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 allow 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 that encode a 307 amino acid polypeptide (Hotta *et al.*, 2000). We show here that Ci-Noto4 contains a C-terminal phosphotyrosine interaction domain (PTB/PID). Proteins encoding PTB/PID function as adaptors or scaffolds that organize signaling complexes involved in a wide range of physiological processes, including neural development, immunity, tissue homeostasis, and cell growth (Uhlik *et al.*, 2005). Although more than 200 proteins in eukaryotes and nearly 60 human proteins with PTB/PID, have been identified, to the best of our knowledge, this is the first report detailing an ascidian protein with a PTB/PID.
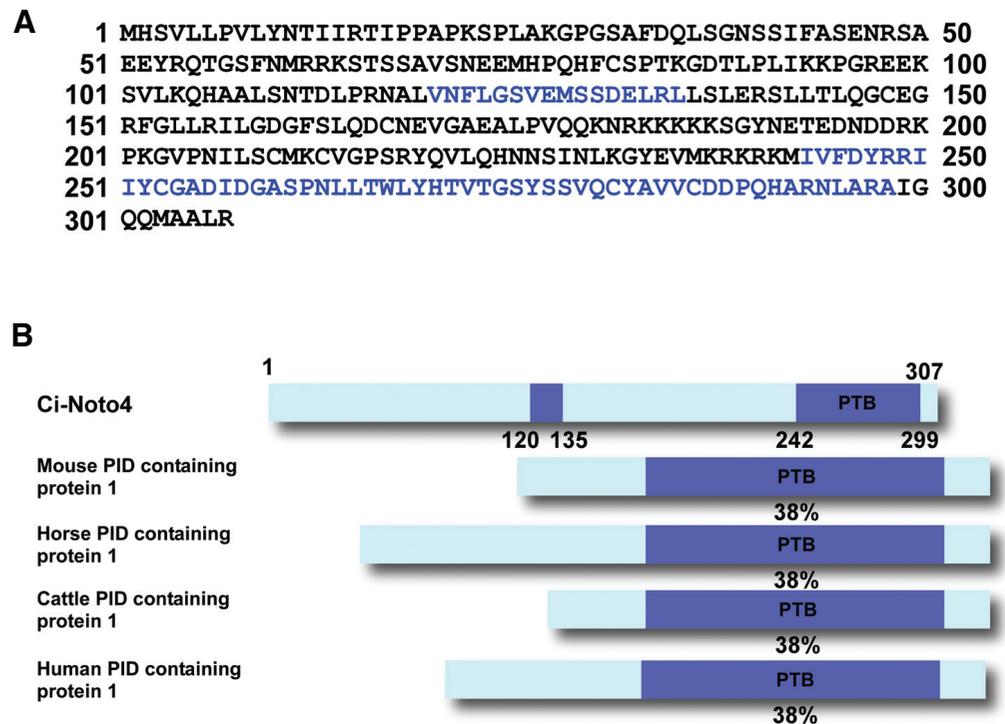
Results and Discussion
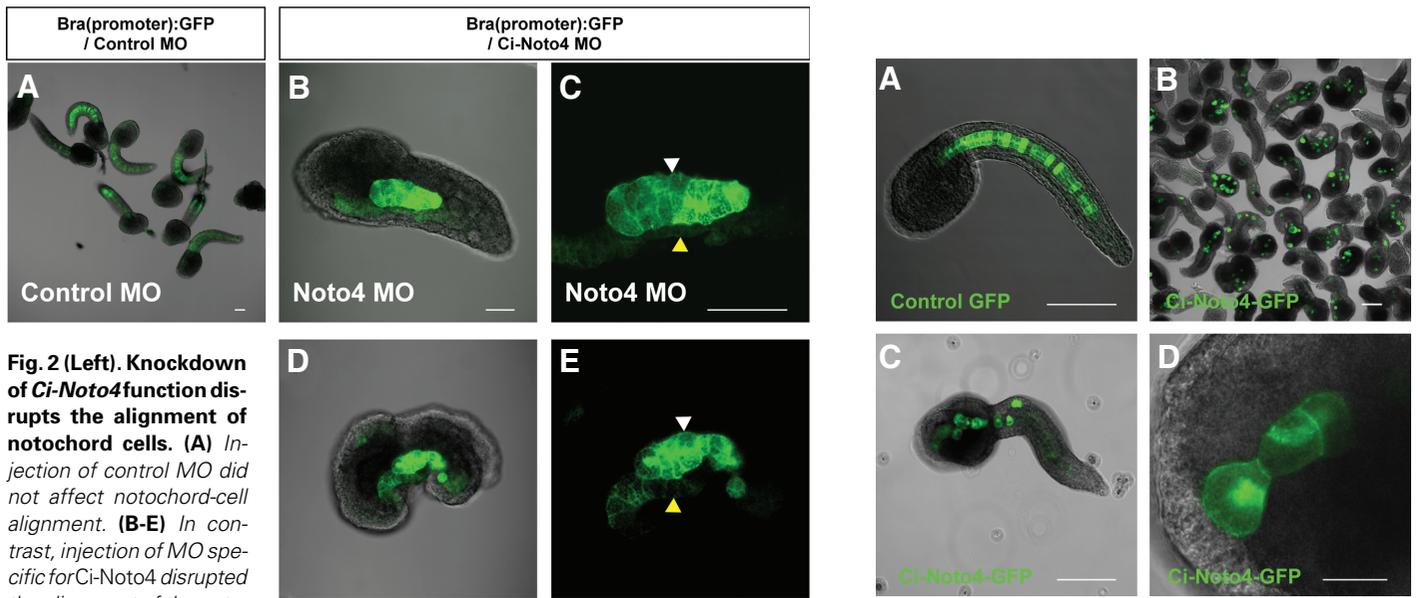
Characterization of Ci-Noto4

