmental Genetics. San Diego: Elsevier Inc; 2007. p. 258-294.

Chen C, Shen MM. Two modes by which Lefty proteins inhibit nodal signaling. Curr. Biol 2004;14:618–624. [PubMed: 15062104]

Cheung M, Chaboissier MC, Mynett A, Hirst E, Schedl A, Briscoe J. The transcriptional control of trunk neural crest induction, survival, and delamination. Dev. Cell 2005;8:179–192. [PubMed: 15691760]

Conlon FL, Lyons KM, Takaesu N, Barth KS, Kispert A, Herrmann B, Robertson EJ. A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. Development 1994;120:1919–1928. [PubMed: 7924997]

Cooper CD, Linbo TH, Raible DW. Kit and foxd3 genetically interact to regulate melanophore survival in zebrafish. Dev. Dyn 2009;238:875–886. [PubMed: 19301400]

Curran K, Raible DW, Lister JA. Foxd3 controls melanophore specification in the zebrafish neural crest by regulation of Mitf. Dev. Biol 2009;332:408–417. [PubMed: 19527705]

De Robertis EM, Kuroda H. Dorsal-ventral patterning and neural induction in Xenopus embryos. Annu. Rev. Cell Dev. Biol 2004;20:285–308. [PubMed: 15473842]

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Ding J, Yang L, Yan YT, Chen A, Desai N, Wynshaw-Boris A, Shen MM. Cripto is required for correct orientation of the anterior-posterior axis in the mouse embryo. Nature 1998;395:702–707. [PubMed: 9790191]

Dottori M, Gross MK, Labosky P, Goulding M. The winged helix transcription factor Foxd3 suppresses interneuron differentiation and promotes neural crest cell fate. Development 2001;128:4127–4138. [PubMed: 11684651]

Dougan ST, Warga RM, Kane DA, Schier AF, Talbot WS. The role of the zebrafish nodal-related genes squint and cyclops in patterning of mesendoderm. Development 2003;130:1837–1851. [PubMed: 12642489]

Feldman B, Dougan ST, Schier AF, Talbot WS. Nodal-related signals establish mesendodermal fate and trunk neural identity in zebrafish. Curr. Biol 2000;10:531–534. [PubMed: 10801442]

Feldman B, Gates MA, Egan ES, Dougan ST, Rennebeck G, Sirotkin HI, Schier AF, Talbot WS. Zebrafish organizer development and germ-layer formation require nodal-related signals. Nature 1998;395:181–185. [PubMed: 9744277]

Freyaldenhoven BS, Freyaldenhoven MP, Iacovoni JS, Vogt PK. Avian winged helix proteins CWH-1, CWH-2 and CWH-3 repress transcription from Qin binding sites. Oncogene 1997;15:483–488. [PubMed: 9242385]

Gritsman K, Zhang J, Cheng S, Heckscher E, Talbot WS, Schier AF. The EGF-CFC protein one-eyed pinhead is essential for nodal signaling. Cell 1999;97:121–132. [PubMed: 10199408]

Guo Y, Costa R, Ramsey H, Starnes T, Vance G, Robertson K, Kelley M, Reinbold R, Scholer H, Hromas R. The embryonic stem cell transcription factors Oct-4 and FoxD3 interact to regulate endodermal-specific promoter expression. Proc. Natl. Acad. Sci. USA 2002;99:3663–3667. [PubMed: 11891324]

Hanna LA, Foreman RK, Tarasenko IA, Kessler DS, Labosky PA. Requirement for Foxd3 in maintaining pluripotent cells of the early mouse embryo. Genes Dev 2002;16:2650–2661. [PubMed: 12381664]

Holley SA. Anterior-posterior differences in vertebrate segments: specification of trunk and tail somites in the zebrafish blastula. Genes Dev 2006;20:1831–1837. [PubMed: 16847343]

Ignatius MS, Moose HE, El-Hodiri HM, Henion PD. colgate/hdac1 repression of foxd3 expression is required to permit mitfa-dependent melanogenesis. Dev. Biol 2008;313:568–583. [PubMed: 18068699]

Kelsh RN, Dutton K, Medlin J, Eisen JS. Expression of zebrafish fkd6 in neural crest-derived glia. Mech. Dev 2000;93:161–164. [PubMed: 10781949]

Kimelman D. Mesoderm induction: from caps to chips. Nat. Rev. Genet 2006;7:360–372. [PubMed: 16619051]

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev. Dyn 1995;203:253–310. [PubMed: 8589427]

Kos R, Reedy MV, Johnson RL, Erickson CA. The winged helix transcription factor FoxD3 is important for establishing the neural crest lineage and repressing melanogenesis in avian embryos. Development 2001;128:1467–1479. [PubMed: 11262245]

Krauss S, Concordet JP, Ingham PW. A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. Cell 1993;75:1431–1444. [PubMed: 8269519]

Lee HC, Huang HY, Lin CY, Chen YH, Tsai HJ. Foxd3 mediates zebrafish myf5 expression during early somitogenesis. Dev. Biol 2006;290:359–372. [PubMed: 16386728]

Lister JA, Cooper C, Nguyen K, Modrell M, Grant K, Raible DW. Zebrafish Foxd3 is required for development of a subset of neural crest derivatives. Dev. Biol 2006;290:92–104. [PubMed: 16364284]

Liu Y, Labosky PA. Regulation of ES cell self renewal and pluripotency by Foxd3. Stem Cells 2008;26:2475–2484. [PubMed: 18653770]

Miller-Bertoglio VE, Fisher S, Sanchez A, Mullins MC, Halpern ME. Differential regulation of chordin expression domains in mutant zebrafish. Dev. Biol 1997;192:537–550. [PubMed: 9441687]

Montero-Balaguer M, Lang MR, Sachdev SW, Knappmeyer C, Stewart RA, De La Guardia A, Hatzopoulos AK, Knapik EW. The mother superior mutation ablates foxd3 activity in neural crest progenitor cells and depletes neural crest derivatives in zebrafish. Dev. Dyn 2006;235:3199–3212. [PubMed: 17013879]

