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Multiple functions of a Zic-like gene in the differentiation of notochord, central nervous system and muscle in *Ciona savignyi* embryos

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SUMMARY

Multiple functions of a Zic-like zinc finger transcription factor gene (*Cs-ZicL*) were identified in *Ciona savignyi* embryos. cDNA clones for *Cs-ZicL*, a β-catenin downstream genes, were isolated and the gene was transiently expressed in the A-line notochord/nerve cord lineage and in B-line muscle lineage from the 32-cell stage and later in a-line CNS lineage from the 110-cell stage. Suppression of *Cs-ZicL* function with specific morpholino oligonucleotide indicated that *Cs-ZicL* is essential for the formation of A-line notochord cells but not of B-line notochord cells, essential for the CNS formation and essential for the maintenance of muscle differentiation. The expression of *Cs-ZicL* in the A-line cells is downstream of β-catenin and a β-catenin-target gene, *Cs-FoxD*, which is expressed in the endoderm cells from the 16-cell stage and is essential for the differentiation of notochord. In spite of its pivotal role in muscle specification, the expression of *Cs-ZicL* in the muscle precursors is independent of *Cs-macho1*, which is another Zic-like gene encoding a *Ciona* maternal muscle determinant, suggesting another genetic cascade for muscle specification independent of *Cs-macho1*. *Cs-ZicL* may provide a future experimental system to explore how the gene expression in multiple embryonic regions is controlled and how the single gene can perform different functions in multiple types of embryonic cells.

Key words: Ascidian, Zic-like gene, Multiple functions, Notochord, CNS, Muscle, *Cs-ZicL*, *Ciona savignyi*

INTRODUCTION

Unfertilized eggs of various groups of animals are maternally provided with a considerable amount of β-catenin protein and mRNA in the cytoplasm. During very early cleavages after fertilization, β-catenin is translocated from the cytoplasm into the nucleus of certain embryonic cells. The nuclear accumulation of β-catenin, together with transcription factor Tcf/Lef, activates many target genes that play pivotal roles in embryonic axis formation and/or embryonic cell specification (reviewed by Cadigan and Nusse, 1997; Moon and Kimelman, 1998; Sokol, 1999).

As in the case of vertebrate embryos, the endoderm of the ascidian embryo is specified autonomously and then induces formation of the notochord and mesenchyme (reviewed by Satoh, 1994; Satoh, 2001; Satou and Satoh, 1999; Nishida, 1997; Jeffery, 2001; Corbo et al., 2001). In a previous study, we showed that during cleavages of the ascidians *Ciona intestinalis* and *C. savignyi*, β-catenin accumulates in the nuclei of vegetal blastomeres by the 32-cell stage, that mis- and/or overexpression of β-catenin induces the ectopic development of endoderm cells, and that downregulation of nuclear β-catenin induced by the overexpression of cadherin results in the suppression of endodermal cell differentiation (Imai et al., 2000). Thus, the accumulation of β-catenin in the nuclei of endoderm progenitor cells is most likely the first step in the process of ascidian embryonic endoderm specification.

To understand the function of endoderm in ascidian embryos, it is necessary to identify the genes that act as direct targets and/or act downstream of β-catenin. We previously took advantage of the availability of β-catenin-overexpressing embryos and cadherin-overexpressing embryos to address this problem; in the former, β-catenin targets may be upregulated and in the latter, β-catenin targets may be downregulated, and subtractive hybridization screening between them performed. We found that a LIM-homeobox gene, *Cs-lhx3*, an otx homolog *Cs-otx*, and an NK-2 class homeobox gene *Cs-ttf1* are downstream genes of β-catenin (Satou et al., 2001a). Inhibition of the function of these genes revealed that inhibition of the possible early embryonic function of *Cs-lhx3* resulted in the suppression of endoderm differentiation. In addition, we found that the nuclear accumulation of β-catenin directly activates *Cs-FoxD*, which encodes a transcription factor with a forkhead domain. *Cs-FoxD* is transiently expressed in endoderm blastomeres at the 16- and 32-cell stages, and this gene function is not associated with endoderm but is necessary for notochord differentiation (K. S. I., unpublished). Here, we showed that another β-catenin downstream gene encodes a Zic-like protein. The gene is expressed in three different domains; namely, A-line notochord and central nervous system (CNS),

and B-line muscle cells, and plays pivotal roles in the differentiation of the three different cell types.

MATERIALS AND METHODS

Ascidian eggs and embryos

Ciona savignyi adults were maintained under constant light to induce oocyte maturation. Eggs and sperm were obtained surgically from the gonoduct. After insemination, eggs were reared at about 18°C in Millipore-filtered seawater (MFSW) containing 50 μg/ml streptomycin sulfate.

Isolation of cDNA clones for a Zic-like gene and sequencing

A cDNA clone for a Zic-like gene (named *Cs-ZicL*) was isolated as one of the β-catenin downstream genes; the procedure for subtractive hybridization screening of cDNA clones for potent β-catenin target genes was described previously (Satou et al., 2001a). The cDNA obtained by subtraction was partial, and cDNA clones that contained the entire coding region were isolated from a *C. savignyi* gastrula cDNA library. Nucleotide sequences were determined for both strands using a Big-Dye Terminator Cycle Sequencing Ready Reaction kit and an ABI PRISM 377 DNA sequencer (Applied Biosystems).

Whole-mount in situ hybridization and histochemical staining for alkaline phosphatase

To examine the spatiotemporal expression patterns of *Cs-ZicL*, RNA probes were prepared with a DIG RNA labeling kit (Roche, Tokyo, Japan). Whole-mount in situ hybridization was performed as described previously (Satou et al., 2001a). Control specimens hybridized with a sense probe did not show any signals above background.

To examine the occurrence of differentiation markers in experimental embryos, in situ hybridization of whole-mount specimens was also carried out. The probes used were for a muscle actin gene, *Cs-MA1* (Chiba et al., 1998), an epidermis-specific gene, *Cs-Epi1* (Chiba et al., 1998), a mesenchyme-specific gene, *Cs-mech1* (DDBJ/GenBank/EMBL database accession number, AB073374), and a CNS-specific gene, *Cs-ETR* (DDBJ/GenBank/EMBL database accession number, AB073375). Notochord differentiation was assessed with probes for the *C. savignyi Brachury* gene (*Cs-Bra*; Imai et al., 2000) and notochord-specific *fibrinogen-like* gene *Cs-fibrn* (DDBJ/GenBank/EMBL database accession number, AB073373).

Differentiation of endodermal cells was monitored by histochemical detection of alkaline phosphatase (AP) activity (Whittaker and Meedel, 1989).

Morpholino oligos and synthetic capped mRNAs

To deduce the function of *Cs-ZicL*, we used morpholino antisense oligonucleotides (hereafter we simply refer to these as "morpholinos"), which have been shown to be very effective in ascidian embryos (Satou et al., 2001b). The 25-mer morpholino for *Cs-ZicL* was order-made (Gene Tools, LLC). The nucleotide sequence of the *Cs-ZicL* morpholino is shown in Fig. 1A. For rescue experiments, in vitro synthesized capped mRNA for *Cs-ZicL* was prepared from *Cs-ZicL* cDNA cloned into pBluescript RN3 vector (Lemaire et al., 1995) using a Megascript T3 kit (Ambion). To obtain a capped mRNA, the concentration of GTP was lowered to 1.5 mM and the cap analog 7mGpppG was added at a final concentration of 6 mM. The synthetic *Cs-ZicL* mRNA was designed to lack the morpholino sequence, and therefore the *Cs-ZicL* morpholino does not recognize the synthetic mRNA. In the present study, we also used morpholinos for *Cs-macho1* (Satou et al., 2002), and *Cs-FoxD* (K. S. I., unpublished).