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).

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. 2 B-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

A

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  1 MHSVLLPVLYNTIIRTIPPAPKSPLAKGPGSAFDQLSGNSSIFASENRSA  50
 51 EEYRQTGSFNMRRKSTSSAVSNEEMHPQHFCSPTKGDTLPLIKKPGREEK 100
101 SVLKQHAALSNTDLPRNALVNFLGSVEMSSDELRLLSLERSLLTLQGCEG 150
151 RFGLLRILGDGFSLQDCNEVGAEALPVQQKNRKKKKKSGYNETEDNDDRK 200
201 PKGVPNILSCMKCVGPSRYQVLQHNNSINLKGYEVMKRKRKMIVFDYRRI 250
251 IYCGADIDGASPNLLTWLYHTVTGSYSSVQCYAVVCDDPQHARNLARAIG 300
301 QQMAALR
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B

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.*

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.*
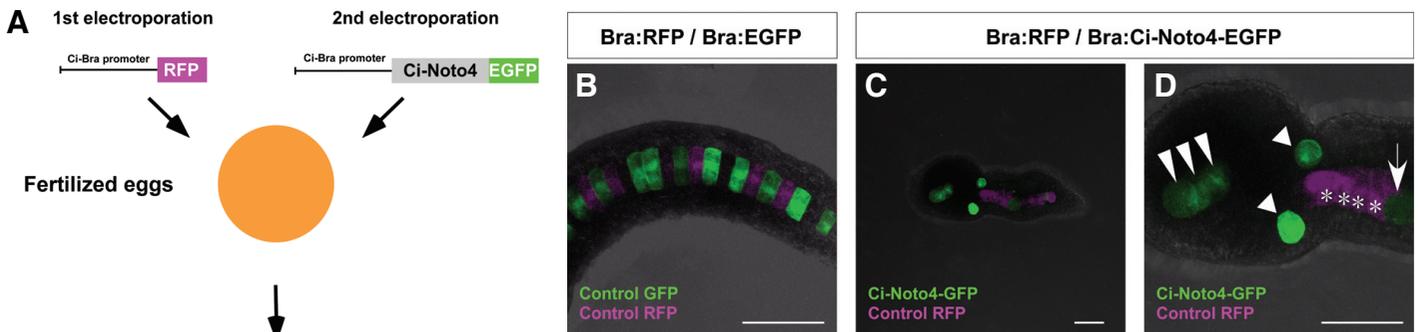
notochord cells (Fig. 2 B-E).