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Mullins MC, Hammerschmidt M, Haffter P, Nusslein-Volhard C. Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. Curr. Biol 1994;4:189–202. [PubMed: 7922324]

Nasevicius A, Ekker SC. Effective targeted gene 'knockdown' in zebrafish. Nat. Genet 2000;26:216–220. [PubMed: 11017081]

Odenthal J, Nusslein-Volhard C. forkhead domain genes in zebrafish. Dev. Genes Evol 1998;208:245–258. [PubMed: 9683740]

Osada SI, Wright CV. Xenopus nodal-related signaling is essential for mesendodermal patterning during early embryogenesis. Development 1999;126:3229–3240. [PubMed: 10375512]

Osada SI, Saijoh Y, Frisch A, Yeo CY, Adachi H, Watanabe M, Whitman M, Hamada H, Wright CV. Activin/nodal responsiveness and asymmetric expression of a Xenopus nodal-related gene converge on a FAST-regulated module in intron 1. Development 2000;127:2503–2514. [PubMed: 10804190]

Pan G, Li J, Zhou Y, Zheng H, Pei D. A negative feedback loop of transcription factors that controls stem cell pluripotency and self-renewal. FASEB J 2006;20:1730–1732. [PubMed: 16790525]

Pohl BS, Knochel W. Overexpression of the transcriptional repressor FoxD3 prevents neural crest formation in Xenopus embryos. Mech. Dev 2001;103:93–106. [PubMed: 11335115]

Rebagliati MR, Toyama R, Haffter P, Dawid IB. cyclops encodes a nodal-related factor involved in midline signaling. Proc. Natl. Acad. Sci. USA 1998;95:9932–9937. [PubMed: 9707578]

Sasai N, Mizuseki K, Sasai Y. Requirement of FoxD3-class signaling for neural crest determination in Xenopus. Development 2001;128:2525–2536. [PubMed: 11493569]

Schier AF. Nodal signaling in vertebrate development. Annu. Rev. Cell Dev. Biol 2003;19:589–621. [PubMed: 14570583]

Schier AF, Neuhauss SC, Helde KA, Talbot WS, Driever W. The one-eyed pinhead gene functions in mesoderm and endoderm formation in zebrafish and interacts with no tail. Development 1997;124:327–342. [PubMed: 9053309]

Schmid B, Furthauer M, Conners SA, Trout J, Thisse B, Thisse C, Mullins MC. Equivalent genetic roles of bmp7/snailhouse and bmp2b/swirl in dorsoventral pattern formation. Development 2000;127:957–967. [PubMed: 10662635]

Schulte-Merker S, Hammerschmidt M, Beuchle D, Cho KW, De Robertis EM, Nusslein-Volhard C. Expression of zebrafish goosecoid and no tail gene products in wild-type and mutant no tail embryos. Development 1994;120:843–852. [PubMed: 7600961]

Schulte-Merker S, Ho RK, Herrmann BG, Nusslein-Volhard C. The protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. Development 1992;116:1021–1032. [PubMed: 1295726]

Shen MM. Nodal signaling: developmental roles and regulation. Development 2007;134:1023–1034. [PubMed: 17287255]

Stachel SE, Grunwald DJ, Myers PZ. Lithium perturbation and goosecoid expression identify a dorsal specification pathway in the pregastrula zebrafish. Development 1993;117:1261–1274. [PubMed: 8104775]

Steiner AB, Engleka MJ, Lu Q, Piwarzyk EC, Yaklichkin S, Lefebvre JL, Walters JW, Pineda-Salgado L, Labosky PA, Kessler DS. FoxD3 regulation of Nodal in the Spemann organizer is essential for Xenopus dorsal mesoderm development. Development 2006;133:4827–4838. [PubMed: 17092955]

Stewart RA, Arduini BL, Berghmans S, George RE, Kanki JP, Henion PD, Look AT. Zebrafish foxd3 is selectively required for neural crest specification, migration and survival. Dev. Biol 2006;292:174–188. [PubMed: 16499899]

Sutton J, Costa R, Klug M, Field L, Xu D, Largaespada DA, Fletcher CF, Jenkins NA, Copeland NG, Klemsz M, Hromas R. Genesis, a winged helix transcriptional repressor with expression restricted to embryonic stem cells. J. Biol. Chem 1996;271:23126–23133. [PubMed: 8798505]

Symes K, Smith JC. Gastrulation movements provide an early marker of mesoderm induction in Xenopus laevis. Development 1987;101:339–349.

Teng L, Mundell NA, Frist AY, Wang Q, Labosky PA. Requirement for Foxd3 in the maintenance of neural crest progenitors. Development 2008;135:1615–1624. [PubMed: 18367558]

Thisse C, Thisse B. Antivin, a novel and divergent member of the TGFbeta superfamily, negatively regulates mesoderm induction. Development 1999;126:229–240. [PubMed: 9847237]

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Tompers DM, Foreman RK, Wang Q, Kumanova M, Labosky PA. Foxd3 is required in the trophoblast progenitor cell lineage of the mouse embryo. Dev. Biol 2005;285:126–137. [PubMed: 16039639]

Westerfield, M. The Zebrafish Book. Eugene: University of Oregon Press; 1993.

Whitlock KE, Smith KM, Kim H, Harden MV. A role for foxd3 and sox10 in the differentiation of gonadotropin-releasing hormone (GnRH) cells in the zebrafish Danio rerio. Development 2005;132:5491–5502. [PubMed: 16291787]

Whitman M. Nodal signaling in early vertebrate embryos: themes and variations. Dev. Cell 2001;1:605–617. [PubMed: 11709181]

Yaklichkin S, Steiner AB, Lu Q, Kessler DS. FoxD3 and Grg4 physically interact to repress transcription and induce mesoderm in Xenopus. J. Biol. Chem 2007;282:2548–2557. [PubMed: 17138566]

Zhang J, Talbot WS, Schier AF. Positional cloning identifies zebrafish one-eyed pinhead as a permissive EGF-related ligand required during gastrulation. Cell 1998;92:241–251. [PubMed: 9458048]