After insemination, fertilized eggs were microinjected with 15 fmole of morpholinos and/or synthetic capped mRNAs. Each injection contained 30 pl of solution, and microinjection was carried out using a micromanipulator (Narishige Sci. Instru. Lab., Tokyo, Japan), as described previously (Imai et al., 2000). Injected eggs were reared at about 18°C in MFSW containing 50 μg/ml streptomycin sulfate. Cleavage of some embryos was arrested at the 110-cell stage with cytochalasin B, and the embryos were further cultured for about 12 hours, when control embryos reached the early tailbud embryo stage.

RESULTS

Isolation and characterization of *Cs-ZicL* cDNA

Subtractive hybridization screening of mRNAs from β-catenin-overexpressing embryos versus cadherin-overexpressing embryos yielded a cDNA fragment of a β-catenin-downstream gene which encoded a zinc finger protein. The gene was named *Cs-ZicL* (*Ciona savignyi* Zic-like). By screening of a gastrula cDNA library with this cDNA fragment as a probe, cDNA clones for *Cs-ZicL* were obtained and the longest was completely sequenced. As shown in Fig. 1, the insert of *Cs-ZicL* cDNA consisted of 1,217 nucleotides, which encoded a predicted polypeptide of 355 amino acids (Fig. 1A: DDBJ/GenBank/EMBL database accession number, AB057747). The predicted polypeptide contained five zinc finger domains (Figs 1 and 2).

Fig. 2A shows a comparison of the amino acid sequences of the five zinc finger domains of Cs-ZicL, Cs-macho1 of *C. savignyi* (Satou et al., 2002), macho-1 of *Halocynthia roretzi* (Nishida and Sawada, 2001) and mouse Zic3 (Aruga et al., 1996). Although there is variation in the first zinc-finger domain, these proteins share comparatively high level of amino-acid identity. Using these amino acid residues within the five zinc finger domains, we constructed a molecular phylogenetic tree using the neighbor-joining algorithm (Fig. 2B). The tree demonstrated that mouse and *Xenopus* Zic gene products form one clade, from which *Ciona* macho1, *Halocynthia* macho-1 and *Ciona* ZicL are distant. Within the three ascidian Zic-like gene products, *Ciona* macho1, *Halocynthia* macho-1 and *Ciona* ZicL did not form a discrete group, but there was a tendency for *Halocynthia* macho-1 and *Ciona* ZicL to form a group. These results suggest that the ascidian Zic-like genes evolved from a common ancestor gene of the Zic family independently of vertebrate Zic genes.

Expression and function of *Cs-ZicL*

Analyses of whole-mount specimens by in situ hybridization revealed that *Cs-ZicL* is expressed in multiple embryonic regions. As shown in Fig. 3, the embryonic expression of *Cs-ZicL* is transient; the transcript was first detected at the 32-cell stage and was downregulated by the early tailbud stage. The expression was found in embryonic cells that give rise to notochord, CNS and muscle. The multiple expression domains and functions of *Cs-ZicL* in each lineage were further examined, with the following results.

(a) Notochord

In ascidians, exactly 40 notochord cells are formed in the larval tail. Of them, 32 are derived from A-line cells and eight from B-line cells. At the 64-cell stage, A7.3 and A7.7 pairs are primordial notochord cells (Fig. 3C′), and they divide three

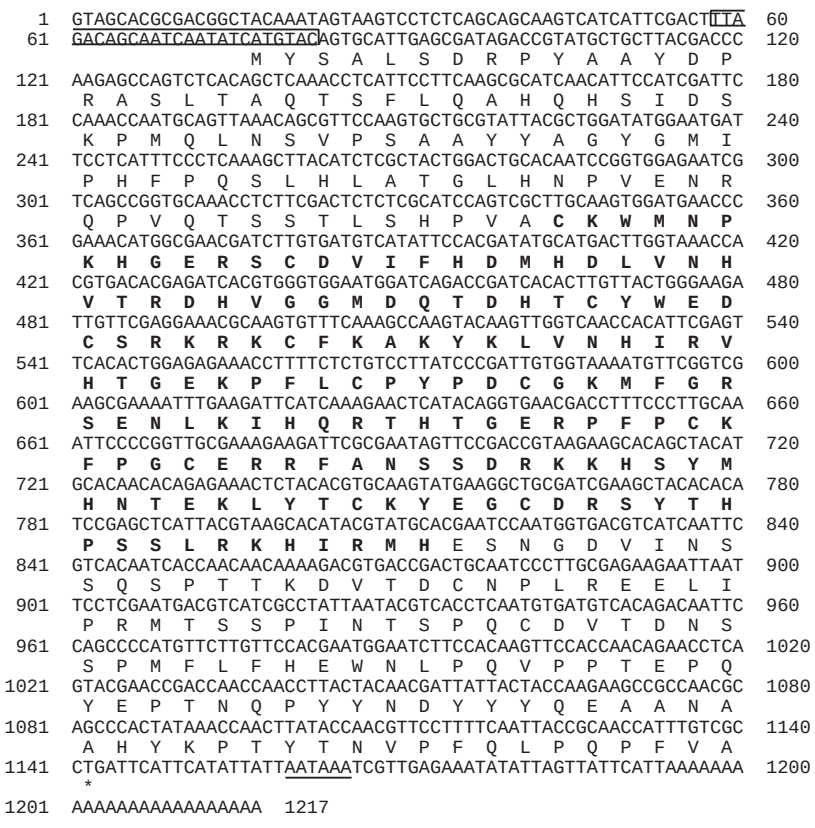

Fig. 1. Nucleotide and deduced amino acid sequences of cDNA for *Cs-ZicL*. The 1217-bp insert includes a single open reading frame that encodes a polypeptide of 355 amino acids. The termination codon is indicated by an asterisk, and polyadenylation signal sequences is underlined. Predicted zinc finger domains are shown by bold letters. The nucleotide sequence of the 5′ region used to prepare the morpholino is boxed.

times to form 32 A-line notochord cells. Pair B8.6 in the 110-cell stage embryo are also primordial notochord cells (Fig. 3D′), and they divide twice to form eight B-line notochord cells.

Expression: In situ hybridization signals were first seen in the A6.2 and A6.4 pairs of the 32-cell stage embryo (Fig. 3B,B′). These cells have the developmental fate to form notochord. Signals were then seen in the A7.3 and A7.7 pairs at the 64-cell stage (Fig. 3C,C′), and A8.5, A8.6, A8.13 and A8.14 pairs at the early gastrula stage (Fig. 3D,D′), although the signals became faint at the early gastrula stage (Fig. 3D,D′).

Function: To deduce the function of *Cs-ZicL*, we suppressed its translation by injection of morpholino into fertilized eggs. Injected embryos developed similarly to normal embryos, but the resultant tailbud embryos looked slightly flat, and the tail was not fully elongated (Fig. 4A′,B′). However, *Cs-ZicL*-suppressed embryos showed differentiation of the endoderm as assessed by histochemical detection of AP (100%, *n*=12; Fig. 4A,A′), of epidermis as assessed by the expression of *Cs-Epi1* (100%, *n*=15; Fig. 4B,B′), and of mesenchyme as assessed by the expression of *Cs-mech1* (97%, *n*=28; Fig. 4C,C′,D,D′), suggesting that the differentiation of endoderm, epidermis and mesenchyme is not dependent on *Cs-ZicL* function.

We next examined *Cs-ZicL* function in notochord differentiation. When we examined the differentiation of the notochord in embryos developed from eggs that had been injected with *Cs-ZicL* morpholino, the manipulated embryos failed to express notochord-specific *Cs-Bra* in A-line notochord cells (100%, *n*=35, data not shown).

