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HrzicN, a new *Zic* family gene of ascidians, plays essential roles in the neural tube and notochord development

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SUMMARY

Two axial structures, a neural tube and a notochord, are key structures in the chordate body plan and in understanding the origin of chordates. To expand our knowledge on mechanisms of development of the neural tube in lower chordates, we have undertaken isolation and characterization of *HrzicN*, a new member of the *Zic* family gene of the ascidian, *Halocynthia roretzi*. *HrzicN* expression was detected by whole-mount in situ hybridization in all neural tube precursors, all notochord precursors, anterior mesenchyme precursors and a part of the primary muscle precursors. Expression of *HrzicN* in a- and b-line neural tube precursors was detected from early gastrula stage to the neural plate stage, while expression in other lineages was observed between the 32-cell and the 110-cell stages. *HrzicN* function was investigated by disturbing translation using a morpholino antisense oligonucleotide. Embryos injected with *HrzicN* morpholino ('*HrzicN* knockdown embryos') exhibited failure of neurulation and tail elongation, and developed into larvae without a neural tube and notochord. Analysis of neural marker gene expression in *HrzicN* knockdown embryos revealed that *HrzicN* plays critical roles in distinct steps of neural tube formation in the a-line- and A-line precursors. In particular *HrzicN* is required for early specification of the neural tube fate in A-line precursors. Involvement of *HrzicN* in the neural tube development was also suggested by an overexpression experiment. However, analysis of mesodermal marker gene expression in *HrzicN* knockdown embryos revealed unexpected roles of this gene in the development of mesodermal tissues. *HrzicN* knockdown led to loss of *HrBra* (*Halocynthia roretzi Brachyury*) expression in all of the notochord precursors, which may be the cause for notochord deficiency. *Hrsna* (*Halocynthia roretzi snail*) expression was also lost from all the notochord and anterior mesenchyme precursors. By contrast, expression of *Hrsna* and the actin gene was unchanged in the primary muscle precursors. These results suggest that *HrzicN* is responsible for specification of the notochord and anterior mesenchyme. Finally, regulation of *HrzicN* expression by FGF-like signaling was investigated, which has been shown to be involved in induction of the a- and b-line neural tube, the notochord and the mesenchyme cells in *Halocynthia* embryos. Using an inhibitor of FGF-like signaling, we showed that *HrzicN* expression in the a- and b-line neural tube, but not in the A-line lineage and mesodermal lineage, depends on FGF-like signaling. Based on these data, we discussed roles of *HrzicN* as a key gene in the development of the neural tube and the notochord.

Key words: *Halocynthia roretzi*, Morpholino antisense oligonucleotide, Neural tube, Notochord, *Zic* family gene

INTRODUCTION

Two axial structures, a neural tube and a notochord, are characteristics of chordates and therefore, information about developmental mechanisms of the neural tube and the notochord in lower chordates is essential for an understanding of the origin of the chordate body plan (Satoh and Jeffery, 1995).

The neural tube of ascidian larvae is composed of about 340 cells, and is divided into three regions along the anteroposterior axis, which are, from anterior to posterior, the sensory vesicle, the visceral ganglion and the caudal neural tube (Nicol and Meinertzhagen, 1991). The sensory vesicle is composed solely of the a-line (anterior-animal) cells (Nishida, 1987). The visceral ganglion present at the junction between the trunk and tail consists of the A-line (anterior-vegetal) cells. The caudal neural tube running along the length of the tail consists of four (dorsal, ventral and two lateral) rows of ependymal cells: the lateral and ventral cells are of A-line origin and the dorsal cells are of b-line (posterior-animal) origin. Beneath the neural tube, a stack of exactly 40 notochord cells runs along the tail. The anterior 32 cells (primary notochord) and the posterior 8 cells (secondary notochord) are derived from A-line and B-line cells, respectively (Nishida, 1987).

Cellular interactions that specify the neural tube and notochord of ascidian embryos have been extensively

demonstrated. Specification mechanisms of neural tube differ between the a- and b-line precursors and A-line precursors. The a-line neural tube precursors require an inductive influence from the vegetal hemisphere cells to form the sensory vesicle (Nishida and Satoh, 1989; Okado and Takahashi, 1990). Upon disturbance of the induction, they adopt epidermal fate, like most other animal hemisphere cells. Although timing of the induction has not been fully understood, it is likely that the induction starts at the 16-cell stage and becomes complete at the early gastrula stage, including multiple sequential steps (Darras and Nishida, 2001b; Nishida and Satoh, 1989; Okado and Takahashi, 1990). The b-line precursors also require an inductive influence from the vegetal cells to differentiate into the caudal neural tube cells (Hudson and Lemaire, 2001). In the induction of a- and b-line neural tube cells, an FGF-like signaling pathway is likely involved, since human recombinant basic FGF mimics the inductive activity of the vegetal hemisphere, and block FGF signaling leads to inhibition of the sensory vesicle formation (Darras and Nishida, 2001b; Hudson and Lemaire, 2001; Inazawa et al., 1998; Kim and Nishida, 2001). This situation is very reminiscent of neural induction in vertebrates. By contrast, specification mechanisms of the A-line neural tube cells seems to be unique (Minokawa et al., 2001). At the 32-cell stage, anterior-most A-line vegetal cells (A6.2 and A6.4 blastomere pairs) have both neural tube and notochord fates, which separate into the daughter cells after the next cleavage. Anteriorly located daughters succeed to the neural tube fate while posterior ones that contact the endoderm precursors assume the notochord fate. A-line neural tube fate is specified autonomously without any cellular interaction (Minokawa et al., 2001).

A-line notochord fate, however, requires inductive influence from the endoderm precursors or the neighboring notochord precursors, which can be mimicked by basic FGF (Darras and Nishida, 2001a; Kim and Nishida, 2001; Nakatani and Nishida, 1994; Nakatani et al., 1996; Shimauchi et al., 2001). Interestingly, all descendants of the isolated A6.2 or A6.4 blastomeres adopt the notochord fate when treated with basic FGF, and conversely, they all adopt the neural tube fate in the absence of the induction. Therefore, binary choice of the alternative fates is involved in specification of the A-line neural tube and the notochord (Minokawa et al., 2001). In contrast to our knowledge about cellular interactions involved in specification of the neural tube, little is known about transcription factors that participate in this process.

Zic was originally identified as a gene encoding zinc finger protein that is expressed abundantly in the adult mouse cerebellum (Aruga et al., 1994). In vertebrates, multiple *Zic* family genes are known: for example, at least six distinct *Zic* family genes have been identified in *Xenopus* (Nakata et al., 2000) and at least four in mouse (Aruga et al., 1996). Vertebrate *Zic* family genes so far identified are very similar to one another both in structure and expression pattern during development (Nagai et al., 1997; Nakata et al., 1998). At the gastrula stage, *Zic* family genes are expressed throughout the presumptive neural plate. Their expression becomes restricted to the lateral edges of the neural plate at the neurula stage and persists in the dorsal region of the forebrain and the midbrain, the roof plate of the spinal cord, the migratory neural crest and additionally in developing somites and limb buds. The expression pattern of vertebrate *Zic* family genes suggests their early roles in neural and neural crest development. In accordance with this, overexpression experiments show that *Xenopus Zic* genes promote differentiation of neural and neural crest derived tissues (Nakata et al., 1998). In mouse, knockout of *Zic1* leads to aplasia of cerebellum and skeletal abnormalities (Aruga et al., 1998). Mutation in *Zic2* and *Zic3* in mouse and/or human cause holoprosencephaly and heterotaxis, respectively (Klootwijk et al., 2000; Nagai et al., 2000). These mutant phenotypes seem to be much weaker than expected from the results of overexpression experiments.

