**Ciona intestinalis** Noto4 contains a phosphotyrosine interaction domain and is involved in the midline intercalation of notochord cells

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ABSTRACT  Brachyury plays a pivotal role in the notochord formation in ascidian embryos. *Ciona intestinalis* Noto4 (Ci-Noto4) was isolated as a gene downstream of Ci-Bra. This gene encodes a 307 amino-acid protein with a C-terminal phosphotyrosine interaction domain (PTB/PID). Expression of Ci-Noto4 commences at the neural plate stage and is specific to notochord cells. Suppression of Ci-Noto4 levels with specific antisense morpholino oligonucleotides resulted in the formation of two rows of notochord cells owing to a lack of midline intercalation between the bilateral populations of progenitor cells. In contrast, overexpression of Ci-Noto4 by injection of a Ci-Bra(promoter):Ci-Noto4-EGFP construct into fertilized eggs disrupted the localization of notochord cells. Ci-Noto4 overexpression did not affect cellular differentiation in the notochord, muscle, mesenchyme, or nervous system. Analysis of Ci-Noto4 regions that are responsible for its function suggested significant roles for the PTB/PID and a central region, an area with no obvious sequence similarity to other known proteins. These results suggested that PTB/PID-containing Ci-Noto4 is essential for midline intercalation of notochord cells in chordate embryos.

KEY WORDS: notochord cell, morphogenetic movement, Ci-Noto4, Ci-Bra down-stream gene

**Introduction**

Notochord formation in ascidian embryos provides an excellent experimental system to explore cellular and molecular mechanisms involved in the development of chordate midline organs and/or the formation of chordate body plan (reviewed by Satoh, 2003; Jiang and Smith, 2007). In ascidian embryos, the notochord forms in the larval midline tail and consists of exactly 40 cells (Satoh, 1994). The lineage of these cells has been extensively detailed (Nishida, 1987). At the 110-cell stage, five pairs of progenitor cells are positioned at both right and left sides of the bilaterally symmetrical embryo. These cells converge toward the midline during gastrulation and neurulation, intercalate with each other, and extend anteroposteriorly to form a notochord consisting of a single row of cells (Miyamoto and Crowther, 1985; Munro and Odell, 2002). Later, the cells produce intracellular and/or intercellular vacuoles, which increase the volume of notochord and are required for elongation of the tail (Cloney, 1990; Jiang and Smith, 2007).

The T-box transcription factor *Brachyury* (*Bra*) plays a pivotal role in the specification of embryonic cells to a notochord fate in ascidian embryos (reviewed by Satoh, 2003; Passaneck and Di Gregorio, 2005). The upstream regulatory network of genes that leads to the transcriptional activation of the *Ciona Brachyury* gene (*Ci-Bra*) (Imai et al., 2006; Shoguchi et al., 2008) and the genes

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downstream of Ci-Bra that govern convergence, intercalation, extension, and differentiation of notochord cells have been extensively studied (Takahashi et al., 1999, Hotta et al., 2000; Jiang and Smith, 2007; Hotta et al., 2007; Hotta et al., 2008; Shoguchi et al., 2008; Yamada et al., 2009). Subtraction screening of normal embryos from Ci-Bra overexpressing embryos allows us to identify and characterize nearly 450 genes that are expressed in developing notochord cells directly or indirectly downstream of Ci-Bra (Takahashi et al., 1999; Hotta et al., 2000; Hotta et al., 2008). Knockdown experiments with specific antisense morpholino oligonucleotides (MO) revealed approximately 10 genes downstream of Ci-Bra that are involved in convergent and extension movements by notochord cells (Hotta et al., 2007).