When we examined the effects of functional suppression of *Cs-ZicL* on notochord formation with a probe for a notochord-specific structural gene, *Cs-fibrn* (*Cs-fibrinogen-like*) (Fig. 5A), the *Cs-fibrn* expression was greatly reduced, and only a few cells on both sides of the tail showed *Cs-fibrn* expression in experimental tailbud embryos (83% of embryos with a few cells expressing *Cs-fibrn*, 17% of embryos with no expression, *n*=23; Fig. 5A′). In order to determine whether differentiation of B-line notochord cells was affected by *Cs-ZicL* morpholino, we took advantage of ‘cleavage-arrest’, in which division of blastomeres is arrested with cytochalasin B but the differentiation of embryonic cells is not disturbed. In control embryos arrested at the 110-cell stage, eight A-line notochord and two B-line notochord cells expressed *Cs-fibrn* (Fig. 5B). In contrast, in *Cs-ZicL* morpholino-injected and 110-cell-stage arrested embryos, only B-line notochord cells, pair B8.6, expressed *Cs-fibrn* (77% of embryos with expression only in B-line notochord cells, 23% of embryos had no expression, *n*=17; Fig. 5B′).

To confirm the specificity of *Cs-ZicL* morpholino, a rescue experiment was preformed. Synthetic mRNA for *Cs-ZicL* lacking the *Cs-ZicL* morpholino recognition sequences (see Fig. 1) was co-injected into fertilized eggs together with *Cs-ZicL* morpholino. This co-injection rescued the expression of *Cs-fibrn* in the experimental embryos (65%, *n*=40; Fig. 5A″). These results clearly indicate that *Cs-ZicL* function is essential for the differentiation of A-line notochord cells but not B-line notochord cells.

Possible cascade of *Cs-ZicL* function in notochord formation: The cDNA clone for *Cs-ZicL* was originally isolated as a β-catenin downstream gene. This suggests that the expression of *Cs-ZicL* is controlled by nuclear accumulation of β-catenin. To examine this issue, we injected in vitro synthesized *Cs-cadherin* mRNA (Imai et al., 2000) into fertilized eggs. Cadherin binds to cytoplasmic β-catenin, and thus downregulates β-catenin nuclear accumulation. As shown in Fig. 6B (arrowheads), the expression of *Cs-ZicL* in two pairs of A-line cells (A6.2 and A6.4) was markedly inhibited (80%, *n*=10). However, the expression of *Cs-ZicL* in two pairs of B-line cells (B6.2 and B6.4) was not blocked by *Cs-cadherin* mRNA injection (arrows in Fig. 6B). This indicates that the expression of *Cs-ZicL* in the A-line cells is regulated by β-catenin nuclear accumulation. However, it should be determined in future studies whether nuclear β-catenin directly activates *Cs-ZicL*, or whether nuclear β-catenin indirectly activates *Cs-ZicL* via some other molecules.

In this regard, we have shown that nuclear β-catenin directly activates a forkhead domain transcription factor gene, *Cs-FoxD*

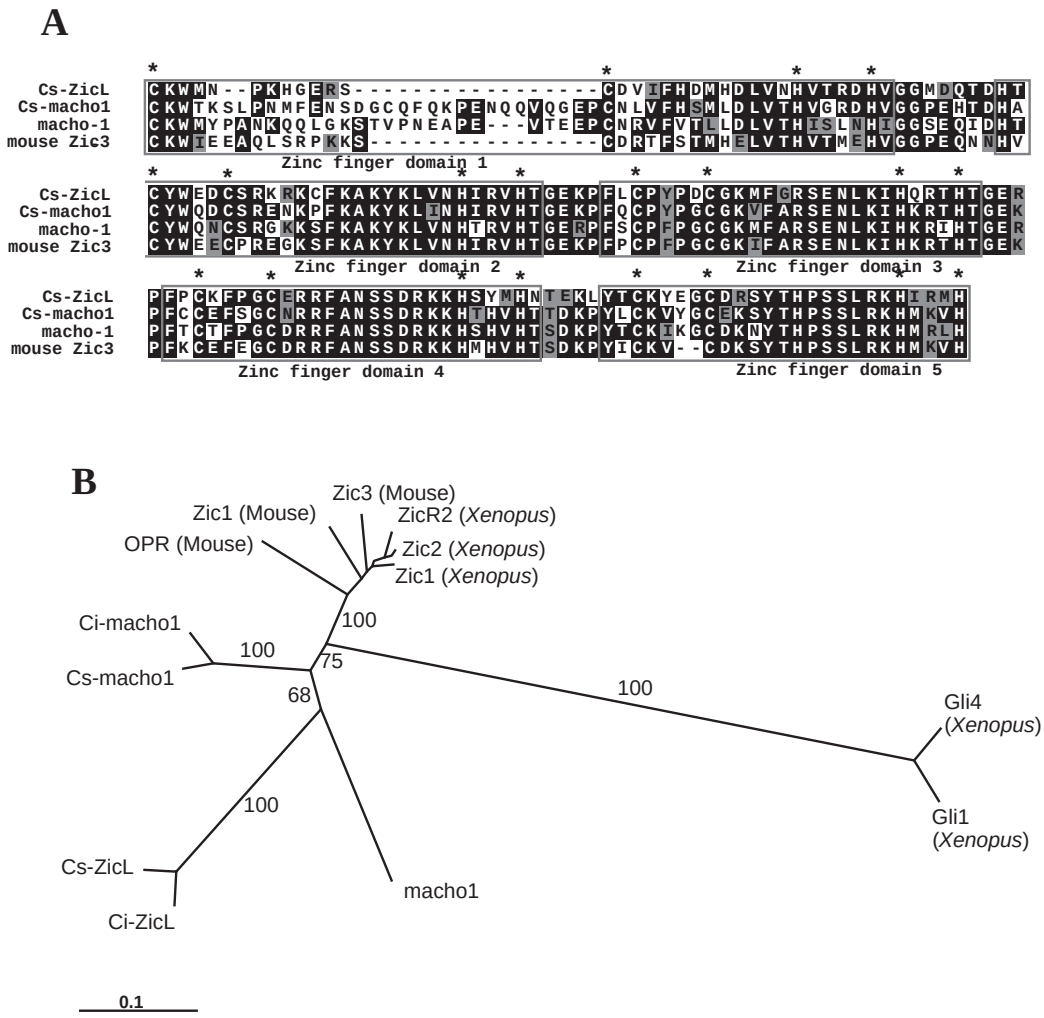

Fig. 2. Molecular relationship of Cs-ZicL with Zic-related proteins. (A) Alignment of amino acid sequences of the zinc finger domains (boxed) of Cs-ZicL, Cs-macho1, macho-1, and mouse Zic3. Identical residues are shaded black, and similar residues are shaded gray. Cysteine and hystidine residues conserved in Zic-related proteins are indicated by asterisks. (B) Unrooted molecular phylogenetic tree constructed by the neighbor-joining method using the zinc-finger domain sequences (Saitou and Nei, 1987).

(K. S. I., unpublished). *Cs-FoxD* is expressed very transiently in A5.1, A5.2 and B5.1 of the 16-cell stage embryo (Fig. 3A′) and A6.1, A6.3 and B6.1 of the 32-cell stage embryo (Fig. 3B′), and the expression is downregulated by the 64-cell stage. This transient expression of *Cs-FoxD* is not associated with endoderm differentiation but is essential for the differentiation of the notochord. Because *Cs-FoxD* is an essential factor for notochord formation, it is possible that the expression of *Cs-ZicL* in the presumptive A-line notochord cells is controlled by *Cs-FoxD*. As we expected, when *Cs-FoxD* function was blocked by the morpholino, the expression of *Cs-ZicL* was not found in A6.2 or A6.4 notochord/nerve cord lineage cells (100%, *n*=19; Fig. 6C), although the expression of *Cs-ZicL* in B6.2 and B6.4 muscle lineage cells was not affected (Fig. 6C). This result suggests that *Cs-ZicL* is downstream of *Cs-FoxD*. If so, ectopic expression of *Cs-FoxD* might induce *Cs-ZicL* expression in cells where it is not normally expressed. As shown in Fig. 6D, microinjection of in vitro synthesized *Cs-FoxD* mRNA into fertilized eggs resulted in ectopic expression of *Cs-ZicL* (90%, *n*=10). The ectopic expression was often seen in the a-line lineage of the anterior animal hemisphere in most of the embryos. These results indicate that in endodermal cells, nuclear accumulation of β-catenin directly triggers the expression of *Cs-FoxD*, which in turn induces the expression of *Cs-ZicL* in notochord/nerve cord lineage cells.