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,
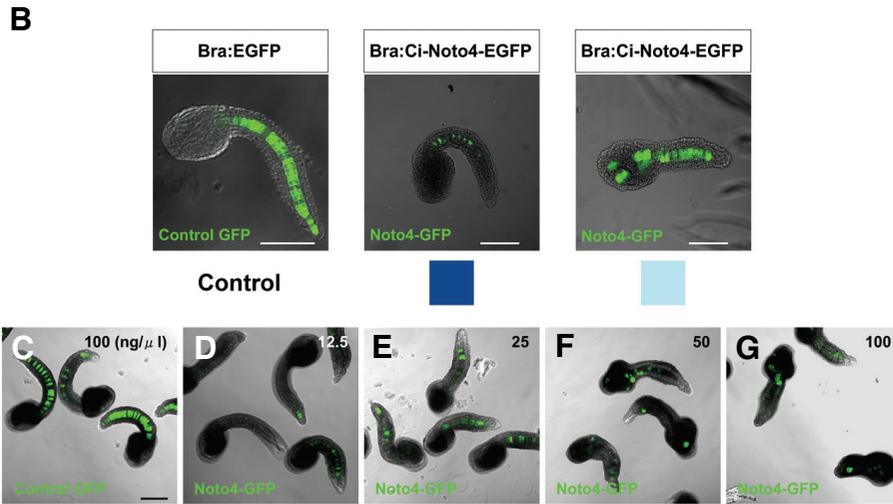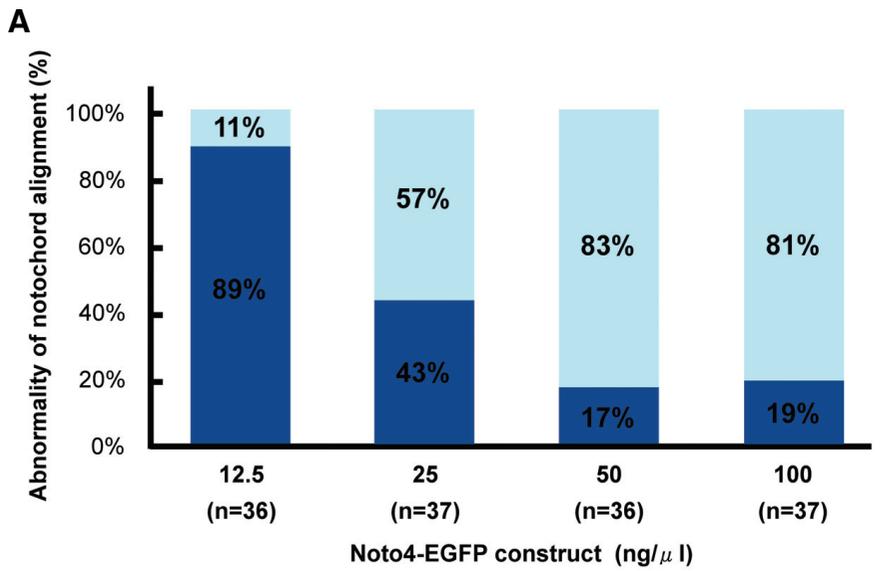
especially their movement, and that these notochord progenitor cells were unable to form a notochord consisting of a single row of cells.