In a previous study, we examined the function of Ci-fibrn, a gene downstream of Ci-Bra that encodes a fibrinogen-like protein (Yamada et al., 2009). Interestingly, although Ci-fibrn mRNA is restricted to notochord cells, the Ci-fibrn protein product is distributed outside of the notochord cells in a filamentous network that resembles the notochord sheath. We previously showed that the correct distribution of Ci-fibrn was dependent on Notch signaling from the overlying neural tube (Yamada et al., 2009). In addition, Ci-fibrn interacts directly with the extracellular domain of Ci-Notch. Aberrant Ci-fibrn distribution caused abnormal positioning of neuronal cells (Yamada et al., 2009).

In the present study, we focused on the function of another gene that is downstream of Ci-Bra, Ci-Noto4, because preliminary experiments suggested that Ci-Noto4 is involved in the midline intercalation of notochord cells. Eighteen genes that were expressed in the notochord and acted downstream of Ci-Bra showed no or weak similarity with other reported genes; we have named these genes Ci-Noto1 to Ci-Noto18 (Hotta et al., 2000; Hotta et al., 2008). Ci-Noto4 is expressed exclusively in notochord cells beginning at the neural plate stage, and its expression persisted until the tailbud stage (see Fig. 1L of Hotta et al., 2000). Ci-Noto4 cDNA consists of 1472 nucleotides with a predicted polypeptide product of 307 amino acids (Fig. 1A; Hotta et al., 2000). Moreover, Ci-Noto4 showed weak similarity to the myb-related protein PP2 from the moss Physcomitrella patens (Hotta et al., 2000). The present search for similarities between mouse proteins and Ci-Noto4 noted some homology with phosphotyrosine interaction domain containing 1 (Accession No. 98496). Notably, Ci-Noto4 contains a short PTB/PID (aa 120-135) and a C-terminal PTB/PID (aa 242-299) (Fig. 1B).

### Functional suppression of Ci-Noto4 disrupted the midline alignment of notochord cells

To examine the possible function of Ci-Noto4, we knocked down its expression levels using MO specific for Ci-Noto4. The division and movements of the notochord cells were examined after Ci-Bra (promoter): EGFP was coinjected into fertilized eggs. Embryos that developed from eggs injected with control MO developed normally and contained GFP-positive notochord cells in a single row in the center of the tail by the late tailbud stage (Fig. 2A). In contrast, Ci-Noto4 MO-injected embryos did not contain a single row of notochord cells (Fig. 2B-E). The progenitor cells on the left (yellow triangle) and right (white triangle) of the embryo independently converged, intercalated, and partially extended, but the left and right progenitor populations did not intercalate at the midline into a single row of cells, resulting in two rows of notochord cells (Fig. 2F-H).

### Results and Discussion

#### Characterization of Ci-Noto4

In our previous study, we showed that Ci-Noto4 cDNA consists of 1472 nucleotides with a predicted polypeptide product of 307 amino acids (Fig. 1A; Hotta et al., 2000). Moreover, Ci-Noto4 showed weak similarity to the myb-related protein PP2 from the moss Physcomitrella patens (Hotta et al., 2000). The present search for similarities between mouse proteins and Ci-Noto4 noted some homology with phosphotyrosine interaction domain containing 1 (Accession No. 98496). Notably, Ci-Noto4 contains a short PTB/PID (aa 120-135) and a C-terminal PTB/PID (aa 242-299) (Fig. 1B).

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### Predicted amino-acid sequence of Ci-Noto4, which consists of 307 amino acids. The short PTB/PID and C-terminal PTB/PID are denoted in blue. B) Comparison of the amino-acid sequences of the Ci-Noto4 C-terminal PTB/PID region with those of other PTB/PID proteins. The identity (%) of the PTB/PID amino-acid residues between Ci-Noto4 and other proteins encoding PTB/PID is shown.

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**Fig. 1. Characterization of Ci-Noto4.**

A) Predicted amino-acid sequence of Ci-Noto4, which consists of 307 amino acids. The short PTB/PID and C-terminal PTB/PID are denoted in blue. B) Comparison of the amino-acid sequences of the Ci-Noto4 C-terminal PTB/PID region with those of other PTB/PID proteins. The identity (%) of the PTB/PID amino-acid residues between Ci-Noto4 and other proteins encoding PTB/PID is shown.