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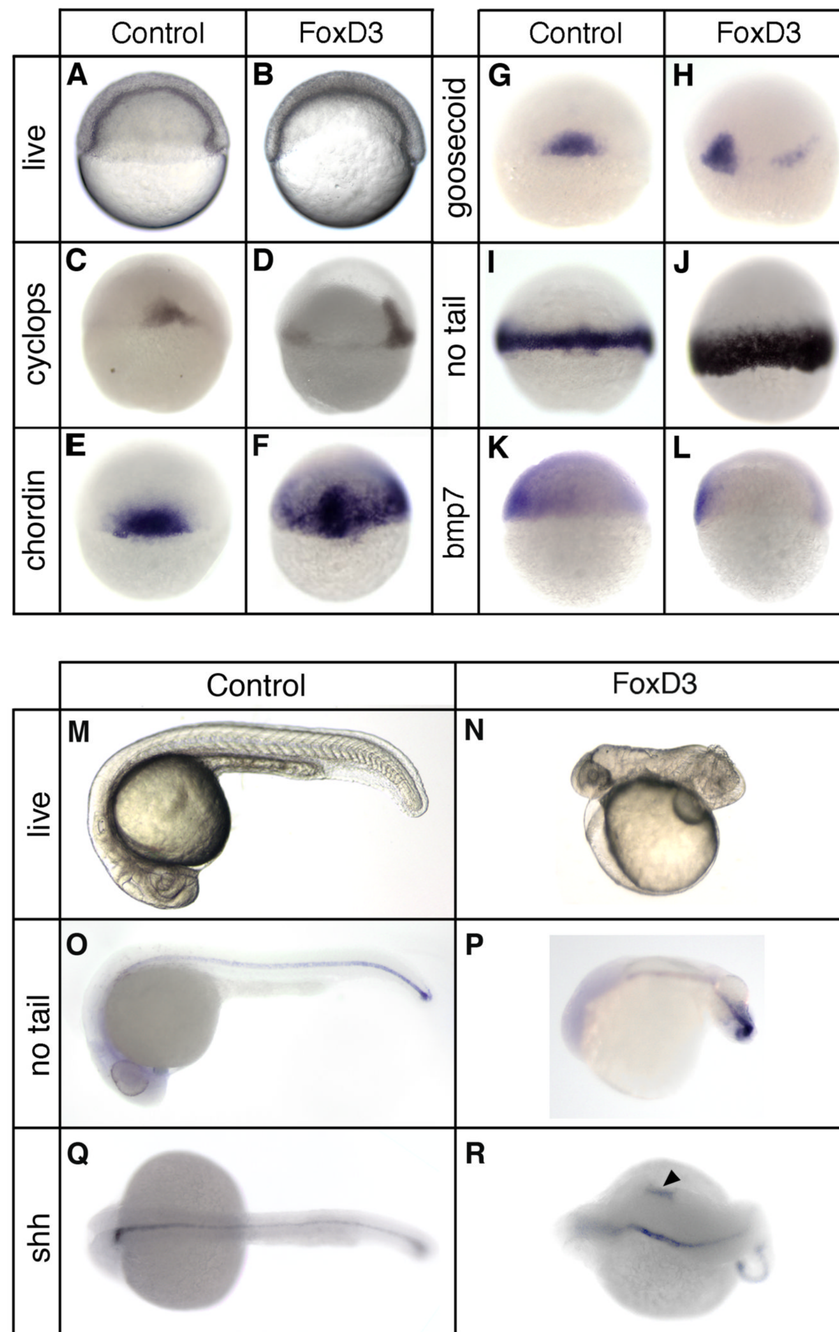

Fig 1.

Foxd3 induction of dorsal mesoderm and axial dorsalization. At the one-cell stage embryos were injected with 25pg of *foxd3* mRNA and analyzed at the shield stage (6hpf) (A–L) or at 24 hpf (M–R). (A,B) Live embryos (lateral views, dorsal right) showing the shield and blastoderm structure in uninjected (A) and *foxd3*-injected (B) embryos. In situ hybridization of uninjected (C,E,G,I,K) and *foxd3*-injected (D,F,H,J,K) embryos showing expression of *cyclops* (C,D), *chordin* (E,F), *goosecoid* (G,H), *no tail* (I,J), and *bmp7* (K,L). Views shown are dorsal (C,E,F,G,I,J), dorsal lateral (D,H), or lateral with dorsal right (K,L). Uninjected (M,O,Q) and *foxd3*-injected (N,P,R) embryos at 24hpf. Shown are live embryos (M,N), and embryos analyzed by in situ hybridization for *no tail* (O,P) or *sonic hedgehog* (Shh) (Q,R)

expression. Views are lateral with anterior left (M–P) or dorsal with anterior left (Q,R). Ectopic expression of *sonic hedgehog* was observed in *foxd3*-expressing embryos (R, arrowhead).