In ascidians, *macho-1*, a recently identified muscle determinant, encodes a zinc finger protein with the zinc finger domain most similar to that of *Zic* family genes (Nishida and Sawada, 2001). Transcripts of *macho-1* are supplied to the eggs maternally, segregated into the primary (the B-line) muscle cells through rounds of cleavage, and have been shown to be necessary and sufficient for primary muscle formation. Thus expression and function of *macho-1* are quite different from those of vertebrate *Zic* family genes. So far, no ascidian *Zic* family gene other than *macho-1* has been reported.

In the present study, toward understanding of the neural development in ascidian embryos, we addressed whether there is another *Zic* family gene that may have a role in the neural development. We have cloned *HrzicN*, a new *Zic* family gene of the ascidian, *Halocynthia roretzi* and studied expression, function and regulation of *HrzicN*. We here report that *HrzicN* plays an essential role in neural tube development. Unexpectedly, we have also found that this gene is required for the formation of the notochord and anterior mesenchyme, representing a novel function of *Zic* genes. Thus, the present study has established that *HrzicN* is a key gene in the development of the two axial structures in the ascidian embryo.

MATERIALS AND METHODS

Embryos

Adult ascidians, *Halocynthia roretzi*, were obtained from fishermen near Asamushi Marine Biological Station, Tohoku University, Aomori, Japan and Otsuchi Marine Research Center, Ocean Research Institute, University of Tokyo, Iwate, Japan. Naturally spawned eggs were fertilized with a suspension of sperm from other individuals. Fertilized eggs were raised at 11-13°C in Millipore-filtered seawater containing 100 μg/ml streptomycin and 100 units/ml penicillin. Nomenclature of the cell lineage is according to Nishida (Nishida, 1987).

Molecular cloning of *HrzicN*

PCR was carried out to amplify DNA fragments of a conserved region within the zinc finger domain of *Zic* family genes. The nucleotide sequences for forward and reverse primers were 5′-GCGAATTCTT(CT)AA(AG)GC(ACGT)AA(AG)TA(CT)AA-3′ and 5′-CGCTGCAGTG(ACGT)AC-(CT)TTCAT(AG)TG(CT)TT-3′, respectively. PCR reaction was carried out through 40 cycles of denaturing at 94°C for 1 minute, annealing at 48°C for 1 minute and elongation at 63°C for 2 minutes using *Halocynthia roretzi* genomic DNA as template. The PCR products were sequenced and two types of candidate clones for *Zic* family genes were identified. One was of *macho-1* and the other represented a novel gene. Therefore, this was used as a probe for screening of a neurula cDNA library. A cDNA clone was isolated and sequenced. 5′- and 3′-most regions for the cDNA were isolated by 5′- and 3′-RACE procedures, respectively, using the RACE System (Gibco BRL).

Construction of expression plasmids for in vitro transcription of mRNA

To generate the expression plasmid for *HrzicN*, a full length *HrzicN* cDNA was cloned into pBluescriptRN3 (Lemaire et al., 1995). To generate the expression plasmid for *lacZ* (pRN3/lacZ), full length *lacZ* of pSV-β-Gal was cloned into pBluescriptRN3. To generate the expression plasmid for *HrzicN/lacZ*, the 5′ UTR and the initiation codon of *lacZ* cDNA were substituted with the 5′ UTR and the first 183 bp of *HrzicN* coding region amplified by PCR. To generate the expression plasmid for *lacZ/HrzicN*, the first 52nd base pair of *HrzicN* cDNA was substituted with the first 123 bp of *lacZ* cDNA. In vitro transcription was carried out using mMessage mMachine (Ambion) as described previously (Wada and Saiga, 1999b).

Design of morpholino oligonucleotide and microinjection

Morpholino oligonucleotides were obtained from Gene Tools. Sequences of HrzicNMO and HrzicNMO2 are 5′-GCTGTTGCGTATGCCATTTTTGCTT-3′ (the underline indicates sequence complimentary to the putative initiation codon) and 5′-ATTCGCTCAATTAAATTACTGTTGT-3′, respectively. As a negative control, ‘standard control oligo’ supplied by Gene Tools was used. Microinjection was carried out as described previously (Wada and Saiga, 1999b). Synthetic RNA and morpholino oligonucleotides to be injected were dissolved in distilled water and 0.1× TE, respectively. Each microinjection experiment was conducted twice or more.

To test the ability of HrzicNMO to inhibit translation, approximately 50 pg of *HrzicN/lacZ* mRNA was injected into fertilized eggs either with or without HrzicNMO (final concentration: 10 μM). Cleavage of the injected embryos was inhibited by treatment with cytochalasin B (Hirano et al., 1984). The injected embryos were tested for β-galactosidase activity at the middle tailbud equivalent stage as described previously (Hikosaka et al., 1994).

For the rescue experiment, a mixture of HrzicNMO (final concentration: 10 μM) and approximately 50 pg of *lacZ/HrzicN* mRNA was injected into fertilized eggs and the injected embryos were examined for *HrBra* expression at the 110-cell equivalent stage.

Treatment with MEK inhibitor

To inhibit the FGF-Ras-MAP kinase signaling pathway, the MEK (MAP kinase kinase) inhibitor (U0126, Promega) was used. Embryos were cultured in Millipore-filtered seawater containing 2 μM U0126 as described previously (Kim and Nishida, 2001).

Whole-mount in situ hybridization

Gene expression was visualized by whole-mount in situ hybridization as described previously (Wada et al., 1995).

RESULTS

Structure of *HrzicN*

The *HrzicN* cDNA (accession no. AB092643) encodes a protein of 468 amino acids with five zinc finger motifs (Fig. 1A). Over the zinc finger domain, HrzicN protein shows significant sequence similarity to the known members of the Zic family proteins. The sequence outside the zinc finger domain shows no similarity to