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**Table 1. Comparison of the amino-acid sequences of the Ci-Noto4 C-terminal PTB/PID region with those of other PTB/PID proteins.**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Identity (%)</th>
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<tbody>
<tr>
<td>Mouse PID containing protein 1</td>
<td>38%</td>
</tr>
<tr>
<td>Horse PID containing protein 1</td>
<td>38%</td>
</tr>
<tr>
<td>Cattle PID containing protein 1</td>
<td>38%</td>
</tr>
<tr>
<td>Human PID containing protein 1</td>
<td>38%</td>
</tr>
</tbody>
</table>

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**Table 2. Predicted amino-acid sequence of Ci-Noto4.**

<table>
<thead>
<tr>
<th>Amino-acid Sequence</th>
<th>Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHSVLLPVLNYNTIIRTIPPAKPSPLAKGPGSAFDQGLSGNSSIFASENRS</td>
<td>50%</td>
</tr>
<tr>
<td>EEYRTGTSFMNRRKSTSSAVSNENMPQHFCSPKTGDTPLLIKPGREE</td>
<td>100%</td>
</tr>
<tr>
<td>SVLQHAALSNTDLPRNLAVNFLGSVEMSDEELRLSSLSSSLQGC</td>
<td>150%</td>
</tr>
<tr>
<td>RFGLLRLIDGDFSLQDCENVGAELPQVQOKRNKKKSNGYETND</td>
<td>200%</td>
</tr>
<tr>
<td>PKGVNLSMCCKGVPSPRYQVLQHNSINLKGYEVKKRRMIVFYRRI</td>
<td>250%</td>
</tr>
<tr>
<td>IYCGADIDGASPNLTTWLYHTVTGSYYSSVQCAYVCDDPQHARNLARIG</td>
<td>300%</td>
</tr>
</tbody>
</table>

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**Table 3. Characterization of Ci-Noto4.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Notch</th>
</tr>
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<tbody>
<tr>
<td>Alignment of notochord cells</td>
<td>Disrupted</td>
</tr>
<tr>
<td>Progenitor cells</td>
<td>Left (yellow triangle) and right (white triangle) independently converged, intercalated, and partially extended</td>
</tr>
</tbody>
</table>

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**Fig. 2. Functional suppression of Ci-Noto4 disrupted the midline alignment of notochord cells.**

A) Embryos that developed from eggs injected with control MO developed normally and contained GFP-positive notochord cells in a single row in the center of the tail by the late tailbud stage (Fig. 2A). In contrast, Ci-Noto4 MO-injected embryos did not contain a single row of notochord cells (Fig. 2B-E). The progenitor cells on the left (yellow triangle) and right (white triangle) of the embryo independently converged, intercalated, and partially extended, but the left and right progenitor populations did not intercalate at the midline into a single row of cells, resulting in two rows of notochord cells (Fig. 2F-H).
Ci-Noto4 contributes to notochord formation

We repeated the Ci-Noto4 MO injection experiments three times and obtained similar results. Ci-Noto4 MO injected embryos showed some range of effects on development, from embryos with normal morphology (Fig. 2 B,C) to those with damaged morphology (Fig. 2 D,E). However, most manipulated embryos showed two rows of notochord cells. Therefore, it is likely that suppression of Ci-Noto4 expression affected the notochord cells, especially their movement, and that these notochord progenitor cells were unable to form a notochord consisting of a single row of cells.

Fig. 2 (Left). Knockdown of Ci-Noto4 function disrupts the alignment of notochord cells. (A) Injection of control MO did not affect notochord-cell alignment. (B-E) In contrast, injection of MO specific for Ci-Noto4 disrupted the alignment of the notochord cells. (B) Some embryos injected with Ci-Noto4 MO show normal outer morphology. (D) While some others show damaged outer morphology. However, (C,E) higher magnification shows that the left (yellow triangle) and right (white triangle) cell populations did not interdigitate. Scale bar, 50 μm.