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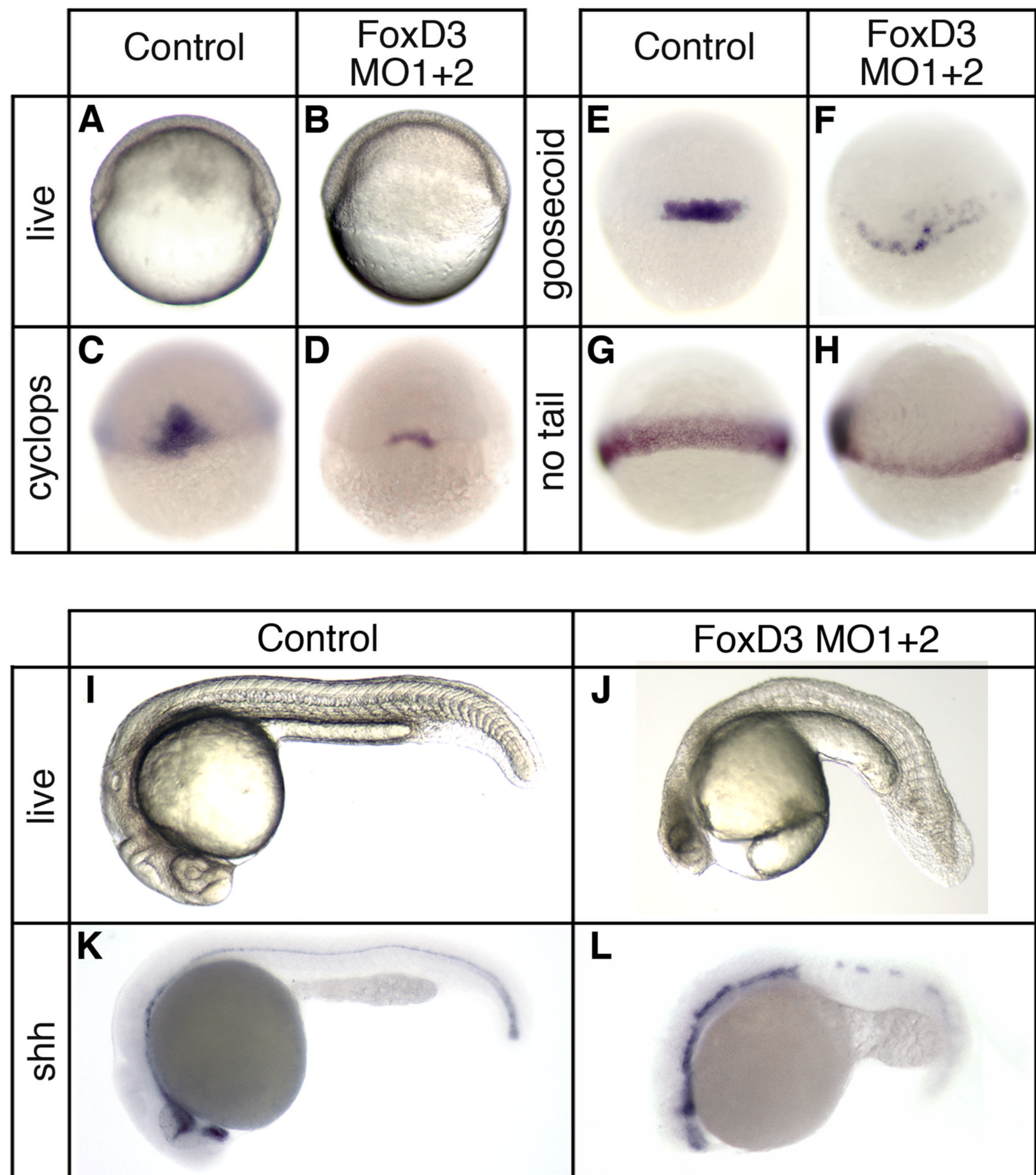

Fig. 2.

Foxd3 is essential for mesodermal development and axis formation. At the one-cell stage embryos were injected with a mixture of two *foxd3*-specific antisense morpholino oligonucleotides (MO1+2, total dosage 20ng) and analyzed at the shield stage (6hpf) (A–H) or at 24 hpf (I–L). (A,B) Live embryos (lateral views, dorsal right) showing the shield and blastoderm structure in uninjected (A) and Foxd3 knockdown (B) embryos. In situ hybridization of uninjected (C,E,G) and FoxD3 knockdown (D,F,H) embryos showing expression of *cyclops* (C,D), *goosecoid* (E,F), and *no tail* (G,H) (dorsal views). Uninjected (I,K) and Foxd3 knockdown (J,L) embryos at 24hpf. Shown are live embryos (I,J), and embryos analyzed by in situ hybridization for *sonic hedgehog* (Shh) (K,L) expression (lateral views with

anterior left). A *foxd3* oligonucleotide with multiple mismatches did not produce mesodermal or axial phenotypes at a dosage (20–40ng) equal to or greater than the perfect match oligonucleotides (data not shown). See Supplementary Materials for rescue experiments (Fig. S2).

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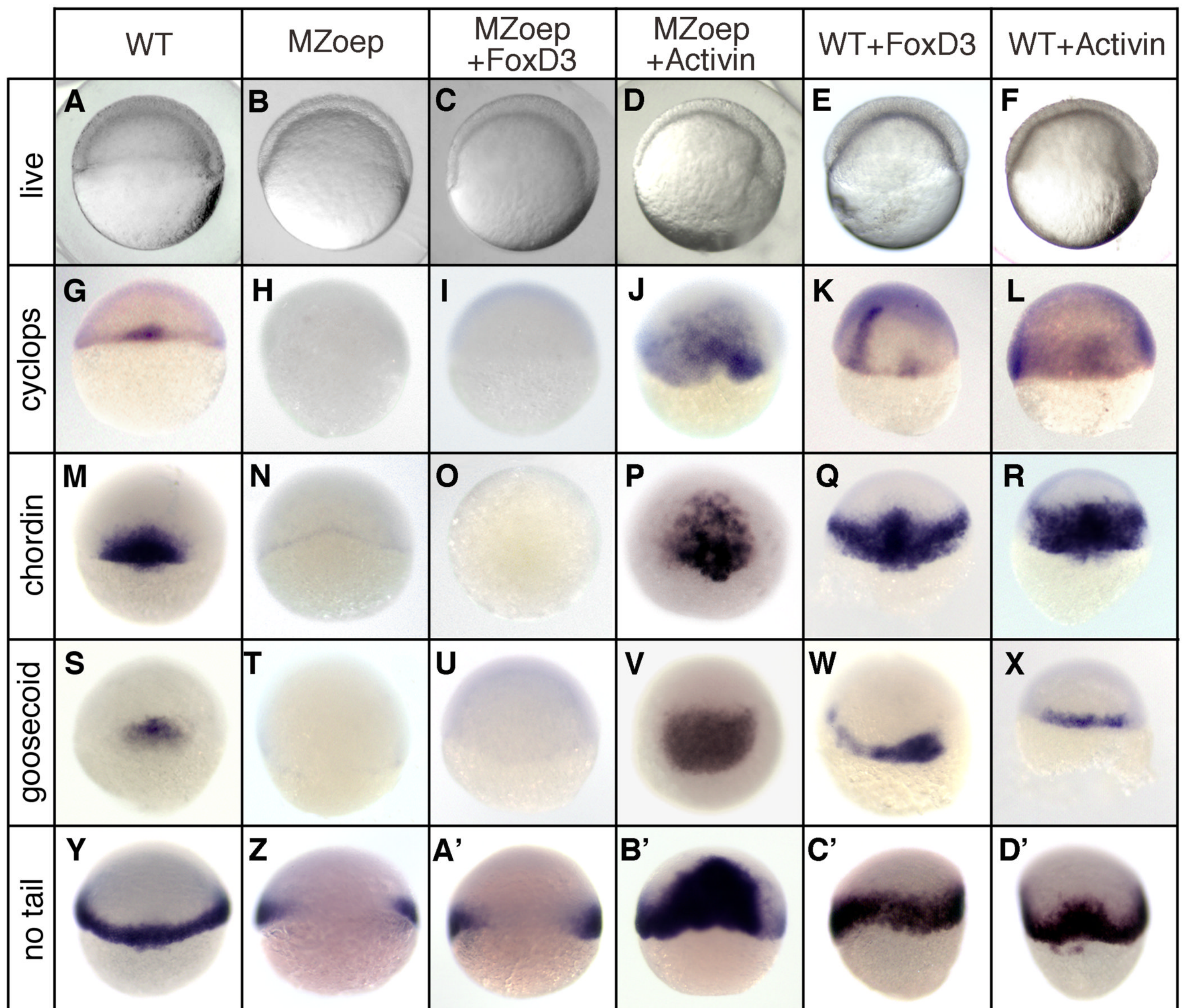

