Nodal regulates neural tube formation in the *Ciona intestinalis* embryo

Kaoru Mita* and Shigeki Fujiwara

Department of Materials Science, Kochi University, 2-5-1 Akebono-cho, Kochi-shi, Kochi 780-8520, Japan

* Corresponding author: Kaoru Mita

Tel: +81-88-844-8317 Fax: +81-88-844-8356

E-mail address: b06d3a05@s.kochi-u.ac.jp

Abstract

Overexpression of a *lefty* orthologue, *Ci-lefty*, caused a failure of neural tube closure in the protochordate ascidian *Ciona intestinalis*. The body bent dorsally and anterior-posterior elongation was inhibited. A similar phenotype was observed in embryos treated with SB431542, an inhibitor of Nodal receptors, suggesting that Ci-Lefty antagonized Nodal signaling as reported in other deuterostome species. Overexpression of *Ci-nodal* also resulted in a similar phenotype, suggesting that a correct quantity and/or a spatial restriction of Nodal signaling are important for the neural tube to form. In addition to known Ci-Nodal target genes, orthologues of *Zic* (*Ci-ZicL*) and *cdx* (*Ci-cdx*) were activated by Ci-Nodal. Expression of a dominant negative *Ci-cdx* caused defects in neural tube formation similar to those obtained on treatment with SB431542 or overexpression of *Ci-lefty*. A regulatory cascade composed of Ci-Nodal, Ci-ZicL, and Ci-Cdx may play an important role in neural tube formation in the *Ciona* embryo.

Keywords: Ascidian, neural tube formation, *lefty*, *nodal*, *cdx*, *ZicL*

Introduction

Inductive cellular interactions mediated by growth factors are important for embryogenesis. *nodal*, a member of the transforming growth factor-β (TGF-β) superfamily, has been identified exclusively in deuterostomes, including vertebrates, amphioxus, ascidians, and sea urchins (Chea et al. 2005). No counterpart is present in genomes of protostomes, such as *Caenorhabditis elegans* and *Drosophila melanogaster* (Boorman and Shimeld 2002). In vertebrates, Nodal signaling is required for multiple developmental processes, such as specification of the endoderm and mesoderm, neural patterning, and the formation of the anterior-posterior and left-right body axes (Whitman 2001; Schier and Shen 2000).

Recently, the expression and functions of *nodal* have been revealed in invertebrate deuterostomes. In ascidians, the first zygotic expression of *nodal* is detected in a pair of lateral blastomeres, named b6.5, as well as six vegetal blastomeres (A6.1, A6.3, and B6.1 pairs) at the 32-cell stage (Morokuma et al. 2002; Imai et al. 2004; see Fig. 1). The expression is maintained in descendant cells of b6.5 blastomeres until the gastrula stage (Morokuma et al. 2002; Imai et al. 2004). *Ci-nodal*, a *nodal*-related gene in the ascidian *Ciona intestinalis*, patterns the neural plate along the medial-lateral axis (Hudson and Yasuo 2005). *Ci-nodal* is also required for induction of a subset of muscle and notochord cell fates (Hudson and Yasuo 2005, 2006). A *nodal*-related gene in another ascidian species

Halocynthia roretzi is expressed only on the left side of the tailbud embryo (Morokuma et al. 2002). A left side-specific expression of *Pitx* is regulated by Nodal signaling in *H. roretzi* (Morokuma et al. 2002). During embryonic development in the sea urchin, Nodal signaling is essential for the establishment of oral-aboral and left-right axes (Duboc et al. 2004, 2005; Flowers et al. 2004).

lefty, also named *antivin*, belongs to the TGF-β superfamily and is known as an antagonist of Nodal in vertebrates (Juan and Hamada 2001). Orthologues of *lefty* have also been reported only in deuterostomes (Duboc et al. 2004; Fujiwara et al. 2002; Imai 2003). Overexpression of sea urchin *Antivin/Lefty* leads to an abnormal oral-aboral axis, indistinguishable from the phenotypes obtained by the injection of *nodal*-specific antisense morpholino oligonucleotides (MO) (Duboc et al. 2004). Therefore, Antivin/Lefty seems to inhibit Nodal signals in sea urchin embryos (Duboc et al. 2004). Expression of *Ci-lefty*, a *C. intestinalis* orthologue of *lefty*, starts from the 16-cell stage (Imai et al. 2004). *Ci-lefty* and *Ci-nodal* show a similar temporal expression pattern, suggesting an interaction. However, the function of *Ci-lefty* is still unclear.

In the present study, we examined the role of the Nodal signal in the formation of the neural tube in *C. intestinalis* embryos. In *Xenopus laevis*, a disturbance of *nodal*-related genes (*Xnr2*, *Xnr3* and *Xnr5*) causes an abnormal neural tube to form (Osada and Wright 1999; Onuma et al. 2002; Yokota et al. 2003). However, there is no report concerning a

possible role of Nodal function in neural tube formation in other deuterostome species. We show here that Ci-Lefty antagonizes Nodal signaling. When *Ci-lefty* was over-expressed in embryos, closure of the neural tube was incomplete and the body curved dorsally. Overexpression of *Ci-nodal* caused similar abnormal phenotypes. The inhibition or promotion of Nodal signaling affected the expression pattern of *Ci-cdx* and *Ci-ZicL* in the A-line neural cells at the late gastrula stage. Overexpression of a dominant negative form of *Ci-cdx* disturbed neural tube formation. These results suggest that Nodal signaling is involved in the formation of the neural tube in the *C. intestinalis* embryo.