Fig. 3 (Right). Effects of Ci-Noto4 overexpression on notochord cells. Distribution of EGFP in embryos electroporated with (A) Ci-Bra (promoter):EGFP (control) or (B-D) Ci-Bra (promoter):Ci-Noto4-EGFP. (A) In control embryos, notochord cells formed a single line. Scale bar, 100 μm. (B) Ci-Noto4 overexpressing embryos showed disrupted notochord-cell alignment. Scale bar, 100 μm. (C) Higher magnification reveals the aberrant morphology and size of the notochord cells. The cells were not properly localized in the trunk and tail regions. Scale bar, 100 μm. (D) Higher magnification demonstrates that although Ci-Noto4-EGFP was localized in the cytoplasm of the notochord cells, strong GFP expression was observed in the nuclei. Scale bar, 20 μm.

notochord cells (Fig. 2 B-E).

We repeated the Ci-Noto4 MO injection experiments three times and obtained similar results. Ci-Noto4 MO injected embryos showed some range of effects on development, from embryos with normal morphology (Fig. 2 B,C) to those with damaged morphology (Fig. 2 D,E). However, most manipulated embryos showed two rows of notochord cells. Therefore, it is likely that suppression of Ci-Noto4 expression affected the notochord cells, especially their movement, and that these notochord progenitor cells were unable to form a notochord consisting of a single row of cells.

Ci-Noto4 overexpression results in notochord cells that are not properly localized in the trunk

We examined the intracellular localization of Ci-Noto4 after electroporation of Ci-Bra (promoter):Ci-Noto4-EGFP expression

Fig. 4. Dual electroporation experiments to examine Ci-Noto4 overexpression in notochord cells. (A) A schematic representation of the dual electroporation experiments. In the first electroporation, the Ci-Bra (promoter):RFP construct was injected into fertilized eggs. In the second electroporation, the Ci-Bra (promoter):Ci-Noto4-EGFP construct was injected into the same fertilized eggs. (B) In control embryos, each notochord cell was distinguished by EGFP or RFP expression. (C,D) Ci-Noto4 overexpressing notochord cells were not properly localized in the trunk region. The morphology of the Ci-Noto4 overexpressing notochord cells also was aberrant. Magenta marks Ci-Bra (promoter):RFP expressing notochord cells. Green marks Ci-Bra (promoter):Ci-Noto4-EGFP expressing notochord cells. (D) Asterisks denote Ci-Bra (promoter):RFP expressing notochord cells. Notochord cells that strongly expressed GFP were round and localized independently (triangles). On the other hand, notochord cells with moderate levels of GFP expression were observed in clusters containing several cells (arrowheads). In contrast, notochord cells with weak levels of GFP expression were aligned with RFP-positive cells in the tail (arrow). Scale bar, 50 μm.
Fig. 5. Abnormal notochord cell behavior is dependent on the Ci-Noto4 levels. (A,D,E,F,G) The Ci-Bra(promoter):Noto4-EGFP construct was electroporated into fertilized eggs at four different concentrations (12.5, 25, 50 and 100 ng/µl). (B,C) The Ci-Bra(promoter):EGFP (control) construct was electroporated into fertilized eggs at concentration of 100 ng/µl. Ci-Noto4 overexpressing notochord cells that were properly or improperly localized in the embryos are represented by dark and light blue, respectively. (A,D,E,F,G) The frequency of abnormal notochord alignment increased in a Ci-Noto4 dose-dependent manner. Scale bar, 100 µm.

constructs. GFP signal was distributed throughout the cell (see Fig. 7, panel of “low level”). Of note, we occasionally observed GFP-positive cells that were abnormally positioned (Fig. 3B). This was in contrast to normal position of GFP-positive cells in embryos injected with Ci-Bra(promoter):EGFP (Figs. 2A and 3A). We thought that the abnormal positioning of the GFP-positive cells is due to overexpression of Ci-Noto4. More detailed analysis showed that the notochord cells of Ci-Noto4 overexpressing embryos were improperly localized in the trunk region (Fig. 3C). These cells were bigger than normal notochord cells, and at times had a spherical morphology (Fig. 3D).