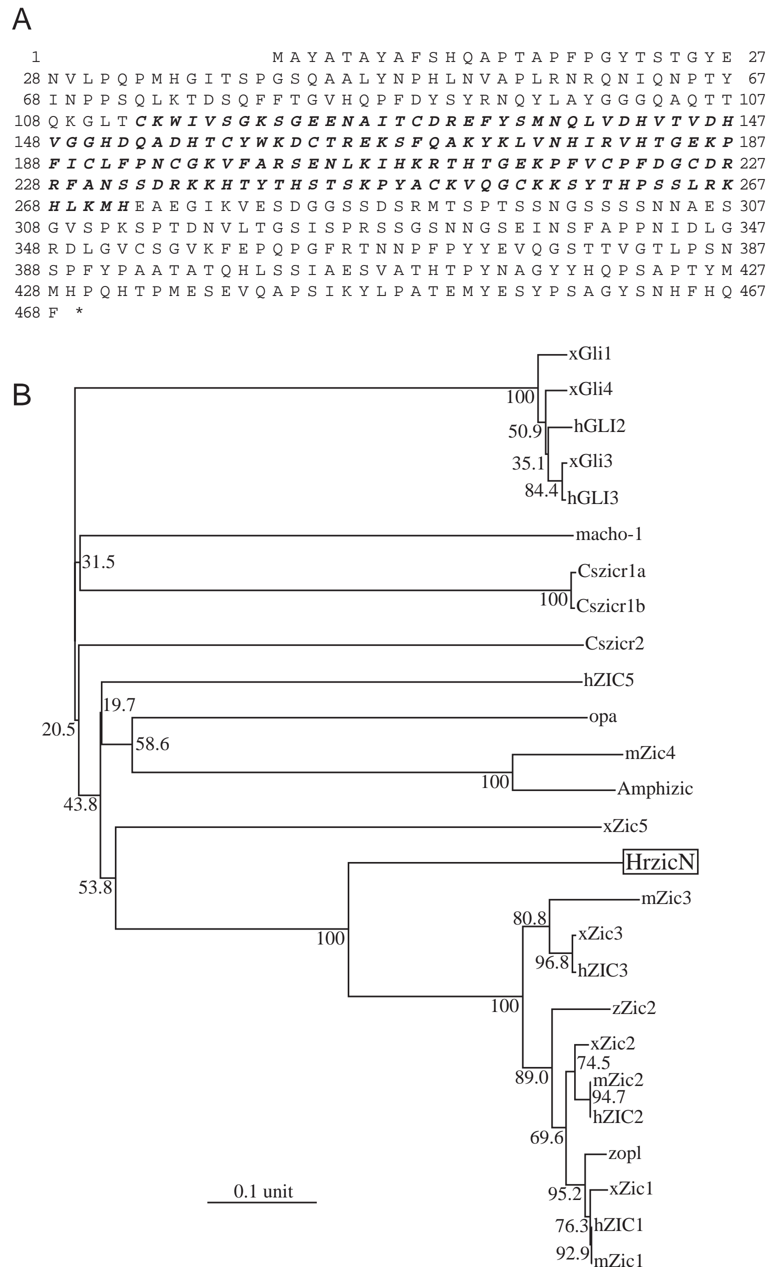

Fig. 1. Structure and phylogenetic analysis of *HrzicN*. (A) The putative amino acid sequence of *HrzicN*. Zinc finger motifs are italicized. (B) Molecular phylogenetic tree of *Zic* genes constructed using the amino acid sequences of the zinc finger motifs by the Neighbor-Joining method and the computer program, ClustalW. Numbers at the nodes represent bootstrap values. *macho-1* is another *Halocynthia Zic* gene isolated as a muscle determinant. *Cszicr1a*, *Cszicr1b* and *Cszicr2* are *Zic* genes of *Ciona savignyi*, another ascidian species. *opa* (*odd-paired*) is a *Zic* gene of *Drosophila*. *Amphizic* is an amphioxus *Zic* gene registered on the database. *xGli1*, *hGli2*, *xGli3*, *hGli3* and *xGli4* are members of vertebrate *Gli* family of zinc finger protein. Other genes given are vertebrate *Zic* family genes. DDBJ/GenBank accession numbers: AB092643(*HrZicN*), BAB19958(*macho-1*), AB057747(*Cszicrla*), AB057748(*Cszicr1b*), AB057740(*Cszicr2*), NP524228(*opa*), AJ252245(*Amphizic*), Q91690(*xGli1*), BAA25668(*hGli2*), Q91660(*xGli3*), CAB59315(*hGli3*), Q91661(*xGli4*), NM_009573(*mZic1*), NM_003412(*hZic1*), AB009564(*xZic1*), NM_009574(*mZic2*), NM_007129(*hZic2*), AB009565(*xZic2*), NM_131558(*zZic2*), NM_009575(*mZic3*), NM_003413(*hZic3*), AB005292(*xZic3*), NM_009576(*mZic4*), AK006747(*mZic5*), AF378304(*hZic5*), AB034983(*xZic5*).

the Zic or other known proteins, lacking a motif conserved between vertebrate Zic and *Drosophila* odd-paired proteins. To see whether *HrzicN* belongs to the *Zic* family, a phylogenic tree was constructed with amino acid sequences of zinc finger motifs of HrzicN and other Zic family proteins, using sequences of vertebrate Gli proteins (Ruiz i Altaba, 1999), which comprise a related family of zinc finger proteins as an outgroup (Fig. 1B). The tree supported close relationship between *HrzicN* and a group consisting of vertebrate *Zic1*, *2* and *3*. Therefore, it is highly likely that *HrzicN* represents a new member of *Zic* family.

Expression pattern of *HrzicN* during embryogenesis

The spatial and temporal expression pattern of *HrzicN* during larval development was examined by whole-mount in situ hybridization (Fig. 2). As described below, *HrzicN* expression was detected in the cells of neural and mesodermal lineages from the early 32-cell stage to the neural plate stage.

Expression in the neural lineage

The larval ascidian neural tube originates from the a4.2, b4.2 and A4.1 cell pairs at the 8-cell stage. *HrzicN* expression was evident in all three lineages of neural tube precursor cells. *HrzicN* expression was first detected in A6.2 and A6.4 blastomere pairs, each of which contains both neural tube and notochord fates, at the early 32-cell stage (Fig. 2A,F,R). At the 44- and 64-cell stages, *HrzicN* expression was found in their daughter cells, the A-line neural tube precursors (A7.4 and A7.8 pairs) and the A-line notochord precursors (A7.3 and A7.7 pairs) (Fig. 2B,C,G,H,R). The expression continued in these cell lineages until the 110-cell stage (Fig. 2D,E,I,J,R) but became undetectable by the early gastrula stage (Fig. 2K,P). At this stage, however, *HrzicN* expression started in all the a- and b-line neural tube precursors (a8.17, a8.19, a8.25, b8.17 and b8.19 pairs; Fig. 2K,P). The expression continued during gastrulation, disappeared by the early neurula stage (Fig. 2L-N,Q) and no longer detected afterwards (Fig. 2O).

Expression in the mesodermal lineage

As mentioned above, *HrzicN* was expressed in the primary notochord lineage cells at the early 32-cell stage through the 110-cell stage. In addition, *HrzicN* was expressed until the 110-cell stage in the B6.2 pair at the late 32-cell stage and their descendants (Fig. 2R), which develop into the secondary notochord, mesenchyme and primary muscle (Fig. 2B-E,G-J,R). Like the expression in the A-line cells, the expression in the B-line cells became undetectable by the early gastrula stage (Fig. 2K,P). In