(b) CNS

The CNS of ascidian tadpole larvae consists of about 330 cells that include sensory receptor cells and neuronal cells in the so-called sensory vesicle (or brain), motoneurons in the visceral ganglion, and ependymal cells in the nerve cord (reviewed by Wada and Satoh, 2001; Meinertzhagen and Okamura, 2001). Cells of the sensory vesicle are derived from a-line cells, while cells of the b- and A-lines contribute to the formation of the visceral ganglion and the nerve cord.

Expression: In situ hybridization signals for *Cs-ZicL* expression in CNS-forming cells were first seen in the A6.2 and A6.4 pairs of the 32-cell stage embryo (Fig. 3B,B′), cells that have the developmental fate to form the nerve cord. Signals were then found in the A7.4 and A7.8 pairs at the 64-cell stage (Fig. 3C,C′). At the early gastrula stage, further zygotic expression of *Cs-ZicL* became evident in the a8.17, a8.19, a8.25, b8.17 and b8.19 pairs (Fig. 3D,D′), in addition to faint expression in the A8.7, A8.8, A8.15 and A8.16 pairs of the nerve cord cells (Fig. 3D,D′). The expression of *Cs-ZicL* in the CNS is evident in the mid-gastrulae (Fig. 3E) and in the anterior tip cells of neurulae (Fig. 3F). No signal was detected at the early tailbud embryo stage (Fig. 3G).

Function: *Cs-ZicL* is expressed in cells of the CNS, suggesting its role in the differentiation of CNS cells. Therefore, we examined the differentiation of the nervous

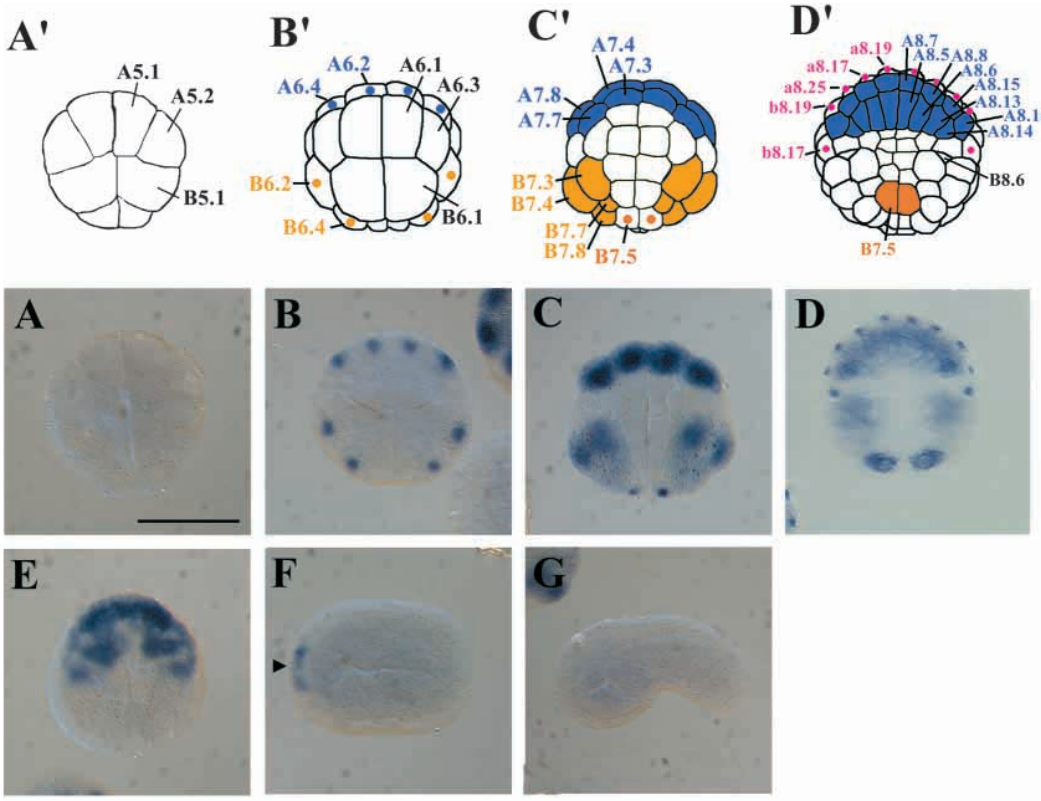

Fig. 3. Zygotic expression of *Cs-ZicL* in *Ciona savignyi* embryos, as revealed by whole-mount in situ hybridization. A′-D′ are drawings of A-D, respectively, to illustrate the *Cs-ZicL* expression. The dots indicate expression in the nucleus. (A) A 16-cell stage embryo, vegetal view with the anterior pole on top. Scale bar represents 100 μm for A-G. (B) A 32-cell stage embryo, vegetal view. Zygotic transcripts of *Cs-ZicL* appear in A6.2, A6.4, B6.2 and B6.4 cell-pairs. (C) A 64-cell stage embryo, vegetal view. Signals are evident in A7.3, A7.4, A7.7, A7.8, B7.3, B7.4, B7.7, B7.8 and B7.5 cell-pairs. (D) A 110-cell stage embryo, vegetal view. Zygotic transcripts are seen in a-line CNS cells and b-line CNS and muscle cells in addition to A-line nerve cord cells and B-line muscle cells. (E) A gastrula, vegetal view. Signals are evident in cells of the CNS. (F) A neurula, dorsal view, showing *Cs-ZicL* transcripts in a few anterior-most cells of the embryo (arrowhead). (G) An early tailbud embryos, lateral view. Signals have become undetectable.

system with a probe for *Cs-ETR*, which is a pan-neural marker of *C. savignyi* embryos (Fig. 7A). As seen in Fig. 7A′, the expression of *Cs-ETR* was greatly reduced in *Cs-ZicL* morpholino-injected embryos examined at the tailbud stage (100%, *n*=12). *Cs-ZicL*, therefore, has an important role in the differentiation of the nervous system.

(c) Muscle

During *Ciona* embryogenesis, 36 unicellular and striated muscle cells are formed in the larval tail: 18 cells on each side of the tail. Of these 36, 28 are derived from B-line cells, four from A-line and four from b-line cells. Regarding the B-line (or primary lineage), B6.2, B6.3 and B6.4 pairs in the 32-cell stage embryo are presumptive muscle cells. At the 64-cell stage, the B7.4 (a daughter cell of B6.2) and B7.8 (a daughter cell of B6.4) pairs are primordial muscle cells, while the B7.5 pair forms larval muscle and adult muscle.

Expression: The first in situ signals for *Cs-ZicL* expression were detected in the B6.2 and B6.4 pairs of the 32-cell stage embryo (Fig. 3B,B′). As mentioned above, these cells have the developmental fate to form muscle. Signals were next seen in the B7.3, B7.4, B7.7 and A7.8 pairs at the 64-cell stage (Fig. 3C,C′). In situ signals in these two muscle lines became faint by the gastrula stage (Fig. 3D,D′). The 64-cell stage embryo also expressed *Cs-ZicL* in the B7.5 pair (Fig. 3C,C′). Signals in B7.5 were strong at the early gastrula (Fig. 3D,D′) but became undetectable by the mid gastrula (Fig. 3E).