Fig. 3.

Foxd3 induction of mesoderm is dependent on an active Nodal signaling pathway. Wild-type (WT) or *MZoep* mutant embryos were injected at the one-cell stage with zebrafish *foxd3* (25pg) or *Xenopus ActivinβB* (25pg) mRNA and analyzed at the shield stage (6hpf). (A–F) Live embryos (lateral views, dorsal right) showing the shield and blastoderm structure in uninjected (A,B), *foxd3*-injected (C,E), and *Activin*-injected (D,F) embryos. In situ hybridization showing expression of *cyclops* (G–L), *chordin* (M–R), *goosecoid* (S–X), and *no tail* (Y–D') (dorsal views).

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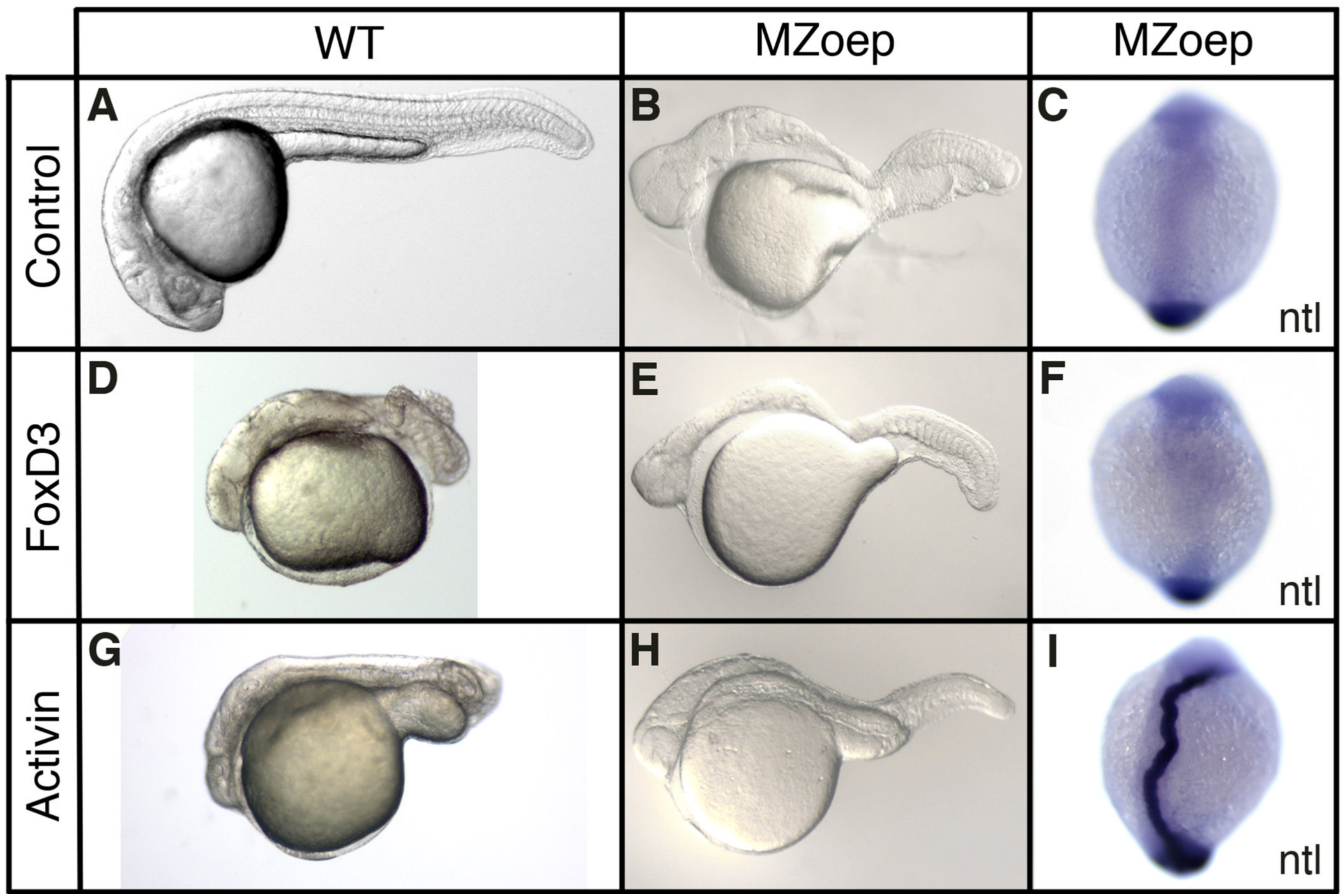

Fig. 4.