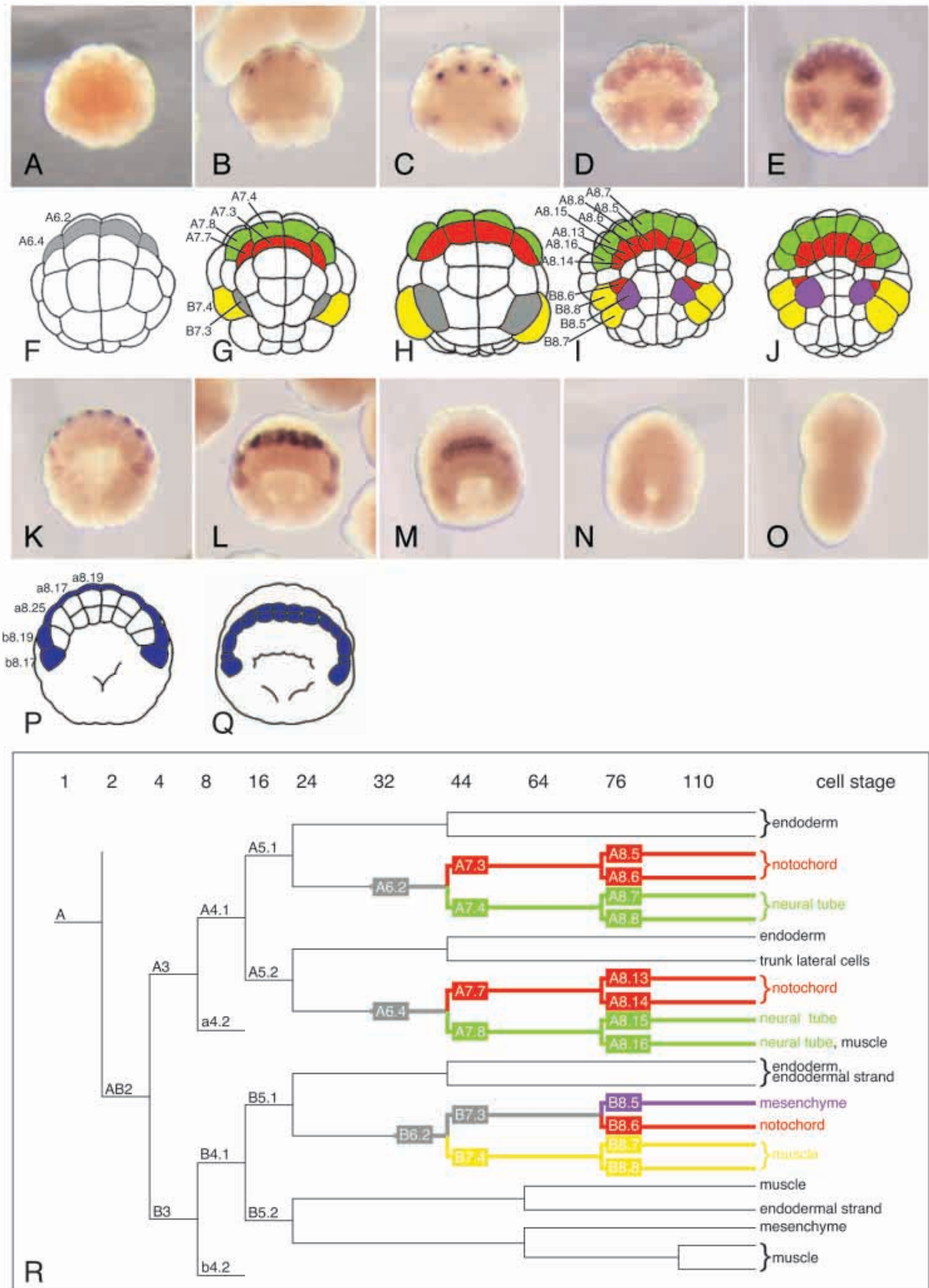

Fig. 2. Expression of *HrzicN* in development. (A-E,K-O) The expression pattern of *HrzicN* and (F-J,PQ) its schematic representation at, the early 32-cell stage (A,F), 44-cell stage (B,G), 64-cell stage (C,H), 76-cell stage (D,I), 110-cell stage (E,J), early gastrula stage (K,P), middle gastrula stage (L,Q), neural plate stage (M), early neurula stage (N) and early tailbud stage (O). (R) Summary of *HrzicN* expression in the various cell lineages up to the 110-cell stage. Lineages positive for *HrzicN* expression are indicated by bold lines.

summary, *HrzicN* expression was detected in all notochord precursors, one of the two pairs of mesenchyme precursors and two out of the five pairs of primary muscle precursors of the 110-cell stage embryo.

Phenotype induced by morpholino antisense oligonucleotide-based translational inhibition of *HrzicN*

Recently, morpholino antisense oligonucleotide-based translational inhibition was shown to be an effective tool for loss-of-function experiments in the ascidian embryos (Satou et al., 2001). We applied this technique to deduce functions of *HrzicN* during embryogenesis. A morpholino oligonucleotide we prepared (HrzicNMO) targets the initiation codon and its flanking regions. The ability of HrzicNMO to inhibit translation was assessed by examining the effect of HrzicNMO on translation of *HrzicN/lacZ* mRNA (a chimeric mRNA in which the 5′ UTR and the initiation codon of *lacZ* mRNA were substituted with the 5′ UTR and the first 183 nucleotides of *HrzicN* mRNA coding region) in cleavage-arrested embryos. As summarized in Table 1, HrzicNMO was capable of disturbing translation of the mRNA with its target site.

To investigate the effect of translational inhibition of *HrzicN* mRNA on ascidian development, we injected HrzicNMO into fertilized eggs to achieve a final concentration of 1, 5 and 10 μM and reared them up to the swimming larva equivalent stage. As a negative control, we injected the 'standard control oligo' supplied by Gene Tools and found that embryos injected at a final concentration of 10 μM or lower developed normally (Fig. 3C,D). Eggs injected with HrzicNMO at 1 or 5 μM developed into normal larvae (data not shown). However, almost all eggs injected with 10 μM developed into larvae with severe defects such as shortening of the tail, no differentiated notochord cells, failure of neural tube formation and lack of sensory pigment cells (Fig. 3B). However, protrusions, which are likely the adhesive organ, formed at the tip of the trunk (arrowhead in Fig. 3B). In the course of development, HrzicNMO-injected embryos seemed to be normal until early gastrula stage but delay in involution became evident in the later half of the gastrula stage (data not shown). The most notable abnormality was that they exhibited no sign of neurulation. Elongation of the tail was also inhibited, although a tail tip-like structure formed (Fig. 3A,B).

Table 1. Effect of HrzicNMO on β-galactosidase activity in cleavage-arrested embryos injected with *lacZ* or *HrzicN/lacZ* mRNA

Injected substances	Number of embryos that showed X-gal staining/Number of embryos injected (%)
lacZ	26/32 (81)
lacZ + HrzicNMO	38/52 (73)
HrzicN/lacZ	29/30 (97)
HrzicN/lacZ + HrzicNMO	3/56 (5.4)

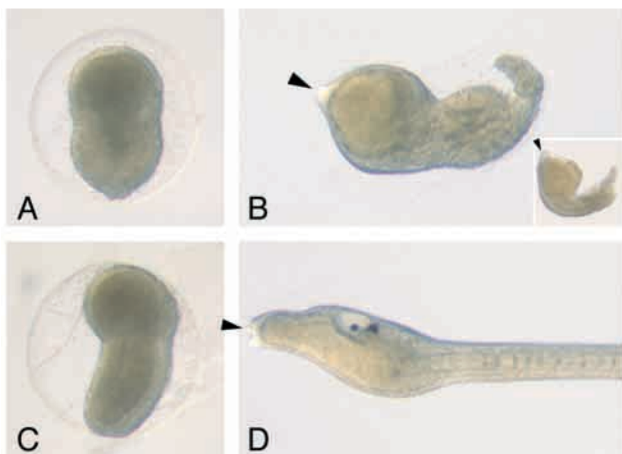

Fig. 3. Phenotypes of *HrzicN* knockdown embryos. (A,B) An embryo injected with HrzicNMO to give a final concentration of 10 μM. Inset in (B) shows an embryo injected with HrzicNMO2 to give a final concentration of 10 μM. (C,D) Embryos injected with the standard control oligo to give a final concentration of 10 μM. (A,C) Tailbud equivalent stage embryos. (B,D) Swimming larvae equivalent stage embryos. Arrowheads indicate protrusions of the adhesive organ. (A) Dorsal view. (B-D) Lateral view.