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
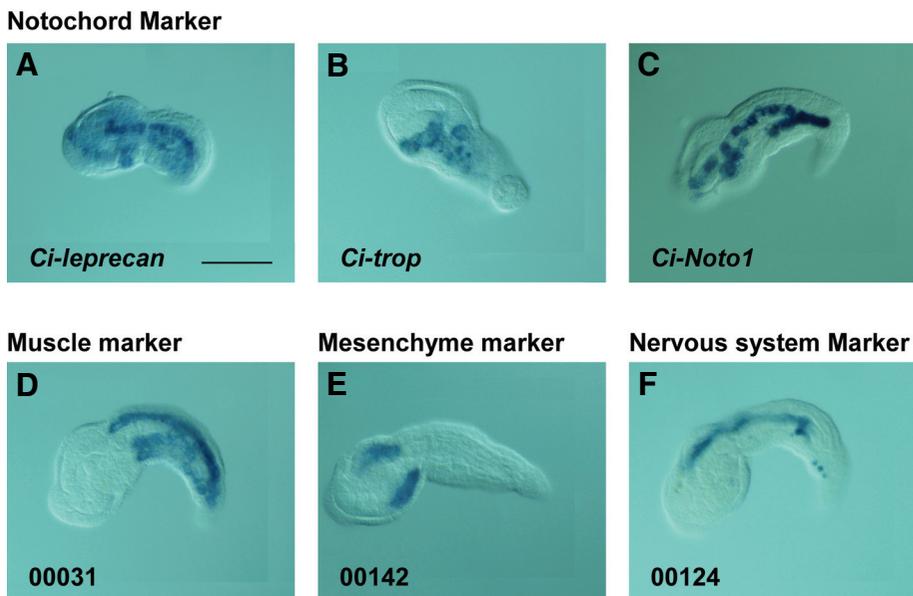

Fig. 4. **Dual electroporation experiments to examine Ci-Noto4 overexpression in notochord cells. (A)** *A schematic representation of the dual electroporation experiments. In the first electroporation, the* Ci-Bra *(promoter):RFP construct was injected into fertilized eggs. In the second electroporation, 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.*