Materials and methods

Biological materials

Adult *C. intestinalis* was collected around the Uranouchi Inlet near the Usa Marine Biological Institute. Juvenile adults were kindly provided by Kazuko Hirayama and Nori Satoh at Kyoto University. They were cultivated for a few months in corves. Gametes were surgically obtained from the gonoducts of mature adults. Eggs were inseminated with non-self sperm. Fertilized eggs were dechorionated with 0.05% actinase E (Kaken Pharmaceutical) and 1% sodium thioglycolate.

Transgenes

A genomic DNA fragment encompassing the 5' flanking region of *Ci-FoxD* was amplified by PCR using the primers 5'-AAAGTCTCGAGCACAAATATAGCGGTTTTGAAGTC-3' and 5'-AAAAACCCGGGCCATCATCACACAACGGATTCGAT-3'. The PCR product was digested with *Xho*I and *Xma*I, and inserted into a *lacZ*-containing plasmid vector, 72-1.27 (Corbo et al. 1997b). This construct was named *Ci-FoxD/lacZ*. A *Ci-nodal* cDNA fragment was amplified by reverse transcription-PCR using the *C. intestinalis* embryonic

RNA as a template. The primers used were 5'-ATATGATTTCTATGTTTAATATCGCTGC-3' and 5'-AAGAATTCTTATCTGCAACCGCATTCG-3'. The PCR product was inserted into pGEM-T (Promega). The cDNA was then excised from pGEM-T using *Eco*RI and *Apa*I, and inserted into pBluescript II SK+ (Stratagene). With this construct as a template, the translated region was amplified by PCR, using 5'-AATGTCCCGGGCGGCTGCTTTCGTCTTCACT-3' and 5'-AAGAATTCTTATCTGCAACCGCATTCG-3'. A *Ci-lefty* cDNA fragment was amplified by PCR using a cDNA clone (ID cicl007p08; Fujiwara et al. 2002) as a template. The primers used were 5'-TATGTCCCGGGCGAAGACTTTCTTACTTATA-3' and 5'-AAGAATTCTTACACACCAAATACACTGTCCAGGGA-3'. The *Ci-lefty* and *Ci-nodal* cDNA fragments were digested with *Xma*I and *Eco*RI. *Ci-FoxD/lacZ* was also excised with *Xma*I and *Eco*RI, and the *lacZ* translated region was substituted with the cDNA fragments. These constructs were named *Ci-FoxD/Ci-lefty* and *Ci-FoxD/Ci-nodal*, respectively.

For the construction of a dominant negative *Ci-cdx*, a *Ci-cdx* cDNA fragment was amplified by PCR using a cDNA clone (ID citb004h19; Satou et al. 2002b) as a template. The primers used were 5-ATGTTACGTAACCCCACCCCGGACCCGAA-3' and 5'-AAACTCGAGGTCATTTCCGTTGCCGTG-3'. The PCR product was digested with *Sna*BI and *Xho*I. The cohesive end produced by *Xho*I was ligated with a cDNA fragment encoding the repression domain of *Drosophila melanogaster* Engrailed (*EnR*) (Jaynes and

O'Farrell 1991). The other end of the *EnR* fragment was cut with *Eco*RI. *Ci-FoxD/lacZ* was digested with *Sma*I and *Eco*RI, and the *lacZ* translated region was substituted with the *Ci-cdx-EnR* fusion cDNA.

Nucleotide sequences were determined using a BigDye Terminator v3.1 cycle sequencing kit and PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). Plasmid DNA was purified using QIAGEN tip-100 (Qiagen). Transgenes were introduced into dechorionated *C. intestinalis* embryos by electroporation as described by Corbo et al. (1997b).

Drug administration

A stock solution of SB431542 (Sigma) was prepared at a concentration of 5 mM in dimethylsulfoxide (DMSO). The stock solution was diluted with filtered seawater. Embryos were continuously treated with 1~5 μM SB431542 from the 16-cell stage until they were fixed. Control embryos were reared in filtered seawater containing 0.1% (v/v) DMSO.

Whole-mount in situ hybridization

RNA probes were labeled with digoxigenin (DIG) as described by Nagatomo et al. (2003). Templates for the synthesis of the probes were cDNA clones obtained from the *C. intestinalis*

Gene Collection Release 1 (Satou et al. 2002b) and from cDNA clones analyzed by Fujiwara et al. (2002). The cDNA clones used were cicl002e04 (*Ci-ZicL*), cicl016e09 (*Ci-chordin*), cieg005o22 (*Ci-Delta-like*), cign044b23 (*Ci-Mnx*), cilv005f18 (*Ci-cdx*), cilv050a24 (*Ci-FoxC*), and citb028e11 (*Ci-ETR*) (Satou et al. 2002a; the *C. intestinalis* cDNA project web site: http://ghost.zool.kyoto-u.ac.jp/indexr1.html). A *Ci-sna* cDNA (Corbo et al. 1997a) and the above-mentioned pBluescript II SK+ containing the *Ci-nodal* cDNA were also used to prepare the probe. T3 RNA polymerase (Promega) was used for the synthesis of an antisense *Ci-nodal* probe. For the other clones T7 RNA polymerase (Takara) was used. The entire translated region of *lacZ* was obtained by cutting pSV-β-galactosidase vector (Promega) using *Bam*HI and *Hin*dIII. This fragment was inserted in pBluescript II SK+. T3 RNA polymerase was used for the synthesis of an antisense *lacZ* probe.