The phenotype generated by HrzicNMO injection seemed to be unique to translational inhibition of *HrzicN*. First, an essentially identical phenotype was observed upon injection of HrzicNMO2, another morpholino nucleotide against *HrzicN* with a different and non-overlapping target site from that of HrzicNMO (see inset in Fig. 3B). This phenotype was completely different from those observed upon injection of a morpholino oligonucleotide against *Hroth* (S. W. and H. S., unpublished) or β-catenin mRNA (S. W., K. W. Makabe and H. S., unpublished). Second, to verify specificity of effects of HrzicNMO, a rescue experiment was carried out using *lacZ/HrzicN* mRNA, in which a translational initiation site was provided by insertion of a *lacZ* fragment into the *HrZicN* plasmid DNA so as to shift the translation initiation site 81 bp upstream to the original initiation site of *HrzicN*. Thus, it is expected that mRNA from this construct is free of translational inhibition by HrzicNMO, since it has been shown that morpholinos that target more than a few bases 3′ to the initiation codon exhibits a quite low efficiency (Summerton, 1999). Co-injection of *lacZ/HrzicN* mRNA and HrzicNMO recovered *HrBra* expression otherwise lost by injection of HrzicNMO (Fig. 6B,G; for details about marker gene expression in HrzicNMO-injected embryos, see below).

Injection of HrzicNMO into eggs at higher than 10 μM resulted in a phenotype similar to that obtained by injection at 10 μM (data not shown), so that injection at 10 μM seemed to be sufficient for inducing a representative phenotype by HrzicNMO. Therefore, we refer to embryos injected with HrzicNMO at 10 μM at the 1-cell stage as "*HrzicN* knockdown embryos".

Neural tube differentiation is disturbed in *HrzicN* knockdown embryos

Since neurulation was blocked in *HrzicN* knockdown embryos, we investigated the neural tube development in these embryos by analyzing expression of neural markers. Initially, we assessed neural tube differentiation in *HrzicN* knockdown embryos at the early tailbud equivalent stage. First, we examined expression of a pan-neural marker, *HrETR-1* (Yagi and Makabe, 2001), which is expressed in the whole neural tube except for the dorsal and ventral walls of the caudal neural tube, and the peripheral neurons in the normal early tailbud

stage embryo (Fig. 4D). In *HrzicN* knockdown embryos, weak *HrETR-1* expression was found in superficial cells of the trunk that seemed to be presumptive a-line neural tube cells, but not in presumptive A-line neural tube precursors (Fig. 4A). Next, we examined expression of another neural marker, *HrTBB2* (*Halocynthia roretzi* β-tubulin gene) (Miya and Satoh, 1997), which is expressed in the neurons of the adhesive organ, the neural tube in the trunk and peripheral epidermal neurons in the tail (Fig. 4E). In *HrzicN* knockdown embryos, expression of *HrTBB2* was evident in the adhesive organ-forming region and the epidermal neurons, but expression in the neural tube-forming region was completely lost (Fig. 4B). We examined expression of *HrTRP* encoding the tyrosinase related protein (Sato et al., 1999) and *Hroth* (*Halocynthia roretzi otx* gene) (Wada et al., 1996). *HrTRP* is expressed in dorsal and lateral parts of the sensory vesicle in the normal early tailbud stage embryo (Fig. 4F), but it was not expressed in *HrzicN* knockdown embryos (Fig. 4C). *Hroth* expression was also abnormal in *HrzicN* knockdown embryos. In the normal early tailbud stage embryo (Fig. 4J) this gene is expressed in the sensory vesicle and the anterior epidermis. In *HrzicN* knockdown embryos, *Hroth* expression was lost from the sensory vesicle precursors, while it was detected only in the anterior epidermis (Fig. 4G). Together, these results indicate that differentiation of the neural tube is severely affected in *HrzicN* knockdown embryos.

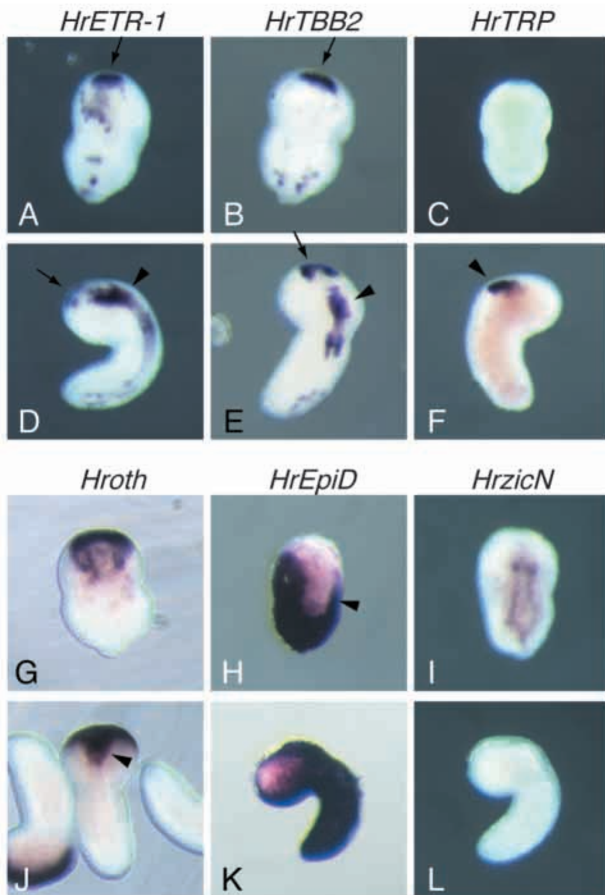

Fig. 4. Expression of epidermal and neuroectodermal markers in *HrzicN* knockdown embryos at the early tailbud equivalent stage. (A-C,G-I) Embryos injected with HrzicNMO. (D-F,J-L) Control uninjected embryos. Expression of *HrETR-1* (A,D), *HrTBB2* (B,E), *HrTRP* (C,F), *Hroth* (G,J), *HrEpiD* (H,K) and *HrzicN* (I,L) was shown. Arrowheads in D-F,J indicate gene expression in the neural tube, which is lost or severely reduced in *HrzicN* knockdown embryos. Arrowhead in H indicates putative neural tube precursor cells without *HrEpiD* expression. Arrows in A,B,D,E indicate gene expression in the adhesive organ, which is unaffected in *HrzicN* knockdown embryos. (A-E,G-J) A dorsal view. (F,K,L) A lateral view.

Neural fate specification occurs in a- but not A-line precursors

In the a- and b-line precursors, *HrzicN* expression starts at the early gastrula stage. Several lines of evidence suggest that fate choice between epidermis and neural tube fates occurs earlier than this stage (Darras and Nishida, 2001b; Ishida et al., 1996; Yagi and Makabe, 2001). However, in the A-line neural tube cells, *HrzicN* expression starts at the early 32-cell stage. Although it is unclear when the neural tube fate is established in these A-line cells, the onset of *HrzicN* expression is well before the appearance of neural properties such as *HrETR-1* expression (this starts at the 110-cell stage). To see when abnormality in the neural tube development arises in *HrzicN* knockdown embryos and whether there is a difference in this process between a-line cells and A-line cells, we next examined expression of these neural markers at earlier stages of development. At the 110-cell stage, in control embryos, *HrETR-1* is expressed in the A-line neural tube precursors and only weakly in the a-line neural tube precursors (Fig. 5F,G). At this stage, *Hroth* is also expressed in the a-line neural tube precursors (Fig. 5H-J). In *HrzicN* knockdown embryos, *HrETR-1* expression was evident in the a-line precursors but not in the A-line neural tube precursors (Fig. 5A,B). The a-line precursors of these embryos were also positive for *Hroth* expression (Fig. 5C-E). These results suggest that initial specification of the neural tube precursors occurs normally in a-line but not A-line precursors in *HrzicN* knockdown embryos.