A

B

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. As

Notochord Marker

Muscle marker   **Mesenchyme marker**   **Nervous system Marker**

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).
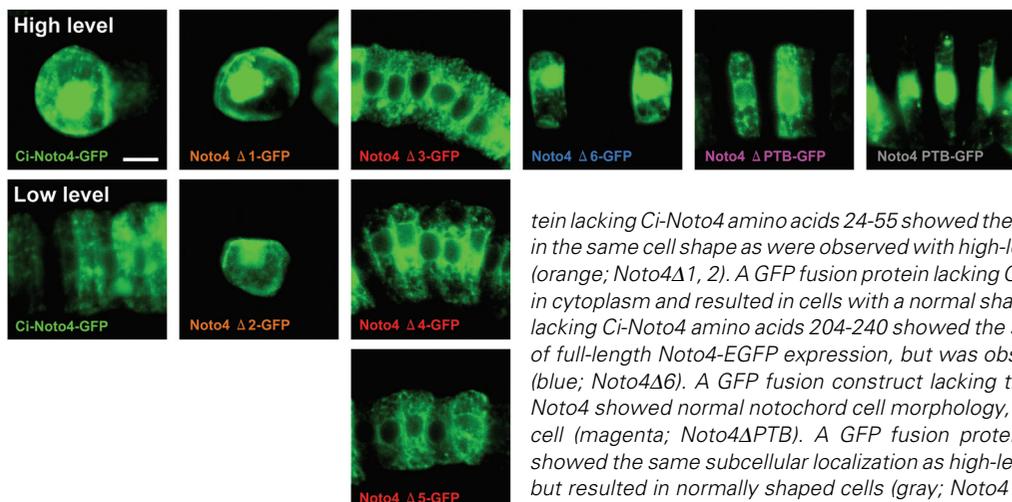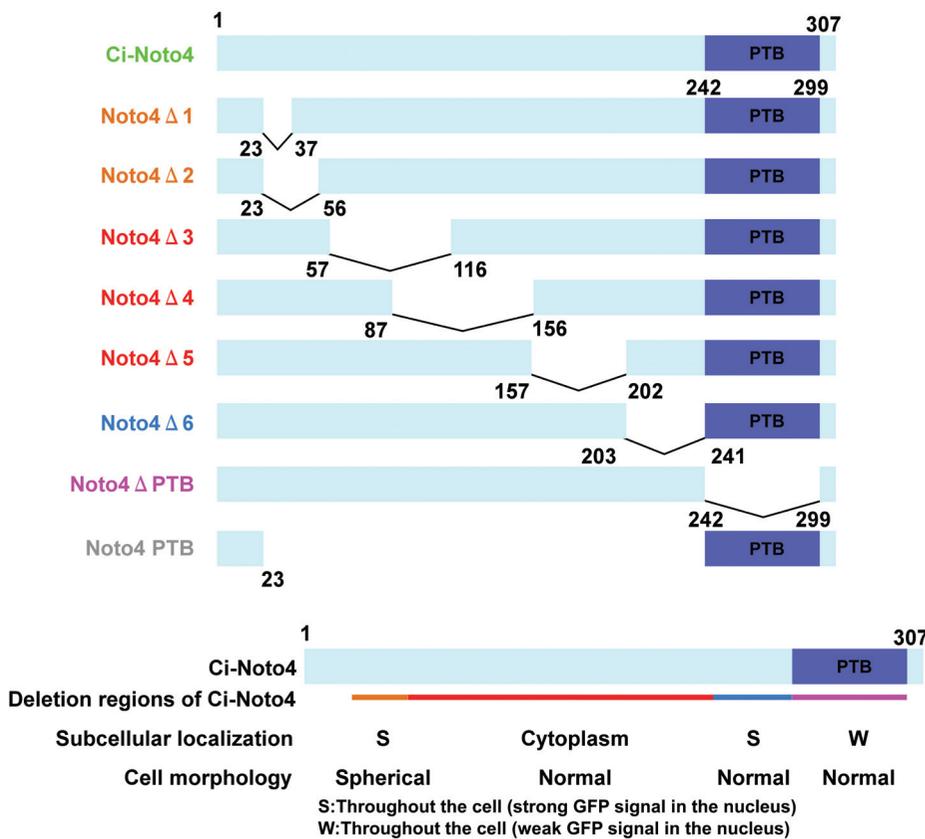
Judging from the intensity of fluorescence, notochord cells with high levels of GFP expression frequently appeared round and were dispersed as single cells (Fig. 4D, triangles). Notochord cells with moderate levels of GFP expression, on the other hand, clustered in abnormal locations (Fig. 4D, arrowheads). 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-

Fig. 7. Domain analysis of Ci-Noto4. *Ci-Noto4-EGFP was distributed throughout the cell, especially in the nucleus by high-level of full-length Ci-Noto4-EGFP expression (high level; green; Ci-Noto4). The cells were bigger than normal notochord cells, and at times had a spherical morphology. In the case of low-level Ci-Noto4-EGFP, Ci-Noto4-EGFP was evenly distributed throughout the cell (low level; green; Ci-Noto4). A GFP fusion protein lacking Ci-Noto4 amino acids 24-55 showed the same subcellular localization and resulted in the same cell shape as were observed with high-level of full-length Noto4-EGFP expression (orange; Noto4Δ1, 2). A GFP fusion protein lacking Ci-Noto4 amino acids 58-201 was localized in cytoplasm and resulted in cells with a normal shape (red; Noto4Δ3-5). A GFP fusion protein lacking Ci-Noto4 amino acids 204-240 showed the same subcellular localization as high-level of full-length Noto4-EGFP expression, but was observed in cells with a normal morphology (blue; Noto4Δ6). A GFP fusion construct lacking the PTB/PID (amino acids 243-298) of Ci-Noto4 showed normal notochord cell morphology, with the protein localized throughout the cell (magenta; Noto4ΔPTB). A GFP fusion protein lacking Ci-Noto4 amino acids 24-241 showed the same subcellular localization as high-level expression of full-length Noto4-EGFP, but resulted in normally shaped cells (gray; Noto4 PTB). Scale bars, 10 μm.*

croscope (Supplementary Fig. S1). The right and left progenitor cells converged toward the midline of the embryo. At the neurula stage, the cells formed a cluster. In Ci-Noto4 overexpressing embryos, notochord progenitor cells were detached from the cluster (Supplementary Fig. S1; arrowhead at 0 min). These cells then stopped moving and did not converge and extend during embryogenesis (Supplementary Fig. S1, arrowheads at 0-210 min). These data suggested that the aberrant position of the Ci-Noto4–overexpressing notochord progenitor cells was caused by decreased movement during embryogenesis.