Function: The role of *Cs-ZicL* in the differentiation of muscle cells was examined by monitoring the expression of muscle actin gene *Cs-MA1* (Fig. 8A,A″). Because *Cs-ZicL* is expressed in all the B-line muscle cells, first in B6.2 and B6.4 at the 32-cell stage (Fig. 3B), and then in B7.5 at the 64-cell stage (Fig. 3C), it is highly likely that *Cs-ZicL* has a role in muscle cell differentiation. When we examined the expression of *Cs-MA1* in *Cs-ZicL* morpholino-injected embryos at the tailbud stage, a certain number of muscle cells in experimental embryos expressed *Cs-MA1* (100%, *n*=10; Fig. 8B″). Because we could not judge the exact number of muscle cells expressing *Cs-MA1*, we checked the expression of *Cs-MA1* in early-gastrula stage (Fig. 8B) and mid-gastrula stage embryos (Fig. 8B′). In both early-gastrula stage and mid-gastrula stage embryos, blastomeres derived from B6.2 (arrowheads in Fig. 8A,A′), which is located at the anterior-most position of all the B-line muscle cells, did not express *Cs-MA1* (96% of embryos at the early-gastrula stage, *n*=24, and 87% of embryos at the mid-gastrula stage, *n*=39; Fig. 8B,B′). However, muscle cells derived from B6.4 (arrows in Fig. 8A,A′) expressed *Cs-MA1* normally in *Cs-ZicL*-function-suppressed embryos (Fig. 8B,B′).

To examine whether this suppression is specifically caused by downregulation of *Cs-ZicL* function, we performed another rescue experiment. As in the case of notochord cells, co-injection of *Cs-ZicL* morpholino and *Cs-ZicL* synthetic mRNA rescued the expression of *Cs-MA1* in the B6.2-derived muscle cells (72%, *n*=50; Fig. 8C).

Possible cascade of *Cs-ZicL* function in muscle formation: Recently, Nishida and Sawada (Nishida and Sawada, 2001) isolated and characterized a Zic-like gene named *macho-1* from *Halocynthia roretzi* (see Fig. 2). *macho-1* is expressed only maternally and its mRNA shows a segregation pattern characteristic of the myoplasm. When

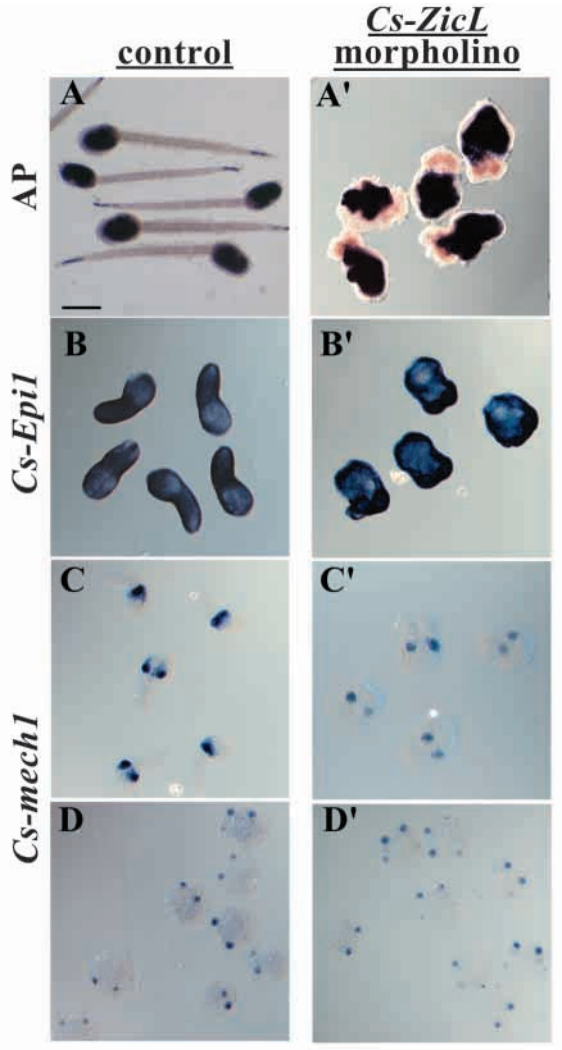

Fig. 4. Effects of suppression of *Cs-ZicL* function on differentiation of (A) endodermal cells, (B) epidermal cells, and (C,D) mesenchyme cells. (A,A′) Histochemical detection of endoderm-specific alkaline phosphatase (AP) activity, and in situ hybridization with probe for (B,B') epidermis-specific gene *Cs-Epi1* and (C,C′,D,D′) mesenchyme-specific gene *Cs-mech1*. (A-C) Control embryos and (A′-C′) embryos developed from eggs injected with *Cs-ZicL* morpholino. (D,D′) Expression of *Cs-mech1* in (D) control and (D′) experimental embryos developed from eggs injected with *Cs-ZicL* morpholino, arrested at the 110-cell stage with cytochalasin B. Scale bar (in A) represents 100 μm for all panels.

macho-1 function is suppressed with antisense oligonucleotides, B-line muscle cell differentiation is blocked. In contrast, when the gene is overexpressed by injection of synthetic mRNA, muscle cells are formed ectopically. Because *macho-1* is considered to be a muscle determinant, it is possible that *Cs-ZicL* is downstream of *macho-1*. Previous characterization of *Cs-macho1* of *Ciona savignyi* showed that its maternal expression pattern coincides with that of *macho-1*, although *Cs-macho1* is also expressed in the CNS zygotically (Satou et al., 2002).

When the expression of *Cs-ZicL* was examined in *Cs-macho1* morpholino-injected 32-cell stage embryos, it was evident that *Cs-ZicL* expression in B-line muscle cells was not affected (100%, *n*=21; Fig. 9A). This indicates that the expression of *Cs-ZicL* is controlled independently of *Cs-macho1*.

Our previous study indicated that suppression of *Cs-macho1* function resulted in blockage of *Cs-MA1* expression at the cleavage and early gastrula stages, but the blockage was not complete, and later transcripts of *Cs-MA1* appeared in muscle cells at the mid-gastrula and later stages (Satou et al., 2002). Therefore, we first re-examined this issue. As shown in Fig. 8D, the expression of *Cs-MA1* at the early-gastrula stage was almost completely inhibited in *Cs-macho1* morpholino-injected embryos (93%, *n*=26). However, *Cs-MA1* expression recovered in embryos by the mid-gastrula stage (80%, *n*=39; Fig. 8D′). This suggests that in *Ciona* embryos, pathway(s) independent of *Cs-macho1* are also responsible for muscle differentiation, and *Cs-ZicL* may be involved in these pathways. To examine this issue, we injected both *Cs-macho1* and *Cs-ZicL* morpholinos together into fertilized eggs. As shown in Fig. 9B,C, this injection completely suppressed the expression of *Cs-MA1* at the mid-gastrula stage (100%, *n*=26) and tailbud stage (100%, *n*=44). These results demonstrate that both *Cs-macho1* and *Cs-ZicL* are essential for muscle differentiation in *Ciona* embryos, and there are at least two independent genetic cascades for muscle differentiation, one that is *Cs-macho1*-dependent, and another that is *Cs-ZicL* dependent.

DISCUSSION

The present study demonstrated that *Cs-ZicL* of *Ciona savignyi* encodes a Zic-like zinc finger protein whose function is associated with the differentiation of three different types of embryonic cells: A-line notochord, CNS, and B-line muscle cells. During early embryogenesis, *Cs-ZicL* is expressed in blastomeres that give rise to these three types of tissue. The present study also demonstrated that the expression of *Cs-ZicL* in the A-line cells is downstream of β-catenin/*Cs-FoxD*, while the expression of *Cs-ZicL* in the B-line muscle cells is independent of another Zic-like gene, *Cs-macho1*.