Embryos were fixed with 4% paraformaldehyde in 0.1 M MOPS (pH 7.5) and 0.5 M NaCl at 4ºC for more than 8 h, and stored in 80% ethanol at -30ºC. After equilibration with phosphate-buffered saline containing 0.1% Tween 20 (PBST), the embryos were treated with 2 μg/ml proteinase K in PBST for 30 min at 37ºC. They were re-fixed with 4% paraformaldehyde in PBS (PBST without Tween 20) at room temperature for 1 h. The specimens were washed with PBST, and incubated in a hybridization solution [50% formamide (FA), 5x SSC (750 mM NaCl and 75 mM sodium citrate, pH 7.0), 100 μg/ml yeast RNA (Roche), 5x Denhardt's solution (Eppendorf), 0.1% Tween 20] at 56˚C for 2 h.

Hybridization with DIG-labeled probes was performed at 56˚C for 18 h. The specimens were washed twice with 4x Wash (4x SSC, 50% FA, and 0.1% Tween 20) at 60˚C for 15 min, and twice with 2x Wash (2x SSC, 50% FA, and 0.1% Tween 20) at 60˚C for 15 min. They were immersed twice in Solution A [0.5 M NaCl, 10 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 0.1% Tween 20] at 60˚C for 10 min. The samples were incubated at 37˚C in Solution A containing 25 μg/ml RNaseA for 30 min. They were washed at 60˚C sequentially with Solution A, 2x Wash, 1x Wash (1x SSC, 50% FA, and 0.1% Tween 20), and a 1:1 mixture of 1x Wash and PBST for 10 min each. They were then washed four times with PBST at 60˚C for 10 min. The hybridization signal was immunologically detected essentially according to Fujiwara et al. (2002), except that blocking was carried out overnight at 4ºC and incubation with anti-DIG antibody (Roche) was performed for 1 h at room temperature.

Results

Morphological abnormality caused by overexpression of *Ci-lefty* and *Ci-nodal*

To analyze the roles of Nodal signaling in the *C. intestinalis* embryo, we caused *Ci-lefty* and *Ci-nodal* to be overexpressed. A single *lefty/antivin* orthologue was identified in the *C. intestinalis* genome (Figure S1; Hino et al. 2003). The gene's ID at the *C. intestinalis* genome project web site (http://genome.jgi-psf.org/Cioin2/Cioin2.home.html) is gw1.03q.154.1 (Dehal et al. 2002). We refer to this gene as *Ci-lefty* in this paper, since "*lefty*" is used in many species. Normal expression of *Ci-lefty* starts at the 16-cell stage in A5.1, A5.2, and B5.1 blastomeres (Imai et al. 2004; Fig. 1d). Normal expression of *Ci-nodal* starts at the 32-cell stage in A6.1, A6.3, B6.1, and b6.5 blastomeres (Imai et al. 2004; Fig. 1f, g). We decided to use the *Ci-FoxD* promoter/enhancer for driving transgene expression. *Ci-FoxD* is expressed at the 16-cell stage in A5.1, A5.2, and B5.1 blastomeres (Imai et al. 2004). The *C. intestinalis* genome contains two closely linked *Ci-FoxD* genes (gw1.08q.1850.1 and gw1.08q.1853.1) on chromosome 8 (Fig. 1a). We isolated a 3-kb genomic DNA fragment that contained a promoter region of gw.1.08q.1850.1, including putative transcription and translation initiation sites (Fig. 1a). Imai et al. (2002) showed that binding sites for a transcription factor Tcf were necessary for activation of a *FoxD* gene in

another ascidian species *Ciona savignyi*. Two of the Tcf-binding sites were also conserved in the promoter region of gw.1.08q.1850.1 (Fig. 1b). This DNA fragment was fused in-frame to *lacZ* and introduced into the 1-cell embryo by electroporation. The transgene, named *Ci-FoxD/lacZ*, was expressed in A5.1, A5.2, and B5.1 blastomeres of the 16-cell embryo, as revealed by in situ hybridization (Fig. 1c, d). The result indicates that the 3-kb upstream region recapitulates initial transcriptional activation of endogenous *Ci-FoxD* gene. The *lacZ* mRNA was detected in all the daughter cells at the 32-cell stage (Fig. 1e-g). This expression pattern was similar to that of endogenous *Ci-lefty* and *Ci-nodal* (Fig. 1c-g). The *lacZ* mRNA was expressed broadly in the vegetal hemisphere at later stages (Fig. 1h, i). The *lacZ* translated region of *Ci-FoxD/lacZ* was substituted with *Ci-lefty* or *Ci-nodal* cDNA fragments. The resultant genes were named *Ci-FoxD/Ci-lefty* and *Ci-FoxD/Ci-nodal*, respectively.

Embryos carrying the *Ci-FoxD/Ci-lefty* transgene showed a slight delay in gastrulation movement (data not shown). The blastopore finally closed, and the alignment of the notochord and muscle cells in the tail region looked normal (Fig. 2d-e). However, the neural tube did not close even at the middle tailbud stage (Fig. 2f-h). The head became round and the tail bent dorsally (Fig. 2f-g). The anterior-posterior elongation was inhibited (Fig. 2d-g). The *Ci-FoxD/Ci-nodal* transgene mediated a similar but slightly less severe phenotype (Fig. 2i-m). The body curved dorsally and the neural tube was open at the early

tailbud stage (Fig. 2i-l). However, gastrulation movement proceeded almost normally (data not shown). The tail nerve cord eventually closed, and only the brain region remained open at the middle tailbud stage (Fig. 2k-m).

Embryos treated with SB431542, an inhibitor of the Nodal receptors, ALK4, ALK5, and ALK7 (Inman et al. 2002), from the 16-cell stage showed dose-dependent defects in morphology (Fig. 2n-q). A high dose (5 μM) of SB431542 strongly inhibited gastrulation movement (Hudson and Yasuo 2005; Fig. 2q). Embryos treated with 1~2 μM SB431542 exhibited a morphology similar to that of embryos electroporated with the *Ci-FoxD/Ci-lefty* transgene (Fig. 2p, q). The neural tube did not close, anterior-posterior elongation was prevented, and the body curved dorsally (Fig. 2o-q). These results suggest that the overexpression of *Ci-lefty* specifically blocked Nodal signaling.