Ci-Noto4 overexpression did not affect cellular differentiation

To determine effects of Ci-Noto4 overexpression on cellular differentiation, we examined the expression of three markers of notochord differentiation: *Ci-leprecan*, *Ci-trop*, and *Ci-Noto1*. Each of these markers was appropriately expressed in notochord cells, suggesting that Ci-Noto4 overexpression did not affect the expression of notochord-specific genes (Fig. 6 A-C). We also examined the expression patterns of markers of muscle differentiation (cDNA cluster ID: 00031; Fig. 6D), mesenchyme tissue (cDNA cluster ID: 00142; Fig. 6E), and the nervous system (cDNA cluster ID: 00124; Fig. 6F). All the three markers were expressed properly (Fig. 6 D-F). These results suggest that cellular differentiation in general was not affected by Ci-Noto4 overexpression.

Analysis of the functional regions in Ci-Noto4

As mentioned above, Ci-Noto4 contains a C-terminal PTB/PID. Several Ci-Noto4:EGFP fusion proteins that contained specific deletions in Ci-Noto4 were employed to examine the protein regions that are responsible for its function. As a result, we identified various sequence elements that contributed to the notochord phenotypes including aberrant cell morphology and cell localization (Fig. 7).

Fusion constructs lacking amino acids 58-201 of Ci-Noto4 were localized in the cytoplasm and were associated with normal notochord cell morphology (Fig. 7, red; Noto4Δ3, Δ4, Δ5). The fusion construct lacking amino acids 204-240 of Ci-Noto4 produced normal notochord cell morphology and the same localization pattern as that was observed with high-level overexpression of full-length Ci-Noto4 (Fig. 7, blue; Noto4Δ6). A fusion construct lacking the PTB/PID (amino acids 243-298) of Ci-Noto4 produced normal notochord cell morphology, with the protein localized throughout the cell, the same localization pattern as that was observed with low-level overexpression of full-length Ci-Noto4 (Fig. 7, magenta; Noto4ΔPTB). Thus, fusion constructs lacking amino acids 58-201 or 243-298 showed altered subcellular Ci-Noto4 localization. Interestingly, deletion of amino acids 204-240 disrupted the function of Ci-Noto4 without changing the subcellular localization of the protein. These data suggested that Ci-Noto4 might play significant roles in the nucleus of the notochord cell.

Like Ci-Noto4, most phosphotyrosine-binding proteins contain a single PTB/PID without any other identifiable modular domains (Uhlik *et al.*, 2005). Nevertheless, the central and C-terminal regions of Ci-Noto4 also play significant roles for the function of the protein, because the deletion constructs lacking these regions did not exert the same effects as the wild-type protein when they were overexpressed under high-level expression condition.

In conclusion, Ci-Noto4 containing PTB/PID is involved 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 (MFSWS) 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* 5mis MO sequences were:
5'- GGCAATAGAACTGAGTGCATGGC-3' and
5'-GGgAATAcAACTcAGTGgATcGC-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 *Ase*I and *Nhe*I, 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 *Hind*III primers were used for PCR amplification. The amplified fragment was then digested with *Hind*III (Takara) and ligated into *Hind*III-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 *Pst*I and Noto4 *BamH*I primers were used for PCR amplification. The amplified fragment was digested with *Pst*I (Takara) and *BamH*I (Takara) and ligated into *Pst*I/*BamH*I-digested *Ci-Bra* promoter/pEGFP-N1/CMV(-). The following PCR primers were used:
T3, 5'-AATTAACCCTCACTAAAGGG-3';
5'-Ci-Bra *Hind*III, 5'-CCCAAGCTTCATTATAGGTTTGTAACTCG-3';
ACC Noto4 *Pst*I F,
5'-AAAACTGCAGACCATGCACTCAGTTCTATTGCCAGTGCTATAC-3';
and Noto4 *BamH*I R,

5'-CGCGGATCCTTGCGCAATGCCGCCATCTGCTGGCCAATTGC-3'.