Cs-ZicL and notochord differentiation

In ascidian embryos in situ hybridization signals for zygotic gene expression are usually first detected in the nucleus, and the signals become distributed throughout the cytoplasm as development proceeds (e.g., Yasuo and Satoh, 1993; Satou et al., 1995). Therefore, we can judge the timing of the gene expression as well as cells exhibiting the gene expression. Based on such criteria, it can be said that the zygotic expression of *Cs-ZicL* takes place in at least three embryonic domains. The signals were first evident in the nuclei of A-line notochord/nerve cord cells at the 32-cell stage (Fig. 3). At the 64-cell and 110-cell stages, the signals were seen in the cytoplasm of this lineage, but the expression was downregulated by the mid-gastrula stage. The fact that injection of cadherin mRNA into fertilized eggs suppressed the *Cs-ZicL* expression in the A-line notochord/nerve cord cells suggests that *Cs-ZicL* expression there is controlled by the nuclear accumulation of β-catenin. There are two possibilities regarding the relationship between β-catenin and *Cs-ZicL* expression: either β-catenin together with Tcf/LEF directly activates the *Cs-ZicL* expression or β-

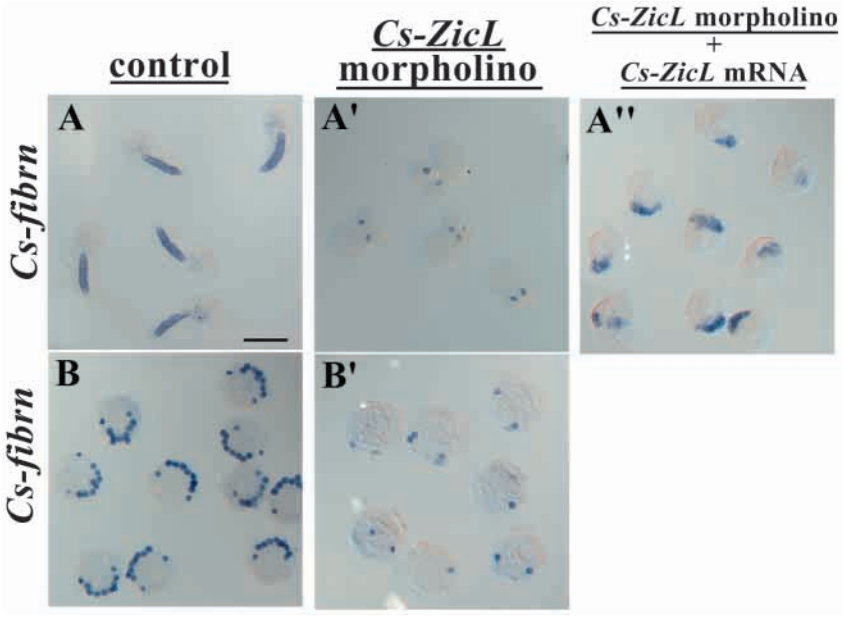

Fig. 5. Effects of suppression of *Cs-ZicL* function on differentiation of notochord cells. (A-A″) Expression of *Cs-fibrn* in (A) control embryos at the early-tailbud stage and (A′) experimental embryos developed from eggs injected with *Cs-ZicL* morpholino. (A″) Expression of *Cs-fibrn* in experimental embryos developed from eggs co-injected with *Cs-ZicL* morpholino and *Cs-ZicL* mRNA. (B,B′) Expression of *Cs-fibrn* in (B) control and (B′) experimental embryos developed from eggs injected with *Cs-ZicL* morpholino, arrested at the 110-cell stage. Scale bar (in A) represents 100 μm for all panels.

catenin indirectly activates the *Cs-ZicL* expression via other molecules. Here we examined the latter possibility by asking whether Cs-*FoxD*, which is a direct target of the nuclear accumulation of β-catenin (K. S. I., unpublished), communicates between the two molecules. Injection of *Cs-FoxD* morpholino into fertilized eggs resulted in the failure of *Cs-ZicL* expression, and injection of *Cs-FoxD* mRNA into fertilized eggs resulted in ectopic expression of *Cs-ZicL*. Therefore, it is highly likely that *Cs-ZicL* is downstream of *Cs-FoxD*. However this does not exclude the first possibility. It is possible that a combinational regulation by β-catenin and *Cs-FoxD* (or its target gene) is required for the expression of *Cs-ZicL*.

At the 16-cell stage, *Cs-FoxD* is expressed in A5.1, A5.2 and B5.1 (Fig. 3A′), while *Cs-ZicL* is not expressed at this stage. At the 32-cell stage, *Cs-FoxD* is expressed in A6.1, A6.3 and B6.1, while *Cs-ZicL* is expressed in A6.2, A6.4, B6.2 and B6.4 (Fig. 3B′). Apparently, the expression of these two genes does not overlap in the 32-cell stage embryo. However, A6.2 and A6.4, which express *Cs-ZicL*, are daughter cells of A5.1 and A5.2, respectively, which express *Cs-FoxD* in the 16-cell stage embryo. Therefore, it is likely that *Cs-FoxD* expression in the 16-cell stage embryo may trigger the activation of *Cs-ZicL* in one daughter cell that gives rise to A6.2 or A6.4, although FoxD proteins are known to be as a transcriptional repressor in *Xenopus* (Pohl and Knöchel, 2001; Sullivan et al., 2001).

An alternative explanation may be that *Cs-FoxD* expression in A6.1 and A6.3 at the 32-cell stage induces *Cs-ZicL* expression in A6.2 and A6.4 through cell-cell communication. This possibility is discussed below. In *Halocynthia* embryos, it has been shown that interaction between the A6.1/A6.3 (primordial endoderm cells) and A6.2/A6.4 (cells with developmental fate to give rise to notochord) takes place in the latter half of the 32-cell stage (Nakatani and Nishida, 1994), and this interaction activates *Brachyury* expression in the primordial notochord cells at the 64-cell stage (Nakatani et al., 1996). In ascidians, *Brachyury* is a key regulatory gene for notochord formation (Yasuo and Satoh, 1993; Yasuo and Satoh, 1998). Therefore, molecular events that take place at the 16-cell or 32-cell stages and to eventually activate *Brachyury* expression are key to understanding the mechanisms of notochord induction. In a previous study, we characterized *Cs-FGF4/6/9* cDNA as a possible notochord inducer, and showed that the corresponding gene is expressed in the endoderm and

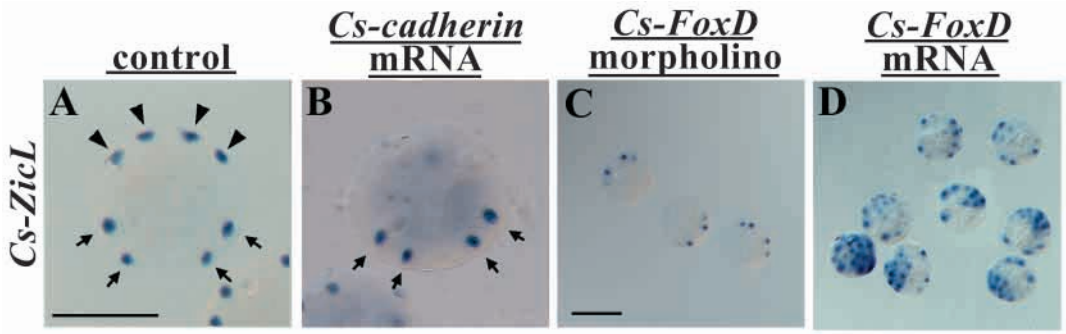

Fig. 6. Relationship between *Cs-ZicL*, β-catenin, and *Cs-FoxD*. (A) Control embryos at the 32-cell stage showing *Cs-ZicL* expression in two pairs of A-line cells (arrowheads) and two pairs of B-line cells (arrows). Scale bar represents 100 μm. (B) Cadherin-overexpressing embryos at the 32-cell stage, showing *Cs-ZicL* expression in the two pairs of B-line cells (arrows), but not in the two pairs of A-line cells. (C) *Cs-ZicL* expression is found only in the two pairs of B-line cells in the 32-cell stage embryos developed from eggs injected with *Cs-FoxD* morpholino. Scale bar represents 100 μm. (D) Ectopic *Cs-ZicL* expression in many cells in the 32-cell stage embryos developed from eggs injected with *Cs-FoxD* mRNA.