Alteration of gene expression pattern by overexpression of *Ci-lefty* and *Ci-nodal*

To confirm that the observed phenotypes were specific effects of transgene functions, we examined the expression pattern of known Ci-Nodal downstream target genes. Ci-Nodal activates expression of *Ci-Delta-like* and *Ci-chordin*, and represses that of *Ci-Mnx* in lateral/dorsal neural-lineage cells (Hudson and Yasuo 2005). The *Ci-FoxD/Ci-nodal* transgene mediated ectopic expression of *Ci-Delta-like* in the ventral nerve cord lineage (Fig.

3a). This result was similar to that observed in embryos injected with *Ci-nodal* mRNA (Hudson and Yasuo 2005). In contrast, *Ci-FoxD/Ci-lefty* mediated repression of *Ci-Delta-like* (Fig. 3a). A similar expression pattern was observed in embryos injected with either mRNA encoding a dominant-negative Ci-ALK4/5/7 or a *Ci-nodal*-specific MO and in embryos treated with SB431542 (Hudson and Yasuo 2005; Fig. 3a). *Ci-FoxD/lacZ* did not affect the expression of *Ci-Delta-like* (Fig. 3a). The expression of *Ci-sna* in the lateral and dorsal nerve cord lineage was similarly affected by overexpression of either *Ci-nodal* or *Ci-lefty* (data not shown). *Ci-chordin* is also expressed in the lateral/dorsal neural lineage as well as in presumptive notochord cells (Hudson and Yasuo 2005). Expression of *Ci-chordin* was activated by *Ci-FoxD/Ci-nodal* and repressed by *Ci-FoxD/Ci-lefty* in all the neural plate cells (Fig. 3b). *Ci-Mnx* is expressed in the ventral nerve cord-lineage cells in normal embryos (Hudson et al. 2003; Fig. 3c). *Ci-FoxC* is normally expressed in a9.35, a9.36, a9.39, and a9.40 blastomeres that give rise to the adhesive organ containing sensory neurons (Imai et al. 2006). The *Ci-FoxD/Ci-nodal* transgene suppressed the *Ci-Mnx* and *Ci-FoxC* expression, while *Ci-FoxD/Ci-lefty* mediated ectopic activation of *Ci-Mnx* and *Ci-FoxC* expression (Fig. 3c, d). The expression patterns of *Ci-chordin*, *Ci-Mnx*, and *Ci-FoxC* obtained on overexpression of *Ci-lefty* were similar to those obtained on treatment with SB431542 or injection of a *Ci-nodal*-specific MO (Hudson and Yasuo 2005; Imai et al. 2006; Fig. 3b-d). The results demonstrate that functional Ci-Nodal and Ci-Lefty proteins were

produced from the *Ci-FoxD*-driven transgenes, and Ci-Lefty antagonizes Nodal signaling in the *C. intestinalis* embryo.

Ci-Nodal activates Ci-ZicL and Ci-cdx expression in A-line nerve cord lineage cells

A *cdx* homologue (*Hrcad*) is required for the neural tube to form in *H. roretzi* (Katsuyama et al. 1999). Embryos injected with a *Hrcad*-specific phosphorothioate antisense oligo or dominant negative form show defects in neural tube formation and tail elongation, and their body curves dorsally (Katsuyama et al. 1999). This phenotype seems similar to that obtained by inhibiting Nodal signaling (see Fig. 2). We examined the expression of a *C. intestinalis* homologue of *cdx* (*Ci-cdx*) in transgenic embryos. *Ci-cdx* is expressed normally in A9.15, A9.29 and A9.31 blastomeres in late gastrulae (Imai et al. 2006). The *Ci-FoxD/Ci-lefty* transgene mediated repression of *Ci-cdx* expression (Fig. 3e). SB431542 had a similar effect on *Ci-cdx* expression (Fig. 3e). In contrast, the *Ci-FoxD/Ci-nodal* transgene induced ectopic *Ci-cdx* expression in A9.13 cells (Fig. 3e).

Expression of *Ci-cdx* in the late gastrula was repressed when the 1-cell embryo was injected with a *Ci-ZicL*-specific MO (Imai et al. 2006). *Ci-ZicL* is expressed normally in A9.15 and A9.29 nerve cord-lineage cells, as well as in some a-line and b-line neural-lineage cells (Imai et al. 2006). In the present study, we found that *Ci-ZicL* was weakly expressed in

A9.13, A9.14, A9.16, A9.30, and A9.32 nerve cord-lineage cells in normal embryos (Fig. 3f). In embryos carrying the *Ci-FoxD/Ci-lefty* transgene, expression of *Ci-ZicL* mRNA in the A-line cells was diminished, while that in the a-line and b-line cells was not affected (Fig. 3f). Suppression was particularly evident in A9.15 and A9.29 cells where the amount of *Ci-ZicL* mRNA became equivalent to that in the other A-line nerve cord-lineage cells (Fig. 3f). SB431542 similarly affected *Ci-ZicL* expression in the A-line cells (Fig. 3f). In contrast, the *Ci-FoxD/Ci-nodal* transgene mediated enhanced expression of *Ci-ZicL* in all the A-line nerve cord-lineage cells (Fig. 3f). *Ci-ZicL* is also expressed in A7.3, A7.4, A7.7, and A7.8 blastomeres at the 64-cell stage (Yagi et al. 2004; Fig. 3g). The A-line nerve cord cells derive from A7.4 and A7.8 blastomeres. Neither promotion nor inhibition of the Nodal signaling affected the expression pattern of *Ci-ZicL* at the 64-cell stage (Fig. 3g). The results indicate that Ci-Nodal is required for later expression of *Ci-ZicL* at the gastrula stage but not for early expression at the 64-cell stage.