Dual electroporation

The experimental scheme for dual electroporation is shown in Fig. 4A. First, the *Ci-Bra*(promoter):RFP construct was introduced into fertilized eggs using electroporation. Then, the *Ci-Bra*(promoter):Noto4-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 PCRs. Ci-Noto4 Δ1 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 *PstI* F, 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 *PstI* F, Noto4 1-22 PTB R, Noto4 1-22 PTB F, and Noto4 *BamHI* R were used for PCR amplification. Ci-Noto4 Δ3 lacked amino acids 57-116. The primers ACC Noto4 *PstI* F, Noto4 Del1 R, Noto4 Del1 F, and Noto4 *BamHI* R were used for PCR amplification. Ci-Noto4 Δ4 lacked amino acids 87-156. The primers ACC Noto4 *PstI* F, Noto4 Del2 R, Noto4 Del2 F, and Noto4 *BamHI* R were used for PCR amplification. Ci-Noto4 Δ5 lacked amino acids 157-202. The primers ACC Noto4 *PstI* F, Noto4 Del3 R, Noto4 Del3 F, and Noto4 *BamHI* R were used for PCR amplification. Ci-Noto4 Δ6 lacked amino acids 203-241. The primers ACC Noto4 *PstI* F, 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
GTTTTCTGAAGCGAAAATGCTCGAATTTCCTTTTGGCGCAGGCGGAATTGTTCGTATAAT-
Del Stb1 Fu F
ATTATACGAACAATTCCGCCTGCGCCAAAAGGAAATTCGAGCATTTTCGCTTCAGAAAAC-
Del Stb2 Fu R
CGTAGATTTTCTTCGCATATTAAAAGAACCTTTTGGCGCAGGCGGAATTGTTCGTATAAT-
Del Stb2 Fu F
ATTATACGAACAATTCCGCCTGCGCCAAAAGGTTCTTTTAATATGCGAAGAAAATCTACG-
Del PTB Fu R
GCGCAATGCCGCCATCTGCTGGCCTTTCCTCTTTCGTTTCATCACTTCGTACCC-
Del PTB Fu F
GGGTACGAAGTGATGAAACGAAAGAGGAAAGGCCAGCAGATGGCGGCATTGCGC-
Noto4 1-22 PTB R
CGGTAATCGAACACGATCATTTTTGGCGCAGGCGGAATTG
Noto4 1-22 PTB
CAATTCCGCCTGCGCCAAAAATGATCGTGTTCGATTACCG
Noto4 Del1 R
AAGAAGTTAACAAGCGCGTTAGTTTGCCTATACTCTTCCG
Noto4 Del1 F
CGGAAGAGTATAGGCAAACTAACGCGCTTGTTAACTTCTT
Noto4 Del2 R
GAGAAACCGTCGCCGAGGATACCTTTAGTTGGAGAGCAAA
Noto4 Del2 F
TTTGCTCTCCAACTAAAGGTATCCTCGGCGACGGTTTCTC
Noto4 Del3 R
GACAGAATATTGGGCACTCCTCGGAGCAAACCGAATCGGC
Noto4 Del3 F

GCCGATTCGGTTTGCTCCGAGGAGTGCCCAATATTCTGTC
Noto4 Del4 R
CGGTAATCGAACACGATCATCTTGGGTTTTCGGTCATCGT
Noto4 Del4 F
ACGATGACCGAAAACCCAAGATGATCGTGTTCGATTACCG

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; Satou *et al.*, 2001), Mesenchyme marker (cDNA cluster ID: 00142; Satou *et al.*, 2001) and Nervous system marker (cDNA cluster ID: 00124; Satou *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.

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