Foxd3 regulation of axis formation is dependent on the Nodal signaling pathway. Wild-type (WT) or *MZoep* mutant embryos were injected at the one-cell stage with zebrafish *foxd3* (25pg) or *Xenopus ActivinβB* (25pg) mRNA and analyzed at the 24hpf stage (A,B,D,E,G,H) or early somitogenesis stage (11hpf) (C,F,I). Uninjected (A,B), *foxd3*-injected (D,E), and *Activin*-injected (G,H) wild-type (A,D,G) and *MZoep* (B,E,H) embryos at 24hpf are shown (lateral views of live embryos with anterior left). In situ hybridization showing notochord expression of *no tail* (ntl) (C,F,I) in uninjected (C), FoxD3-injected (F), and Activin-injected (I) *MZoep* embryos at early somitogenesis stage (11hpf) (dorsal views with anterior up).

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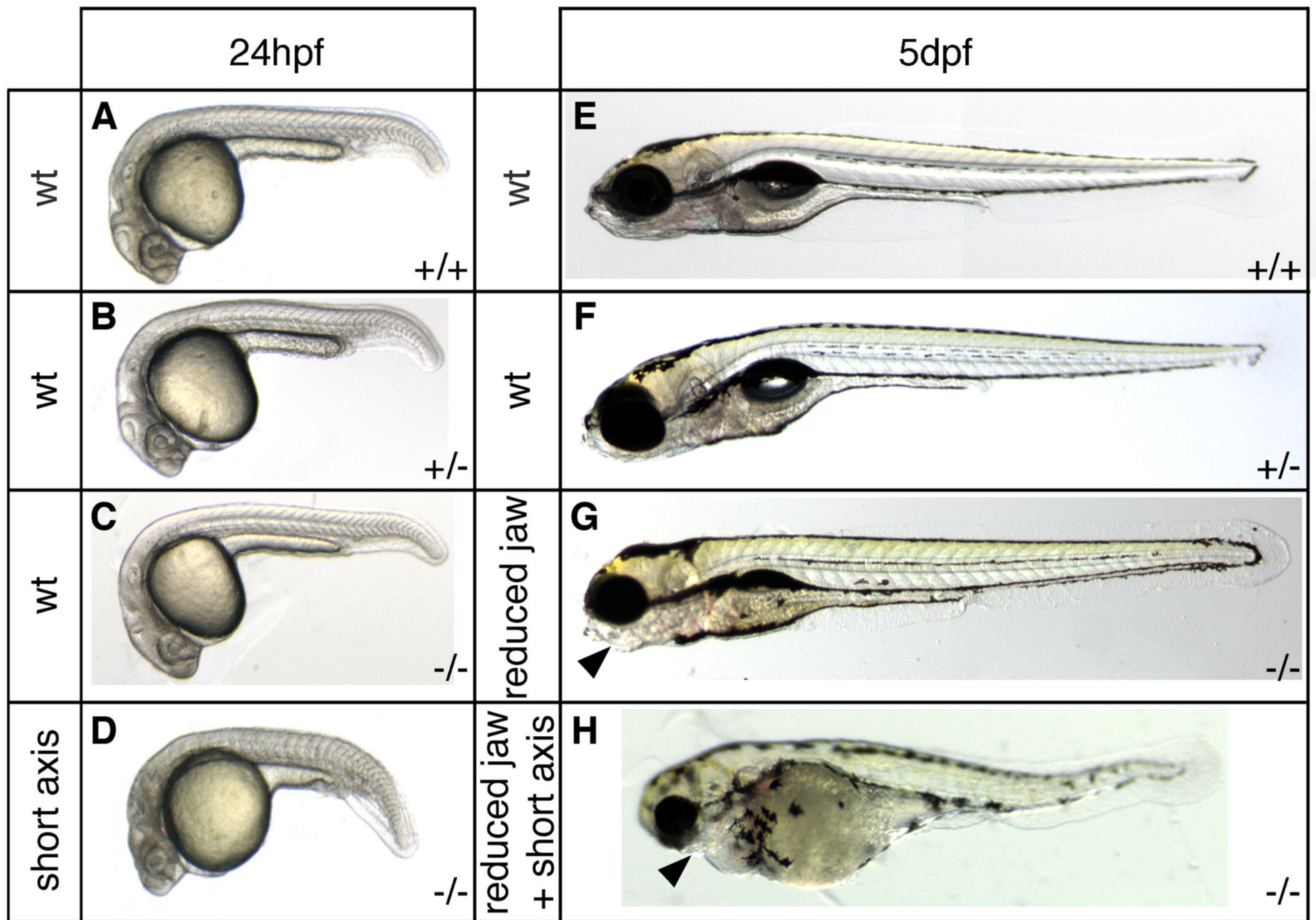

Fig. 5.

Axis formation defects in *sym1* embryos. Heterozygous *sym1* adults were crossed and axial development of progeny was analyzed at ~24hpf and ~5dpf. Representative samples of the two phenotypic classes observed at 24hpf (n=119): wild-type (90%) (A,B,C), and axial defects (10%) (D), and representative samples of the three phenotypic classes observed at 5dpf (n=494): wild-type (77%) (E,F), craniofacial defects (14%) (G), and craniofacial defects together with axial defects (9%) (H). Genotyping analysis indicated that at 24hpf (A–D) the wild-type phenotypic class consisted of genotypically wild-type (+/+), *sym1* heterozygotes (+/−), and *sym1* homozygotes (−/−), while axial defect class consisted only of *sym1* homozygotes (−/−). At 5dpf (E–H) the wild-type phenotypic class consisted of both genotypically wild-type (+/+) and *sym1* heterozygotes (+/−), while the craniofacial defect and the craniofacial with axial defect classes consisted only of *sym1* homozygotes (−/−). Arrowhead indicates reduced jaw structures in the craniofacial defect class (G) and in the craniofacial defects together with axial defect class (H). Phenotypic class is indicated to the left of each panel and genotype is shown on the bottom right of each panel.