To determine when Ci-Nodal is required for expression of *Ci-ZicL* and *Ci-cdx*, SB431542 treatment was started at different developmental stages. Expression of *Ci-ZicL* and *Ci-cdx* were diminished in the A-line nerve cord-lineage cells, when embryos were treated with SB431542 from the 16-cell stage, (Fig. 4). In contrast, SB431542 treatment from the 64-cell stage did not affect the expression pattern of both genes (Fig. 4). In this experiment, few embryos showed defects in neural tube formation (data not shown). These

results suggest that the Nodal signaling before the 64-cell stage is necessary for activation of *Ci-ZicL* and *Ci-cdx* at the gastrula stage. Similarly, expression of *Ci-sna*, *Ci-Delta-like* and other known target genes was not affected by SB431542 treatment after the 64-cell stage (data not shown).

A dominant negative *Ci-Cdx* inhibits neural tube formation

A dominant negative *Ci-cdx* cDNA was constructed. A cDNA fragment encoding the repression domain of *Drosophila* Engrailed (*EnR*) was fused in-frame to the 3' end of the *Ci-cdx* translated region. This fusion cDNA was ligated downstream to the *Ci-FoxD* promoter/enhancer. The resultant gene was named *Ci-FoxD/Ci-cdx-EnR*. In embryos carrying the *Ci-FoxD/Ci-cdx-EnR* transgene, the blastopore did not close even at the neurula stage (Fig. 5). A neural-lineage marker, *Ci-ETR*, is expressed in presumptive neural cells (Fig. 5), suggesting that neural differentiation partially occurred. However, these neural-lineage cells did not converge on the dorsal midline, indicating that the neural fold did not form (Fig. 5). At the early tailbud stage, the neural tube did not close and the body curved dorsally (Fig. 5). Differentiation of the notochord and muscle was normal (data not shown). The morphological abnormality of these embryos was similar to that obtained by SB431542 treatment (see Fig. 2p-q) or overexpression of *Ci-lefty* (see Fig. 2d-e). This

phenotype was also similar to that observed in the *H. roretzi* embryo, in which the Hrcad function was suppressed (Katsuyama et al. 1999).

Discussion

Ci-Lefty antagonizes Nodal signaling

Lefty functions as a competitive inhibitor of Nodal in vertebrates (Juan and Hamada 2001). A similar function of a Lefty orthologue was suggested in the sea urchin (Duboc et al. 2004). In the *C. intestinalis* embryo, the *Ci-FoxD/Ci-lefty* transgene and SB431542 had almost the same effect on the spatial expression of Nodal target genes. These altered expression patterns are consistent with those obtained by injection of a *Ci-nodal*-specific MO (Hudson and Yasuo 2005; Imai et al. 2006). These results suggest that Ci-Lefty antagonizes Nodal signaling in the *C. intestinalis* embryo. This is also supported by the observation that the *Ci-FoxD/Ci-lefty* transgene and SB431542 caused a similar morphological abnormality. Pasini et al. (2006) observed a similar malformation by overexpression of *Ci-lefty* in the ectodermal region. The function of Lefty as an antagonist of Nodal seems conserved in deuterostomes.

Ci-Nodal activates Ci-ZicL and Ci-cdx in A-line lateral nerve cord lineage cells

The *Drosophila caudal* gene and its orthologues, designated *cdx*, in vertebrates play

an important role in the establishment of the posterior structures in the embryo (Epstein et al. 1997; Isaacs et al. 1998; Macdonald and Struhl 1986; Marom et al. 1997; van den Akker et al. 2002). Expression of *cdx* in the posterior region of developing embryos is regulated by extracellular signals, such as retinoic acid, Wnt, and FGF (Lohnes 2003). There is no report to suggest that Nodal regulates the expression of *cdx*. In the *C. intestinalis* embryo, injection of a *Ci-SoxC*-specific MO induced ectopic expression of *Ci-nodal* and *Ci-cdx* in A-line nerve cord-lineage cells (Imai et al. 2006). Based on this observation, Ci-Nodal was speculated to be a candidate activator of *Ci-cdx* expression (Imai et al. 2006). In the present study, *Ci-FoxD/Ci-nodal* mediated ectopic activation of *Ci-cdx*, while *Ci-FoxD/Ci-lefty* and SB431542 repressed *Ci-cdx*. From these results, we conclude that Ci-Nodal is an activator of *Ci-cdx* expression.

Transcriptional activation of *Ci-ZicL* in A-line cells is intricately regulated (Anno et al. 2006). *Ci-ZicL* is first activated in A6.2 and A6.4 cells at the 32-cell stage (Yagi et al. 2004). The expression becomes weak at the early gastrula stage and is resumed at the middle/late gastrula stage (Yagi et al. 2004; Anno et al. 2006). The present study demonstrated that only this re-activation requires Nodal signaling. A *Ci-ZicL*-specific MO suppressed the expression of *Ci-cdx* at the late gastrula stage, suggesting that Ci-ZicL is an activator of *Ci-cdx* expression (Imai et al. 2006). This raised the possibility that Ci-Nodal activates *Ci-ZicL*, which in turn activates *Ci-cdx*. Overexpression of *Ci-lefty*, as well as

treatment with SB431542, diminished *Ci-ZicL* expression in A9.15 and A9.29 blastomeres where *Ci-cdx* is normally expressed. This is consistent with our hypothesis, although this does not exclude the possibility of direct activation of *Ci-cdx* by Ci-Nodal.