However, it was possible that the observed effects were an artifact of the electroporation method to introduce Ci-Bra(promoter):Ci-Noto4-EGFP rather than Ci-Noto4 overexpression. To examine this possibility, we performed dual electroporation experiments in which two different expression constructs were separately introduced into the same embryos. We first introduced Ci-Bra(promoter):RFP (magenta) into the embryos followed by Ci-Bra(promoter):Noto4-EGFP (green) (Fig. 4A). As a control, we electroporated the embryos first with Ci-Bra(promoter):RFP and then with Ci-Bra(promoter):EGFP (Fig. 4B). When the two constructs were each incorporated into opposing cells in a 2-cell embryo, a half of the notochord progenitor cells expressed GFP whereas the other half expressed RFP.

Fig. 6. Expression of differentiation markers in Ci-Noto4 overexpressing embryos. Cell differentiation was examined using the expression of Ci-leprecan (A), Ci-trop (B), and Ci-Noto1 (C). (D-F) Markers for muscle differentiation (cDNA cluster ID: 00031 (D), mesenchyme tissues (cDNA cluster ID: 00142 (E) and the nervous system (cDNA cluster ID: 00124) (F) were examined in whole mount in situ hybridizations. (A-F) Ci-Noto4 overexpression in notochord cells did not affect their cell fate. Scale bar, 50 µm.
shown in Fig. 4B, dual electroporation with the control constructs did not affect embryogenesis; half of the notochord cells expressed GFP and intermingled with the other notochord cells expressing RFP, and both sets of cells were aligned in a single row. In contrast, when one of two embryonic cells received Ci-Bra(promoter):Noto4-EGFP, the RFP expressing control cells were aligned in the midline of the tail (Fig. 4C, asterisks in D), whereas the GFP expressing cells were improperly localized in the trunk region (Fig. 4C, triangles and arrowheads in D).

Judging from the intensity of fluorescence, notochord cells with high levels of GFP expression frequently appeared round and were dispersed as single cells (Fig. 4D, triangles). Notochord cells with moderate levels of GFP expression, on the other hand, clustered in abnormal locations (Fig. 4D, arrowheads). In contrast, notochord cells with low levels of GFP expression were aligned with RFP-positive cells in the tail (Fig. 4D, arrow). These data suggest that change in the notochord cell morphology was dependent on the level of Ci-Noto4 expression within the cell.

To further examine the dose-dependent effects of Ci-Noto4 overexpression, we introduced Ci-Bra(promoter):Ci-Noto4-EGFP into fertilized eggs by electroporation at four different concentrations (12.5, 25, 50, and 100 ng/μl). We counted embryos that contained abnormally located Ci-Noto4-overexpressing notochord cells. In Fig. 5, dark blue shows a normal phenotype with no dispersed notochord cells, and light blue denotes a phenotype with improperly localized Ci-Noto4-overexpressing notochord cells. Injection of 12.5 ng/μl of the construct did not affect so much on the position of notochord cells (Fig. 5A, D). Injection of 50 or 100 ng/μl resulted in abnormal position of notochord cells in more than 80% of manipulated embryos (Fig. 5 A,F,G). The number of embryos with aberrant localization of Ci-Noto4 overexpressing notochord cells increased in a Ci-Noto4 dose-dependent manner (Fig. 5 A,D,E,F,G).