We then examined expression of the markers at the neural plate equivalent stage. At this stage, in control embryos expression of *HrETR-1* and *Hroth* continues in the a- and A-line neural tube precursors and in the a-line precursors, respectively (Fig. 5Q,R). In the a-line precursors, *HrTRP* is also expressed (Fig. 5S). In *HrzicN* knockdown embryos, *HrETR-1* expression was again absent from the A-line neural tube precursors (Fig. 5L) but evident in the a-line neural tube precursors, although the level of the expression was lower than that in control embryos (Fig. 5L,Q). Similarly, the expression of *Hroth* in the a-line neural tube precursors was reduced in *HrzicN* knockdown embryos (Fig. 5M,R). Furthermore, *HrTRP* expression was lost in them (Fig. 5N). These results suggest that the neural fate is once specified but not maintained at later stages of development in the a-line neural tube precursors.

Since early specification of the neural fate likely occurs in the a-line neural tube precursors of *HrzicN* knockdown embryos, it is expected that the epidermal fate is excluded from these cells. To test this possibility, we first examined *HrzicN* knockdown embryos for expression of *HrEpiG* (Ishida et al., 1996), which occurs only in the epidermis precursors after the 76-cell stage in normal development. *HrEpiG* expression was normal, being excluded from both of the a- and b-line neural

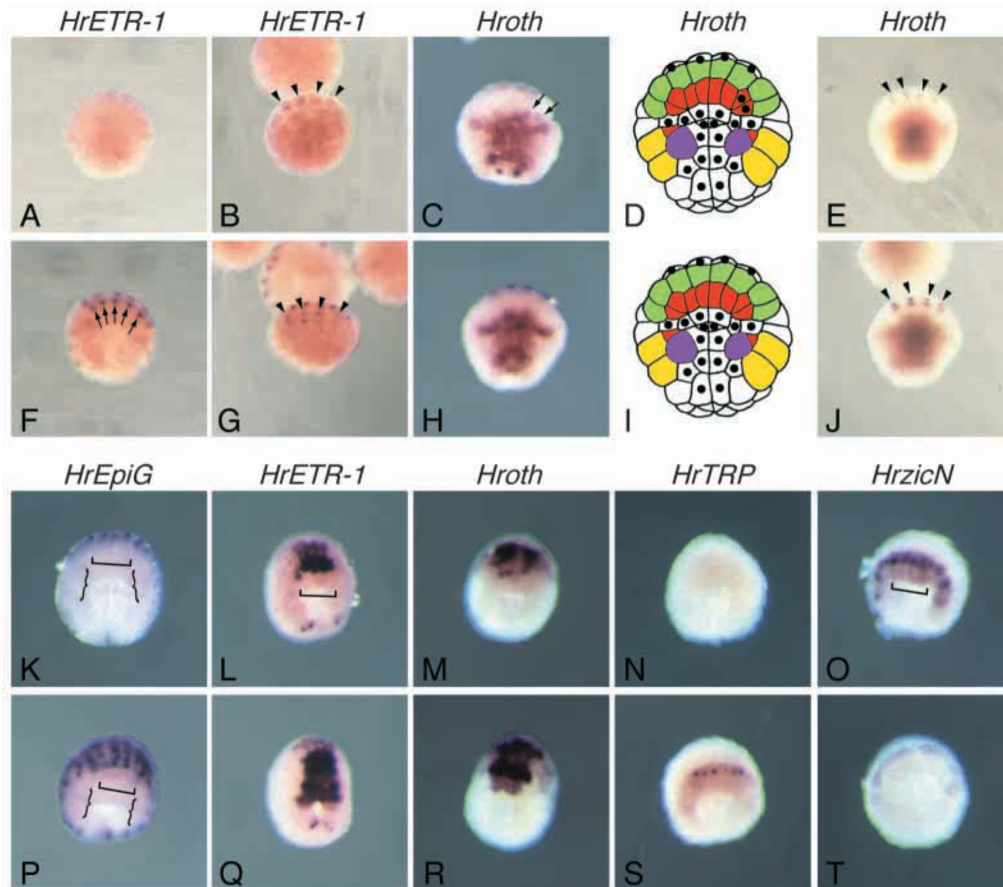

Fig. 5. Expression of epidermal and neuroectodermal markers in *HrzicN* knockdown embryos examined before the tailbud stage. (A-C,E,K-O) Embryos injected with HrzicNMO. (F-H,J,P-T) Control uninjected embryos. (A,B,F,G,L,Q) Expression of *HrETR-1*, (C,E,H,J,M,R) *Hroth*, (K,P) *HrEpiG*, (N,S) *HrTRP* and (O,T) *HrzicN*. D and I are schematic representations of C and H, respectively; dots indicate blastomeres that exhibited *Hroth* expression. (A,C,F,H) Vegetal view of 110-cell stage embryos. (B,E,G,J) Animal view of 110-cell stage embryos. (K-T) Vegetal views. (K,P) Early gastrula equivalent stage embryos. (L-N,Q-S) Neural plate equivalent stage embryos. (O,T) Middle gastrula equivalent stage embryos. Arrowheads in B,E,G,J indicate gene expression in the sensory vesicle precursors. Arrows in C indicate ectopic expression of *Hroth* in the notochord precursors. Arrows in F show *HrETR-1* expression in the A-line neural tube precursors, which is absent in *HrzicN* knockdown embryos (A). Brackets and braces in K and P indicate the region of the neural tube precursor cells of a-line and b-line, respectively, from which *HrEpiG* expression is excluded. Braces in L and O indicate A-line neural tube precursors without *HrETR-1* expression and with *HrzicN* expression, respectively.

tube precursors in *HrzicN* knockdown embryos at the early gastrula equivalent stage (Fig. 5K,P). Next, we examined *HrzicN* knockdown embryos at the early tailbud equivalent stage for expression of *HrEpiD* (Ishida et al., 1996), which is another epidermis-specific gene expressed in the whole epidermis except for a small area around the neuropore in the normal embryo at this stage (Fig. 4K). *HrEpiD* expression was excluded from a group of cells at the dorsal side of *HrzicN* knockdown embryos. These cells were thought to be descendants of the original neural tube precursors that had rejected the epidermis fate (Fig. 4H). These results indicate that the fate choice between neural tube and epidermis is made successfully in *HrzicN* knockdown embryos.

Finally, we examined expression of *HrzicN* itself in *HrzicN* knockdown embryos. At the 110-cell stage, *HrzicN* expression in *HrzicN* knockdown embryos seemed to be identical to that in control embryos, indicating that *HrzicN* is not required for the maintenance of its early expression (data not shown). As mentioned previously, *HrzicN* expression disappears from the vegetal cells by the early gastrula stage in the normal development. By contrast, *HrzicN* expression was detected not only in the a- and b-line but also in the A-line neural tube precursors in *HrzicN* knockdown embryos at the middle gastrula equivalent stage (Fig. 5O,T). Thus, *HrzicN* expression in the A-line neural tube precursors failed to be suppressed in *HrzicN* knockdown embryos. Furthermore, *HrzicN* expression continued in dorsal superficial cells until early tailbud equivalent stage (Fig. 4I,L). From their position, these cells were thought to be descendants of the neural tube precursors that failed to form a neural tube. Thus, *HrzicN* expression in *HrzicN* knockdown embryos continued until a much later stage of development. This suggests that the activity of *HrzicN* is required for proper suppression of its own transcription.