Role for Ci-Nodal in neural tube formation

In the present study, both the inhibition and accentuation of Nodal signaling caused defects in neural tube formation. This suggests that the appropriate dose and/or spatial restriction of the Ci-Nodal influence are important for normal morphogenesis. We propose two possible mechanisms that mediate Ci-Nodal-dependent neural tube formation. (1) Ci-Nodal induces the lateral/dorsal fate and suppresses the ventral fate in the nerve cord (Hudson and Yasuo 2005). Consequently, both the inhibition and enhancement of Nodal signaling lead to interference in the pattern formation of the neural plate (Hudson and Yasuo 2005). This may affect the morphogenetic movement required for the neural groove to form. (2) Ci-Cdx may be responsible for the morphogenesis of the nerve cord. Inhibition of the function of Ci-Cdx caused defects in gastrulation, anterior-posterior elongation, and neural tube formation. This phenotype was similar to that observed in embryos caused by SB431542 treatment or overexpression of *Ci-lefty*. These two possibilities are not mutually exclusive, although the involvement of Ci-Cdx was demonstrated. Early Ci-Nodal target

genes, such as *Ci-Delta-like* and *Ci-sna*, may activate *Ci-cdx* in late gastrulae. Functional analysis of these early target genes is important for understanding of the entire molecular network that regulates neural tube morphogenesis.

Ci-cdx is also expressed in the tail epidermis (Imai et al. 2004). However, in the present study, the *Ci-cdx-EnR* transgene was expressed under the control of the *Ci-FoxD* enhancer that is not activated in the epidermis. This suggests that the Ci-Cdx function is required in the nerve cord lineage. Ci-Nodal is also necessary for specification of the notochord and muscle (Hudson and Yasuo 2006). However, possible involvement of these mesodermal tissues in neural tube formation is now unclear. Katsuyama et al. (1999) proposed a hypothesis that Hrcad regulates cell motility and intercalation in the neuroectoderm and notochord, which is essential to body elongation. Convergence and intercalation in the neuroectoderm and notochord are important for closure of the blastopore and neural tube in vertebrates (Wallingford and Harland 2001). It is therefore likely that the neural tube's formation depends, at least in part, on a regulatory cascade composed of Ci-Nodal, Ci-ZicL, and Ci-Cdx, in addition to genes involved in the medial-lateral patterning of the neural plate.

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Figure legends

Fig. 1 The *Ci-FoxD* promoter/enhancer activity. (a) The cluster of two *Ci-FoxD* genes (gw1.08q.1850.1 and gw1.08q.1853.1) on chromosome 8 of the *C. intestinalis* genome. Exons are indicated by boxes. The genomic DNA fragment used for constructing transgenes is shown by a bracket. (b) The nucleotide sequence of the enhancer element of *Cs-FoxD* (Imai et al. 2002) was compared with the corresponding region of *Ci-FoxD*. Tcf-binding elements (TBE1, TBE2, and TBE3) were indicated by green shade. (c, e, h and i) Expression of the *Ci-FoxD/lacZ* transgene was examined by in situ hybridization using a *lacZ*-specific probe. Stained embryos are oriented to display the vegetal hemisphere, with the anterior side up. (c) The 16-cell embryo. (d) A schematic diagram of the 16-cell embryo. Blastomeres that express the *Ci-FoxD/lacZ* transgene are shown in pink. Those that express the endogenous *Ci-lefty* gene are indicated by red circles (left) and names (right). (e) The 32-cell embryo. (f and g) Schematic diagrams of the 32-cell embryo, viewed from the vegetal (f) and animal (g) hemispheres. Blastomeres that express *Ci-FoxD/lacZ* are shown in pink. Those that express *Ci-nodal* and *Ci-lefty* are indicated by blue and red circles, respectively (left). They are also indicated by names (right). (h) Early gastrulae. (i) Late gastrulae.

Fig. 2 Morphological abnormality caused by inhibition or enhancement of Nodal signaling. The anterior side of the embryo is oriented to the left. Arrows indicate the neural groove that failed to close. (a-m) Phenotypes observed in embryos electroporated with *Ci-FoxD/lacZ* (a-c), *Ci-FoxD/Ci-lefty* (d-h), and *Ci-FoxD/Ci-nodal* (i-m). The dorsal side is up in all panels, except for (b, e, and j), where the dorsal side is shown. mu, muscle; no, notochord. (a, b, d, e, i, j) Early tailbud embryos. (c, f, g, h, k, l, m) Middle tailbud embryos. (h and m) High magnification view of the boxed region in (g) and (l), respectively. (n-q) SB431542-treated embryos, with the dorsal side up. (n) The DMSO-treated control embryo. (o-q) Embryos treated with 1 μM (o), 2 μM (p), or 5 μM (q) SB431542.

Fig. 3 Spatial expression pattern of Ci-Nodal target genes. The expression of *Ci-Delta-like* (a), *Ci-chordin* (b), *Ci-Mnx* (c), *Ci-FoxC* (d), *Ci-cdx* (e), and *Ci-ZicL* (f and g) was examined in embryos that expressed *Ci-FoxD/lacZ*, *Ci-FoxD/Ci-nodal*, or *Ci-FoxD/Ci-lefty*. The expression pattern was also examined in SB431542-treated embryos. Stained embryos are oriented to display the vegetal hemisphere. Embryos in Panel a are at the early gastrula stage, and those in Panel g are at the 64-cell stage. Those in the other panels are at the late gastrula stage, with the anterior side up. Red arrowheads indicate ectopic expression, while blue arrowheads indicate blastomeres in which the expression was diminished. Asterisks indicate *Ci-chordin* expression in the presumptive notochord cells, which was not dependent

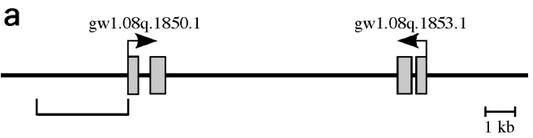
on the Nodal signal.