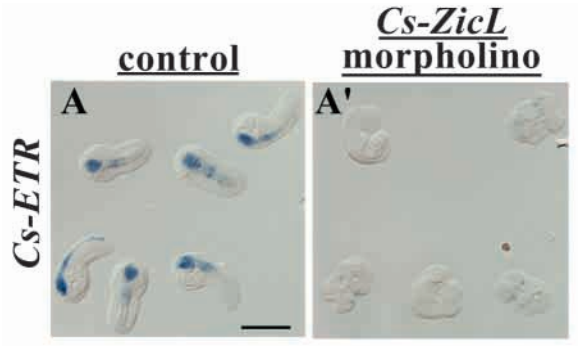

Fig. 7. Effects of suppression of *Cs-ZicL* function on differentiation of cells of the CNS. (A) Control embryos showing expression of CNS-specific *Cs-ETR* gene. Scale bar represents 100 μm. (A′) Experimental embryos developed from eggs injected with *Cs-ZicL* morpholino.

notochord cells at the 32-cell stage (Imai et al., 2002). *Cs-FGF4/6/9* is essential for the induction of mesenchyme. Although the expression of *Cs-fibrn* was reduced in *Cs-FGF4/6/9* morpholino-injected embryos, the function of *Cs-FGF4/6/9* in the notochord induction was partial. In addition, *Cs-FGF4/6/9* is not downstream of *Cs-FoxD* nor upstream of *Cs-ZicL*, suggesting that the function of *Cs-FGF4/6/9* in the formation of the notochord is independent of the *Cs-FoxD/Cs-ZicL* cascade. Therefore the notochord inducer downstream of *Cs-FoxD* is still unknown. Whether Cs-FoxD activates the expression of *Cs-ZicL* directly or via an unknown inducer should be determined in future studies. It should be noted here that *Cs-ZicL* is necessary for A-line notochord formation, but is not involved in B-line notochord formation. *Cs-FoxD* is expressed in both A-line and B-line endoderm cells and its function is essential for the differentiation of both A-line and B-line notochord cells. Therefore, in the formation of the B-line notochord, a different genetic cascade, which does not

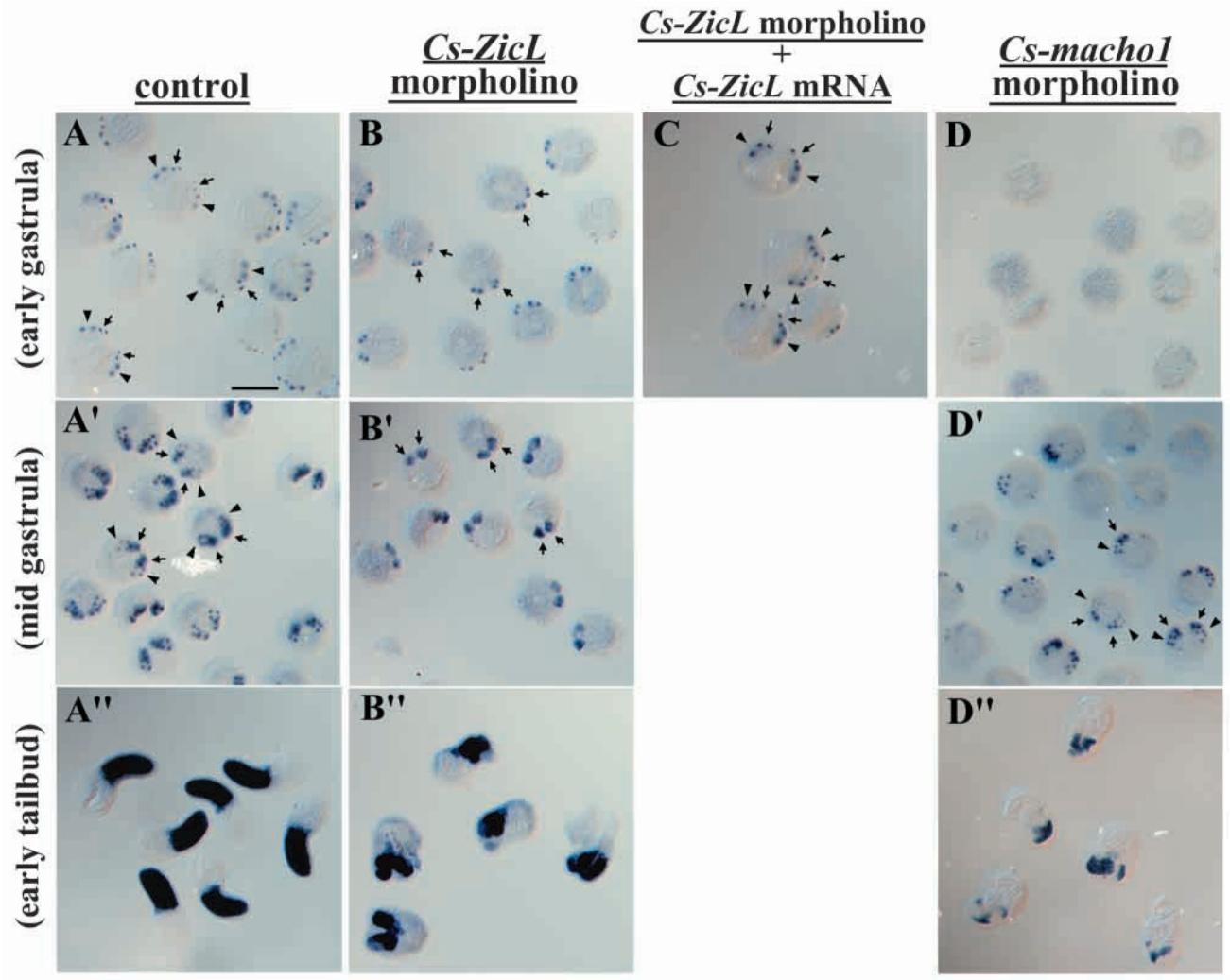

Fig. 8. Effects of functional suppression of *Cs-ZicL* and/or *Cs-macho1* on the differentiation of muscle cells assessed by in situ hybridization with a probe for muscle actin gene *Cs-MA1*. (A-A″) Control embryos at (A) the early-gastrula stage, (A′) mid-gastrula stage and (A″) early-tailbud stage. (B-B″) *Cs-ZicL* morpholino-injected embryos at (B) the early-gastrula stage, (B′) mid-gastrula stage and (B″) early-tailbud stage. (C) Experimental embryos developed from eggs co-injected with *Cs-ZicL* morpholino and *Cs-ZicL* mRNA, showing recovery of *Cs-MA1* expression in B8.7 and B8.8 cells (arrowheads). (D-D″) *Cs-macho1* morpholino-injected embryos at (D) the early-gastrula stage, (D′) mid-gastrula stage and (D″) early-tailbud stage. Arrowheads indicate the expression of *Cs-MA1* in B6.2-derived muscle cells and arrows indicate the expression in B6.4-derived muscle cells. Scale bar (in A) represents 100 μm for all panels.