Development of mesodermal tissues in *HrzicN* knockdown embryos

As mentioned above, *HrzicN* was expressed in precursors for mesodermal tissues, and *HrzicN* knockdown larvae exhibited a shortened tail phenotype without differentiated notochord cells as judged by morphological criteria. Therefore, we

investigated development of the notochord and other mesodermal tissues in *HrzicN* knockdown embryos by examining marker gene expression. First we examined expression of *HrBra* (*Halocynthia roretzi Brachyury*) (Yasuo and Satoh, 1993) to test whether the notochord fate was specified in *HrzicN* knockdown embryos. Normally, *HrBra* is expressed in the A- and B-line notochord precursors after the 64- and 110-cell stages, respectively (Fig. 6F). We found *HrBra* was not expressed in *HrzicN* knockdown embryos at the 110-cell stage (Fig. 6A) or at the neural plate equivalent stage (data not shown). We also noticed another abnormality in the gene expression profile of the notochord precursors in *HrzicN* knockdown embryos. Expression of *Hroth*, which is excluded from the notochord precursors in normal development (Fig. 5H,I), was observed ectopically in some notochord precursors in *HrzicN* knockdown embryos (Fig. 5C,D). These suggest that the notochord fate is not successfully specified in *HrzicN* knockdown embryos.

HrzicN is also expressed in the anterior pair (B8.5 pair) of the two mesenchyme precursor pairs and two (B8.7 and B8.8 pairs) of the five muscle precursor pairs at the 110-cell stage (Fig. 2R). Therefore, expression of the muscle-specific actin gene was examined in *HrzicN* knockdown embryos at the 110-cell stage. In control embryos, all five pairs of the primary muscle precursors expressed the actin gene (Fig. 6J). This expression pattern was also observed in *HrzicN* knockdown embryos (Fig. 6E), suggesting that the muscle fate is properly specified in B8.7 and B8.8 pairs without *HrzicN* function.

To test this possibility further and to investigate development of the mesenchyme precursors, we examined expression of *Hrsna* (*Halocynthia roretzi snail*) (Wada and Saiga, 1999a) at the 110-cell stage. Normally, *Hrsna* is expressed in all notochord precursors, two A-line neural tube precursor pairs (A8.15 and A8.16 pairs), both mesenchyme precursor pairs and all primary muscle precursors (Fig. 6H,I). In *HrzicN* knockdown embryos, *Hrsna* expression was lost from all the notochord precursors, the two A-line neural tube precursor pairs (A8.15 and A8.16 pairs) and the anterior mesenchyme precursor pair (B8.5 pair; Fig. 6C,D). By contrast, expression in the primary muscle precursors and the posterior mesenchyme precursor pair (B7.7 pair) was unaffected (Fig. 6C,D). This observation strengthens the idea that the muscle fate is properly specified in B8.7 and B8.8 pairs in *HrzicN* knockdown embryos. Also this result points to a possibility that specification of the anterior mesenchyme precursor pair (B8.5 pair) requires *HrzicN* function.

In summary, these results suggest that among *HrzicN*-expressing cells, specification of all notochord precursors and anterior mesenchyme precursors was disturbed, while specification of muscle precursors was unaffected in *HrzicN* knockdown embryos.

HrzicN overexpression promotes neural development but not notochord development

As described above, *HrzicN* seems to be essential for development of the neural tube and notochord. To verify this idea, we next carried out overexpression of *HrzicN* as a complementary experiment. Eggs were injected with approximately 50 pg of *HrzicN* mRNA, cultured up to the middle gastrula equivalent stage and examined for expression of marker genes. We found that *HrETR-1* was expressed in a half of the body of *HrzicN*-overexpressing embryos. Judging from the size of cells, the expression domain seemed to correspond to the animal hemisphere and its level of expression was higher than that in control embryos (Fig. 7A,B,D). Thus, *HrETR-1* expression was upregulated in *HrzicN*-overexpressing embryos. This suggests that *HrzicN* promotes neural development by activating downstream neural genes, directly or indirectly. However, expression of *HrBra* was not detected in embryos injected with 50 pg of *HrzicN* mRNA (Fig. 7C,E). Upon injection with approximately 5 or 15 pg of *HrzicN* mRNA, *HrBra* expression was reduced as compared with that in the control embryos (data not shown). Thus, *HrzicN* overexpression affects development of the notochord and neural tube differently. *HrzicN* alone may be insufficient to promote the notochord fate. Alternatively, it is possible that the level of *HrzicN* expression must be controlled precisely and/or that temporal down-regulation of *HrzicN* expression may be important for this gene to promote the notochord fate.

Regulation of *HrzicN* expression by FGF-like signaling pathway

In ascidians, FGF-like signaling has been thought to be involved in inductive interactions that are responsible for formation of the neural tube, the notochord and the mesenchyme (Darras and Nishida, 2001a; Darras and Nishida, 2001b; Hudson and Lemaire, 2001; Inazawa et al., 1998; Kim and Nishida, 2001; Kim et al., 2000; Minokawa et al., 2001;

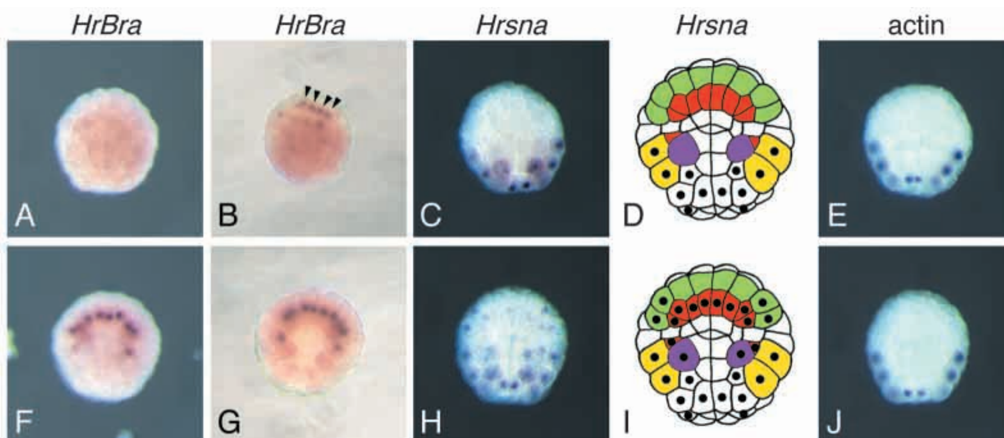

Fig. 6. Expression of mesodermal markers in *HrzicN* knockdown embryos. (A,C,E) Embryos injected with HrzicNMO. (B) An embryo co-injected with *lacZ/HrzicN* mRNA and HrzicNMO. Arrowheads indicate rescued expression of *HrBra*. (F-H,J) Control uninjected embryos. Expression of (A,B,F,G) *HrBra*, (C,H) *Hrsna* and (E,J) the actin gene, at the 110-cell stage. D and I are schematic representation of C and H, respectively; dots indicate blastomeres that exhibited *Hrsna* expression. All specimens, vegetal view.

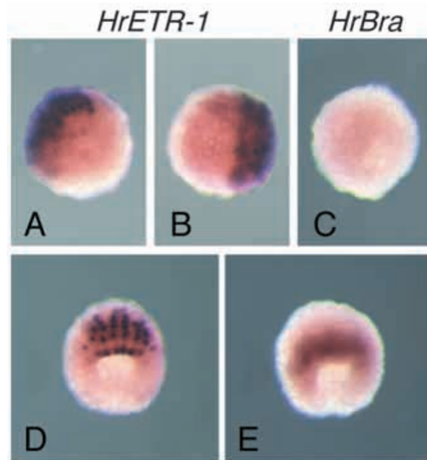

Fig. 7. Expression of markers in embryos overexpressing *HrzicN*. (A-C) Embryos injected with *HrzicN* mRNA. (D,E) Vegetal view of control uninjected embryos. Expression of *HrETR-1* (A,B,D) and *HrBra* (C,E) at the middle gastrula equivalent stage. A and B show opposite sides of the same specimen.