Fig. 4 Requirement of Ci-Nodal at different developmental stages for activation of the *Ci-cdx* and *Ci-ZicL* expression. The expression of *Ci-cdx* (a) and *Ci-ZicL* (b) was examined in embryos treated with SB431542 from the 16- or 64-cell stage. Control embryos were treated with DMSO from the 16-cell stage. Embryos are oriented to show the vegetal hemisphere, with the anterior side up. Blue arrowheads indicate blastomeres in which the expression was diminished.

Fig. 5 Effect of the *Ci-cdx-EnR* transgene on neural tube formation. Expression of a neural-lineage marker, *Ci-ETR*, was examined by in situ hybridization. The anterior side of the embryo is oriented to the left. (a) Embryos carrying the *Ci-FoxD/lacZ* transgene. (b) Embryos carrying the *Ci-FoxD/Ci-cdx-EnR* transgene. bp, blastopore.

Figure S1. Characterization of a *lefty/antivin* orthologue in *C. intestinalis*. (a) The amino acid sequence predicted from the *Ci-lefty* cDNA is aligned with other Lefty protein sequences obtained from mouse (m), zebrafish (z), and the sea urchin *Paracentrotus lividus* (Pl). The putative signal peptide region is indicated by a bar. Cysteine residues conserved among Lefty proteins are shown by green shade. An arrowhead indicates the position where