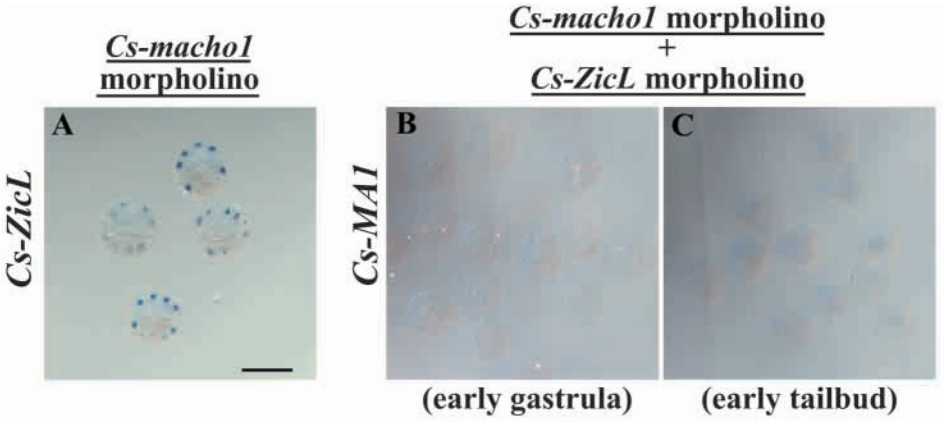

Fig. 9. Relationship between *Cs-macho1* and *Cs-ZicL*. (A) The 32-cell stage embryos developed from eggs injected with *Cs-macho1* morpholino, showing that *Cs-ZicL* expression is not affected by functional inhibition of *Cs-macho1*. Scale bar represents 100 μm. (B,C) Expression of *Cs-MA1* is suppressed in (B) early-gastrulae and (C) early-tailbud embryos, developed from eggs co-injected with *Cs-macho1* morpholino and *Cs-ZicL* morpholino.

include *Cs-ZicL*, exists between the expression of *Cs-FoxD* and *Cs-Bra*.

Cs-ZicL and muscle differentiation

Cs-ZicL is expressed in two anterior B-line muscle cells, B6.2 and B6.4, at the 32-cell stage, and later at the 64-cell stage the gene is expressed in posterior B-line muscle cells, B7.5 (Fig. 3). Injection of *Cs-ZicL* morpholio resulted in the failure of *Cs-MA1* expression in B6.2-line muscle cells (Fig. 8), suggesting that the initiation of the muscle-specific structural gene in B6.2 is controlled by *Cs-ZicL*.

Cs-macho1 is a *C. savignyi* homolog of *Halocynthia macho-1*, which is a maternal muscle determinant gene (Nishida and Sawada, 2001). The maternal transcript of *Cs-macho1* is distributed like a *posterior end mark* (Satou et al., 2002); it is localized to the posterior-most blastomeres throughout early embryogenesis (Yoshida et al., 1996; Satou and Satoh, 1997). However, Cs-macho1 protein is expected to be distributed in all the B-line muscle cells, because injection of *Cs-macho1* morpholino blocks the initiation of *Cs-MA1* expression in all of the B-line muscle cells (Fig. 8D). Therefore, the distribution of the products of the *Cs-macho1* and *Cs-ZicL* genes may be overlapping in B-line muscle cells. As mentioned above, embryos injected with *Cs-macho1* morpholino cannot initiate the expression of *Cs-MA1* in any of the B-line muscle cells, while embryos injected with *Cs-ZicL* morpholino failed to initiate *Cs-MA1* expression only in B6.2-line muscle cells. This suggests that the activity of *Cs-macho1* is required for the initiation of *Cs-MA1* expression in B6.4 and B7.5, and the combined activity of *Cs-macho1* and *Cs-ZicL* is sufficient for the initiation of *Cs-MA1* expression in B6.2.

However, even if the initiation of *Cs-MA1* expression is blocked by *Cs-macho1* morpholino, transcripts of *Cs-MA1* are later detected in muscle cells, suggesting that in *Ciona* embryos, *Cs-macho1* is required but not sufficient for *Cs-MA1* expression. In other words, the initiation of *Cs-MA1* expression is governed by *Cs-macho1* but the activity of other genes such as *Cs-ZicL* is required for the *Cs-MA1* expression. Actually, muscle cells are not formed when the function of both genes is suppressed with morpholinos, indicating that *Cs-macho1* is not the only muscle determinant and there are other muscle determinants govering the expression of *Cs-ZicL* in muscle cells in *Ciona* embryogenesis. In *Halocynthia, macho-1* is essential and sufficient for the muscle cell differentiation (Nishida and Sawada, 2001), suggesting that determination mechanism of the muscle cells are slightly different in these two species. It will be interesting to study how these two different mechanisms were evolved.

Cs-macho1 and Cs-ZicL share highly similar zinc finger domains. It is also of interest to ask what are their own specific functions, and how do they cooperate with each other. One possibility is that these two factors recognize the same binding sequece and control the same genes. In this case, Cs-ZicL seems to work as a backup factor of Cs-macho1. Another possibility is that Cs-ZicL and Cs-macho1 do not share their recognition sequences. Our preliminary results showing these two factors have different recognition sequences (K. Yagi, N. S. and Y. S., unpublished data) support this hypothesis. In this latter case, two different pathways using Zic-like factors are working in muscle differentiation.

Control of *Cs-ZicL* expression

Cs-ZicL is expressed in embryonic domains that give rise to three different cell types. The first is A-line notochord/nerve cord cells at the 32-cell stage, the second is B-line muscle cells at the 32-cell stage, and the third is CNS cells at the 110-cell stage. In addition, *Cs-ZicL* expression in the two lineages at the 32-cell stage is regulated by independent mechanisms. The expression of *Cs-ZicL* in the A-line notochord/nerve cord cells at the 32-cell stage is downstream of β-catenin/*Cs-FoxD* but independent of *Cs-macho1*. The expression of *Cs-ZicL* in the B-line muscle cells at the 32-cell stage is not regulated by β-catenin/*Cs-FoxD*, and is also independent of *Cs-macho1*. It is very important to determine the *cis*-regulatory elements of *Cs-ZicL*, and this analysis is now being conducted. *Cs-ZicL* has at least three different regulatory functions in embryogenesis: the differentiation of the notochord, the central nervous system and muscle cells. This means that the same factor can work in different ways depending on the context of the cells. Therefore, *Cs-ZicL* provides a good experimental system for studying how the same factor recognizes different targets in different cell lineages.

Zic-like genes in ascidians and vertebrates

The molecular phylogenetic analysis based on the comparison of amino acid sequences of the zinc finger domains demonstrated that mouse and *Xenopus Zic*-related genes form one clade, from which *Cs-macho1, macho-1* and *Cs-ZicL* are distant (Fig. 2B). Blast search indicated that the zinc finger domains of Cs-ZicL and Cs-macho1 show the highest

similarity to those of vertebrate Zic family gene products. In addition, a *Ciona intestinalis* EST project has characterized nearly 150 zinc finger transcription factor genes (L. Yamada, N. S. and Y. S., unpublished data), and only *Ci-ZicL* and *Ci-macho1* have high similarity to vertebrate *Zic* family genes. This indicates that *Cs-ZicL* and *Cs-macho1* and vertebrate *Zic* might have originated from a common ancestral gene. In ascidians, the expression and function of this gene diverged as *macho-1* for a maternal muscle determinant gene, while *ZicL* diverged as a zygotic gene with multiple functions.

The expression pattern of *Cs-ZicL* resembles that of vertebrate *Zic*. It has been reported that the expression and function of vertebrate *Zic* genes are mainly associated with the nervous system. However, some vertebrate *Zic* genes are also expressed in embryonic mesoderm. For example, *Xenopus Zic3* is expressed in involuting mesoderm at the early gastrula stage (Kitaguchi et al., 2000), and mouse *Zic1, Zic2*, and *Zic3* are expressed in embryonic mesoderm at the early primitive streak stage (Nagai et al., 1997). Because the present study demonstrated the function of *Cs-ZicL* in mesoderm formation, the function of vertebrate *Zic* genes in mesoderm formation should be addressed in future studies.

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