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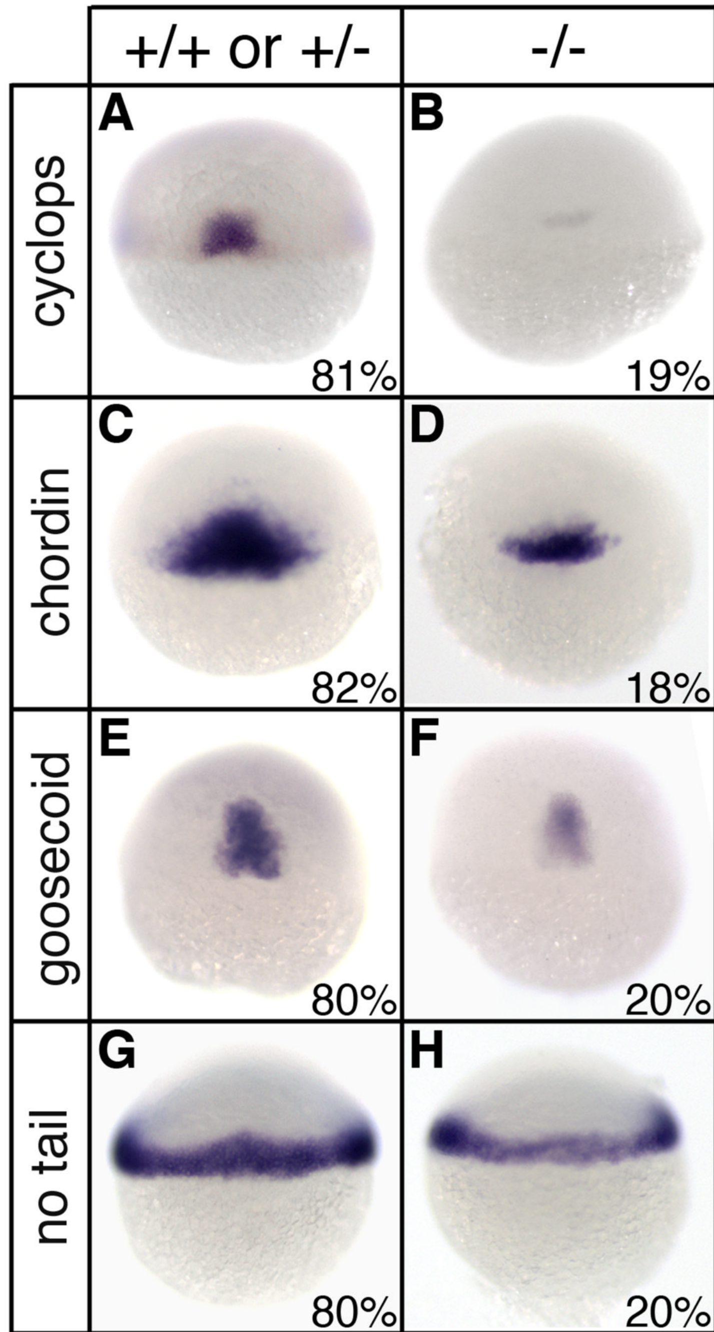

Fig. 6.

Reduced mesodermal gene expression in *sym1* mutants. Heterozygous *sym1* adults were crossed and progeny were examined for mesodermal gene expression by in situ hybridization at shield stage (6hpf). For *cyclops* (A,B), *chordin* (C,D), *goosecoid* (E,F), and *no tail* (G,H), two phenotypic classes were observed: wild-type (A,C,E,G) and reduced gene expression (B,D,F,H). For each mesodermal gene analyzed, distribution of progeny into the two classes was ~80% for the wild-type class and ~20% for the reduced class (exact distribution for each gene is indicated in lower right of each panel). Representative embryos were selected for genotyping analysis following in situ hybridization and this indicated that the wild-type phenotypic class consisted of both genotypically wild-type (+/+) and *sym1* heterozygotes (+/

−), while the reduced gene expression class consisted only of *sym1* homozygotes (−/−). Dorsal views are shown.

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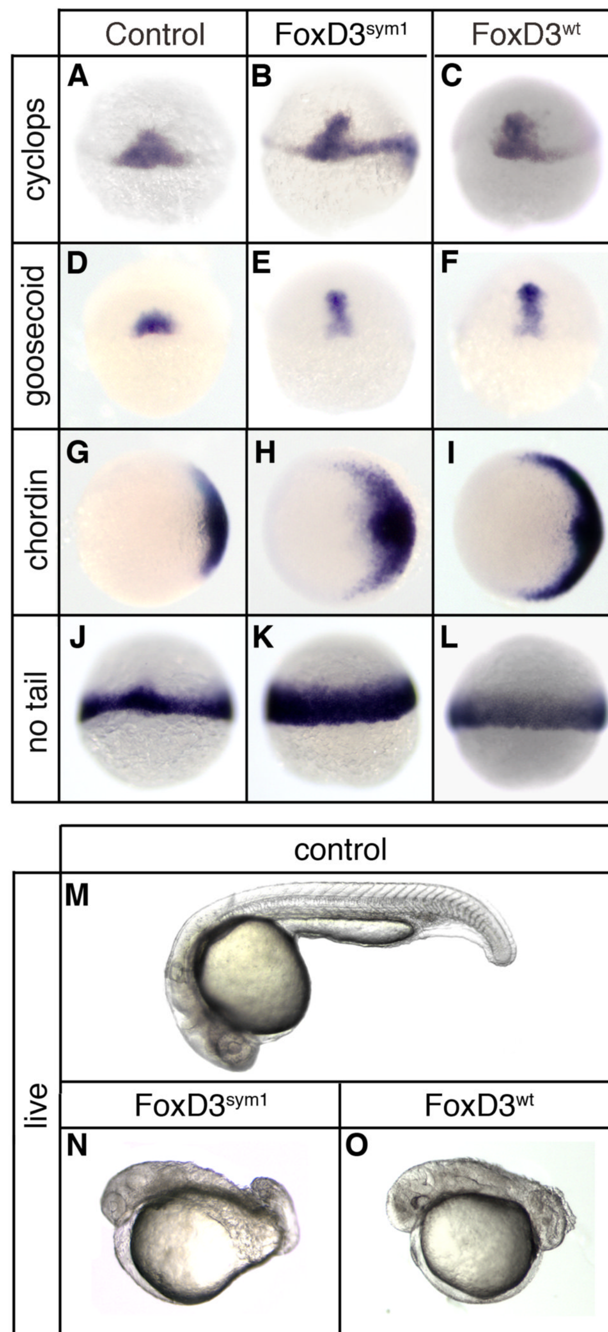