cysteine is conserved in other TGF-β superfamily proteins but not in Lefty proteins. Putative protein product of *Ci-lefty* consists of 385 amino acids. A characteristic configuration of seven cysteine residues, called a cysteine knot, is conserved among TGF-β superfamily members (Kingsley 1994). Among the family, only the Lefty/Antivin group lacks the fourth cysteine residue (Thisse and Thisse 1999; Juan and Hamada 2001). A predicted amino acid sequence of Ci-Lefty showed the Lefty/Antivin-type cysteine knot. The N-terminal signal peptide sequence was observed in the Lefty proteins, including Ci-Lefty. (b) A phylogenetic tree of TGF-β superfamily proteins constructed by the maximum likelihood method (Felsenstein 1981), using full-length amino acid sequences. Zebrafish and mouse TGF-β proteins were defined as an outgroup. Ci, *C. intestinalis*; m, mouse; Pl, *P. lividus*; z, zebrafish. Orthologues of *lefty* formed a monophyletic cluster. Within the cluster, the branching pattern was consistent with widely accepted phylogenetic relationships of deuterostome groups. Paralogous subgroups in vertebrates (e.g. Lefty1 and Lefty2 in the mouse) diverged within the vertebrate clade.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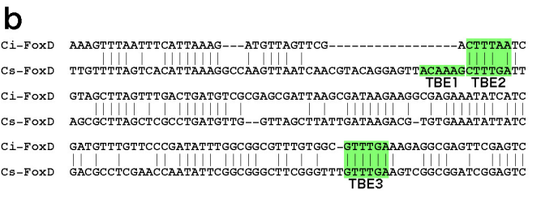
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TBE1 TBE2
Ci-FoxD GTAGCTTAGTTTGACTGATGTCGCGAGCGATTAAGCGATAAGAAGGCGAGAAATATCATC
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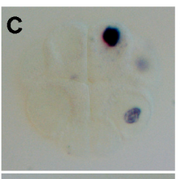
TBE3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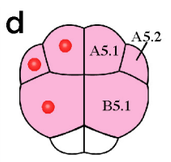
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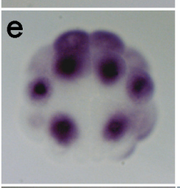
A5.2
B5.1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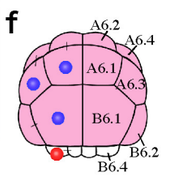
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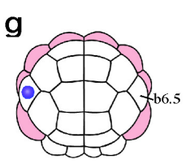
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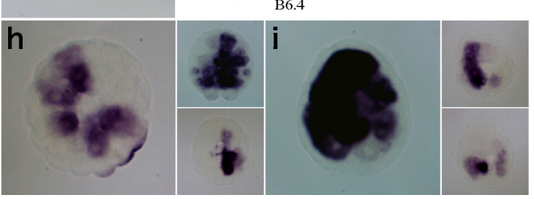
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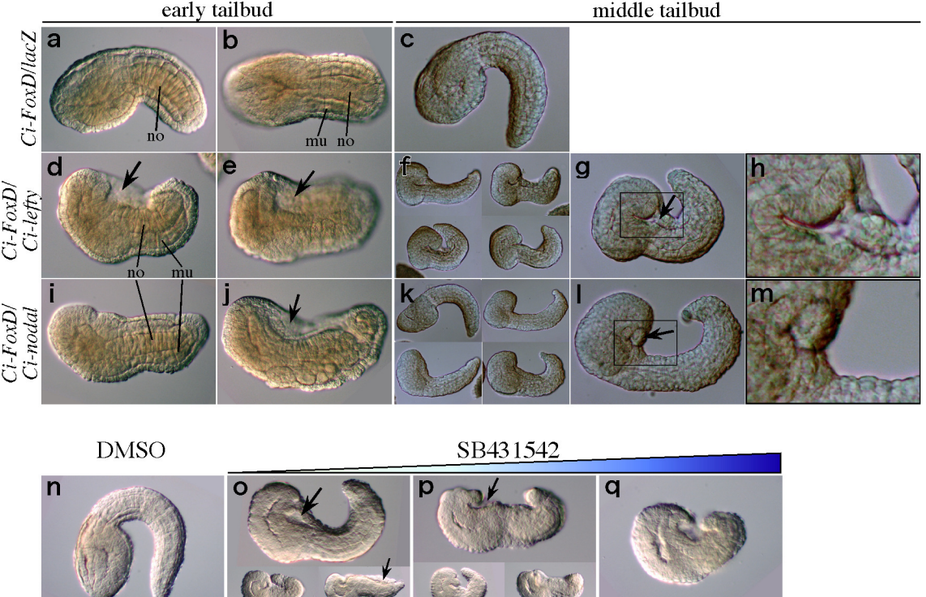
early tailbud
middle tailbud
Ci-FoxD/lacZ
Ci-FoxD/
Ci-lefty
Ci-FoxD/
Ci-nodal
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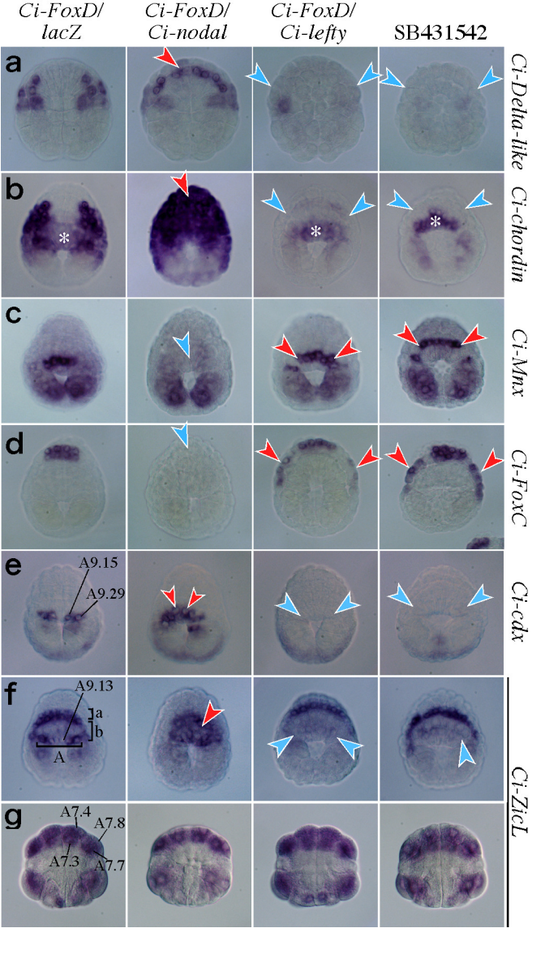
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Ci-Mnx
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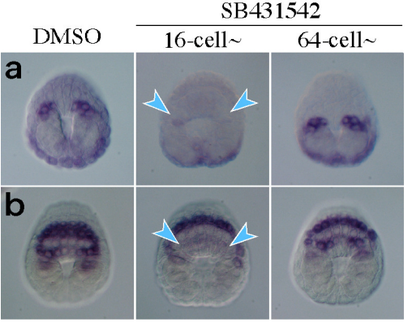
Ci-ZicL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64-cell~
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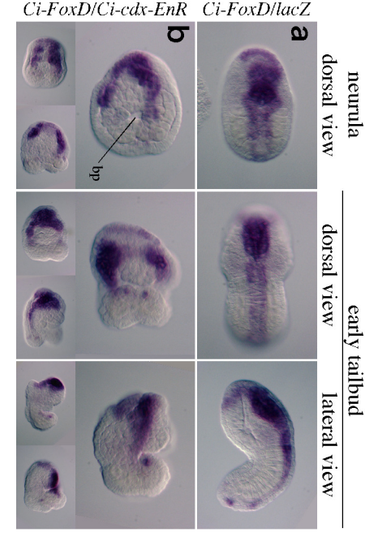
b
Ci-cdx
Ci-Zic1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early tailbud
dorsal view
lateral view
Ci-FoxD/lacZ
Ci-FoxD/Ci-cdx-EnR
a
b
bp

a

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mLefty-2  MEQRLPPNSELVQAVLRLFQEPVPRTAL-----------------------RRFERLS
Ci-lefty  MEGKIPDKTTITMAELRLFKKLPNHSRLGAYTVKHRATSGS------RNDVERPSVRRSP
Pl_Lefty  ISS-IPEGSEVIMAELKVYKERPNHSIFKPEGEEGEAPHSNNHDHVHSALVSIKQLVDQE

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Pl_Lefty  ALHIDPIEG--GHHAQQVVDEMVFATDFFPETPDSPDSRPVLVIYTTKYAPASDEPNECR

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Ci-lefty  SASRHRRHRKCCREKRFISFREMEWAKDWIIEPSGYDAYQCAGGCSSSRRKNSK------
Pl_Lefty  YEGE--EEHRCCRRRKYVDFRDLSWTSRWIIEPAGFEAFDCYGPCHNPRSRHIRDVFRLP

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Ci-lefty  ----------------RSPRSCTVAESSSLPVMYLVKD-GDGTKVEVSEFPNMVVEKCAC
Pl_Lefty  FFGASSSGSSIFGAGSGGHRTCGVSRSSSLPMMYLSETPSGTVELKVEEIPNMIVEDCGC

zLefty-1  AMDNISVV-----
zLefty-2  SMDKIPPV-----
mLefty-1  ASDGALIPRRLQP
mLefty-2  ASDGALIPRGIDL
Ci-lefty  SLDSVFGV------
Pl_Lefty  QL-----------
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b