The abnormally localized notochord cells in Ci-Noto4 overexpressing embryos were examined using time-lapse video analysis. We began observing the embryos at the neurula stage under a confocal laser-scanning mi-
were overexpressed under high-level expression condition. Like Ci-Noto4, most phosphotyrosine-binding proteins contain a C-terminal PTB/PID and the central region play significant roles in the midline intercalation of notochord cells in Ciona embryos. Loss of Ci-Noto4 function in knockdown experiments and gain of function after Ci-Noto4 overexpression did not always result in completely different phenotypes. Rather, functional suppression produced a weak phenotype with a notochord containing two rows of cells and normal notochord cell morphology, whereas functional overexpression resulted in a strong phenotype with a complete dispersal of the individual notochord progenitor cells and spherical cell morphology. The PTB/PID and the central region play significant roles in Ci-Noto4 function. Future studies should elucidate the proteins interact with Ci-Noto4 and how Ci-Noto4 functions during the midline intercalation of notochord cells.

Materials and Methods

Experimental design

Ciona intestinalis adults were obtained form several places including Mikawa Bay, Gamagohri, Aichi, Maizuru Fisheries Research Station of Kyoto University, Maizuru Bay, Kyoto. Ciona intestinalis eggs and sperm were handled as described previously (Corbo et al., 1997, Mita-Miyazawa et al., 1985).

MOs were microinjected into dechorionated fertilized eggs as described below. Fertilized eggs were electroporated with reporter constructs as previously described (Takahashi et al., 1999). Eighty-microgram aliquots of plasmid DNA were used for electroporation. After electroporation, the embryos were maintained in agar-coated dishes with Millipore-filtered seawater containing 50 μg/ml streptomycin sulfate (MFWS) at 18°C. Embryos were fixed at room temperature for 30 min in 4% paraformaldehyde dissolved in 0.5 M NaCl and 0.1 M MOPS at pH 7.5. Fluorescence was observed using an LSM 510 META confocal microscope (Carl Zeiss).

MO and microinjection

MOs were made-to-order (Gene Tools). The Ci-Noto4 MO and control Ci-Noto4 5′s and 3′s MO sequences were: 5′-GGCAATAGAACTGAGCTGATGCATGGC-3′ and 5′-GGGATACACTAAGTGAGCTGC-3′, respectively. Microinjections were carried out as described previously (Yamada et al., 2009). The control MO did not produce any marked effect.

Ci-Noto4-EGFP constructs

An 885-bp upstream sequence of Ci-Bra was fused in-frame with DNA encoding Ci-Noto4-EGFP (monomer EGFP). This upstream sequence of Ci-Bra contains the basal promoter and enhancers that are required for specific notochord expression. Ci-Noto4 and EGFP were fused to remove the CMV promoter from pEGFP-N1 (BD Biosciences); the vector was digested with Asel and Nhel, blunted with Klenow fragment (Takara), and religated. To create a construct containing the Ci-Bra promoter region (first 885 bp upstream from the start codon) fused to EGFP, the T3 and 5′-Ci-Bra HindIII primers were used for PCR amplification. The amplified fragment was then digested with HindIII (Takara) and ligated into HindIII-digested pEGFP-N1/CMV(-). To create a construct containing Ci-Noto4 fused with EGFP, the Ci-Noto4-coding sequence was amplified by PCR. Sequences flanking Ci-Noto4 were converted to a Kozak consensus translation initiation site. The ACC Noto4 Psfl and Noto4 BamHI primers were used for PCR amplification. The amplified fragment was digested with Psfl (Takara) and BamHI (Takara) and ligated into Psfl/BamHI-digested Ci-Bra promoter/pEGFP-N1/CMV(-). The following PCR primers were used: T3, 5′-ATTAACCCCTACTAAAGG-3′; 5′-Ci-Bra HindIII, 5′-CCCAAGCTTCTTATAGTTTGTGAATCTG-3′; ACC Noto4 Psfl F, 5′-AAACCTGACAGCATGACCTGTTTTGCTATAC-3′; and Noto4 BamHI R.
Ci-Noto4 contributes to notochord formation

5'-CGCGGATCCCTGGCAATGGCACCATCTGGCAGGGCATTGC-3'.