Fig. 7.

sym1 retains mesoderm induction and axis dorsalizing activity. At the one-cell stage embryos were injected with 150pg of *sym1* mRNA (Foxd3^{sym1}) (B,E,H,K,N) or 25pg of wild-type *foxd3*mRNA (Foxd3^{wt}) (C,F,I,L,O) and analyzed at the shield stage (6hpf) (A–L) or at 24 hpf (M–O). In situ hybridization at shield stage of uninjected (A,D,G,J), *sym1*-injected (B,E,H,K), and *foxd3*-injected (C,F,I,L) embryos showing expression of *cyclops* (A–C), *goosecoid* (D–F), *chordin* (G–H), and *no tail* (J–L). Views shown are dorsal (A–F, J–L) or animal with dorsal right (G–I). Uninjected (M), *sym1*-injected (N), and *foxd3*-injected (O) embryos at 24hpf. Shown are live embryos (lateral views with anterior left).

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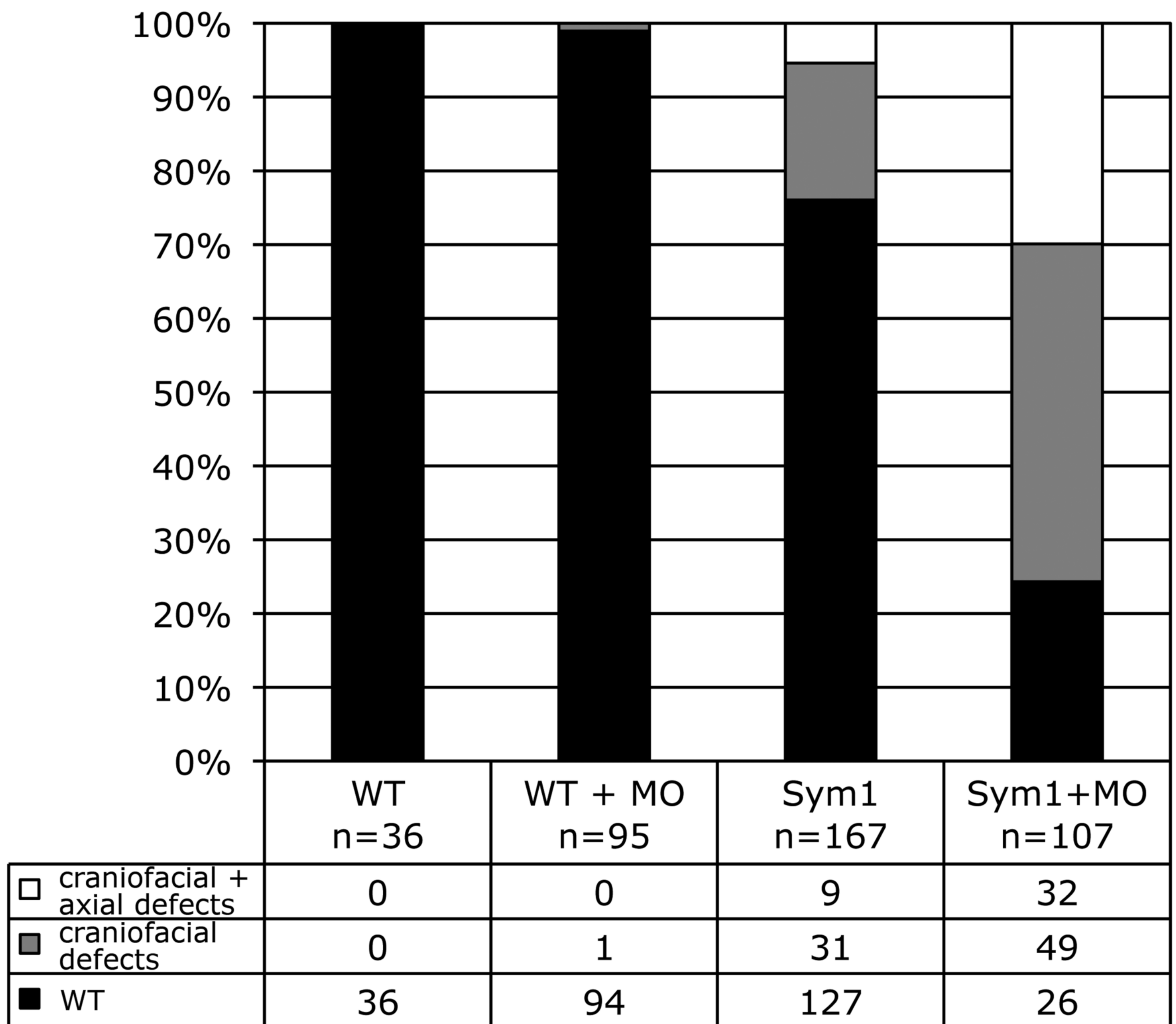

	WT n=36	WT + MO n=95	Sym1 n=167	Sym1+MO n=107
□ craniofacial + axial defects	0	0	9	32
■ craniofacial defects	0	1	31	49
■ WT	36	94	127	26

Fig. 8.

Foxd3 knockdown in *sym1* embryos enhances penetrance of mesodermal defects. Cross progeny of wild-type (WT) or *sym1* heterozygous (Sym1) adults were injected at the one-cell stage with a mixture of *foxd3*MO1 and *foxd3*MO2 (MO) (total dosage 2.5ng) and craniofacial and axial phenotypes were assessed at 5dpf. Quantification of the three phenotypic classes is shown as a percentage of total: Wild-type (wt, black), craniofacial defect (reduced jaw, gray), craniofacial defects together with axial defects (short, white).