**Dual electroporation**

The experimental scheme for dual electroporation is shown in Fig. 4A. First, the Ci-Bra(promoter):EGFP construct was immediately introduced into the same eggs. After the second electroporation procedure, embryos were raised in MFSWS at 18°C in agar-coated culture dishes.

**In vivo time-lapse imaging**

For observation, embryos were transferred to agar coated glass based dish (Iwaki glass based dishes, Asahi Techno Glass Co.) with MFSWS. Morphogenetic movements of notochord cells were followed in time-lapse movies obtained using a 10x Plan-Neofluar objective lens mounted on a LSM 510 META laser scanning microscope (Carl Zeiss). Images were obtained every 2.5 min.

**Ci-Noto4-EGFP expression constructs for domain analysis**

A full-length Ci-Noto4 construct was used as a template for PCR. Ci-Noto4-Δ11 lacked amino acids 23-37. The ACC Noto4 PstI, Del Stb1 Fu R, Del Stb1 Fu F, and Noto4 BamHI R primers were used for PCR amplification. Ci-Noto4-Δ2 lacked amino acids 23-56. The primers ACC Noto4 PstI, Del Stb2 Fu R, Del Stb2 Fu F, and Noto4 BamHI R were used for PCR amplification. Ci-Noto4-ΔPTB lacked amino acids 242-299. The primers ACC Noto4 PstIF, Del PTB Fu R, Del PTB Fu F, and Noto4 BamHI R were used for PCR amplification. Ci-Noto4 PTB lacked amino acids 24-241. The primers ACC Noto4 PstIF, Noto4 1-22 PTB R, Noto4 1-22 PTB F, and Noto4 BamHI R were used for PCR amplification. Ci-Noto4-Δ6 lacked amino acids 157-202. The primers ACC Noto4 PstIF, Del Stb2 Fu R, Del Stb2 Fu F, and Noto4 BamHI R were used for PCR amplification. Ci-Noto4-Δ6 lacked amino acids 203-241. The primers ACC Noto4 PstIF, Noto4 Del4 R, Noto4 Del4 F, and Noto4 BamHI R were used for PCR amplification. 

The sequences of the primers (in 5’-3’ order) were as follows:

- Del Stb1 Fu R
- Del Stb1 Fu F
- Del Stb2 Fu R
- Del Stb2 Fu F
- Noto4 BamHI R

**Dose-dependent effects of Ci-Noto4 protein expression**

The Ci-Bra(promoter):Noto4-EGFP DNA construct was introduced into fertilized eggs at four different concentration (12.5, 25, 50 and 100 ng/μl) using electroporation. The embryos were raised in MFSWS in agar-coated culture dishes for 12 hours at 18°C. We counted the number of embryos that contained aberrantly localized Ci-Noto4 overexpressing notochord cells.

**In situ hybridization**

cDNAs used were for Ci-leprecan (Hotta et al., 2000), Ci-trop (Hotta et al., 2000), Ci-Noto1 (Hotta et al., 2000), Muscle marker (cDNA cluster ID: 00031; Satoh et al., 2001), Mesenchymal marker (cDNA cluster ID: 00142; Satoh et al., 2001) and Nervous system marker (cDNA cluster ID: 00124; Satoh et al., 2001). RNA probes were prepared with a DIG RNA labeling kit (Roche Diagnostics). Whole-mount in situ hybridizations were performed using digoxigenin-labeled antisense probes as described previously (Hotta et al., 1999). Control embryos hybridized with sense probes did not show signals above background.

**Acknowledgments**

We thank Dr. Chuya Shinzato for useful comment; Kazumi Taniyama and Kazuko Hirayama for technical support; Mr. Suzuki of Kaneya Seiko co., LTD for his cooperation in the equipment (ropes) of gathering the ascidians. This work was supported by Grants-in-Aid from MEXT (N.S.) and JSPS (H.T.), Japan. This study was also supported by the National Bio-Resource Project (NBRP) of MEXT/Japan, and BiRD of Japan Science and Technology Agency.

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