Nakatani and Nishida, 1997; Nakatani et al., 1996; Shimauchi et al., 2001). For example, treatment of embryos with a MEK inhibitor, U0126, blocks formation of these tissues (Darras and Nishida, 2001a; Kim and Nishida, 2001). Since our analyses so far showed significant correlation between *HrzicN* expression and the neural tube, notochord and mesenchyme fates, we addressed whether *HrzicN* expression is dependent on FGF-like signaling. Embryos were treated with U0126 from the 1-cell stage onward and fixed at the 76-cell stage or the middle gastrula equivalent stage to examine their *HrzicN* expression as well as *HrBra* expression, which has been shown to depend on FGF-like signaling as control (Nakatani et al., 1996). *HrzicN* expression in the vegetal hemisphere at the 76-cell stage was normal in U0126-treated embryos (Fig. 8A,E). By contrast, expression of *HrzicN* in the a- and b-line neural tube precursors at the middle gastrula stage was inhibited in U0126-treated embryos (Fig. 8B,F). As expected, *HrBra* expression was not detected in U0126-treated embryos at both stages (Fig. 8C,D,G,H). These data indicate that *HrzicN* expression in the vegetal cells is independent of FGF-like signaling, while *HrzicN* expression in a- and b-line neural tube precursors depends on FGF-like signaling.

DISCUSSION

In the present study, we have isolated a new member of *Zic* family gene from *Halocynthia roretzi*. This gene is distinct from *macho-1*, the muscle determinant, and plays multiple roles in neural tube formation and notochord development as well as mesenchyme specification as discussed below.

HrzicN is required for maintenance, but not for initial specification, of neural tube fate in the a-line precursors

It has been shown that an inductive signal from the vegetal hemisphere cells is required for formation of the a- and b-line neural tube (Darras and Nishida, 2001b; Nishida and Satoh, 1989; Okado and Takahashi, 1990). The induction likely occurs between the 16-cell and the early gastrula stage and is mediated by FGF-like molecules (Darras and Nishida, 2001b; Hudson and Lemaire, 2001; Inazawa et al., 1998; Kim and Nishida, 2001; Nishida and Satoh, 1989). In response to the induction, specification of the neural tube fate takes place with activation of early neural genes such as *HrETR-1* and *Hroth* and suppression of the epidermis fate. *HrzicN* expression starts in the a- and b-line precursors later than the onset of expression of these genes and therefore, *HrzicN* may be unnecessary for specification of the neural tube fate in these lineages. We further tested this possibility by examining gene expression in *HrzicN* knockdown embryos and found this is the case with the a-line precursors (Fig. 9A). Furthermore, our data suggest that *HrzicN* in a-line neural cells may be involved in maintenance of the neural tube fate by keeping expression of early neural genes active and by activating late neural genes such as *HrTRP* (Fig. 9A). This view is supported by the results of the overexpression experiment, showing that *HrzicN* enhances *HrETR-1* expression. It should be noted, however, that some aspect of the genetic program for sensory vesicle formation may be kept active in the knockdown embryo, because expression of *HrETR-1* did not vanish completely in the putative sensory vesicle region of the knockdown embryo.

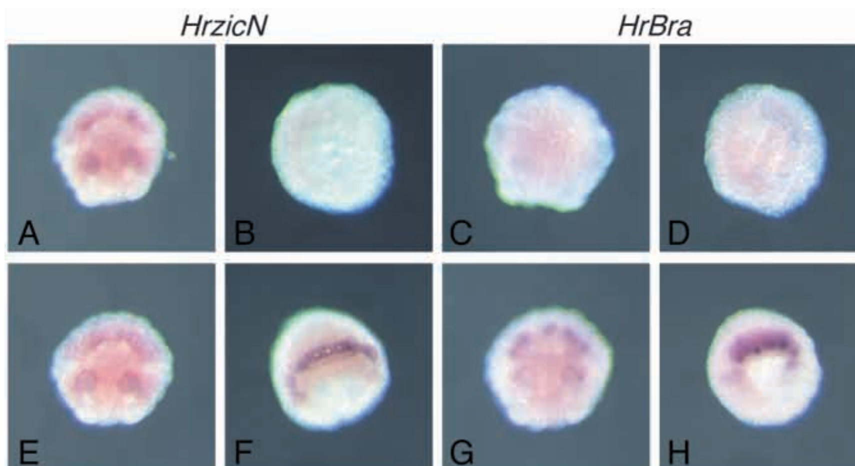

Fig. 8. Effect of a MEK inhibitor, U0126, on the expression of *HrzicN* and *HrBra*. (A-D) Embryos cultured in Millipore-filtered seawater containing 2 μM U0126. (E-H) Control embryos cultured in Millipore-filtered seawater containing 0.02% DMSO. Expression of *HrzicN* (A,B,E,F) and *HrBra* (C,D,G,H). (A,C,E,G) 76-cell stage embryos. (B,D,F,H) Middle gastrula equivalent stage embryos. All specimens, vegetal view.

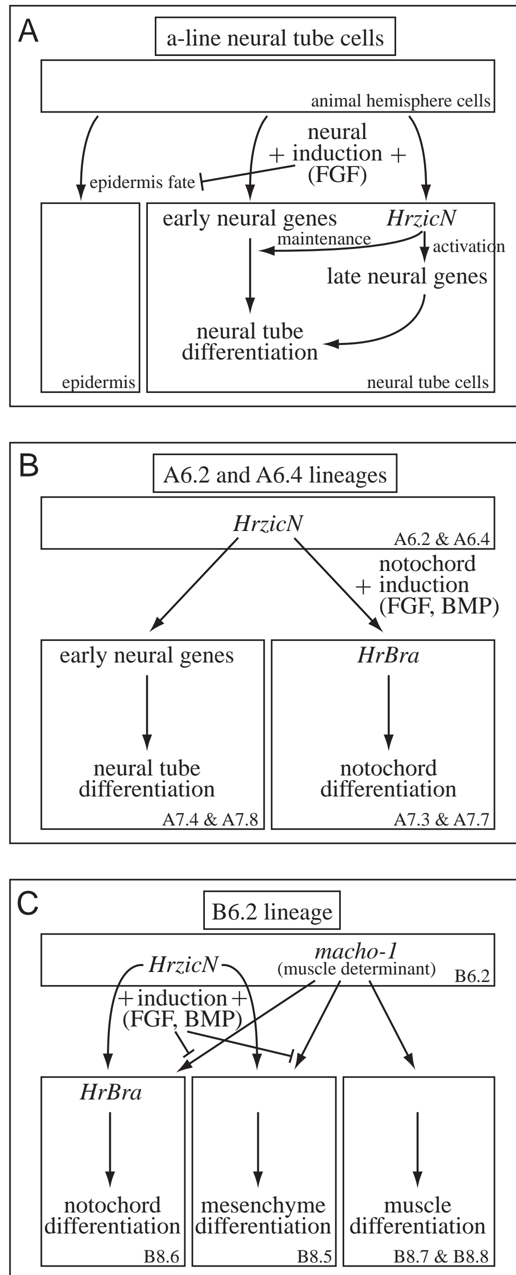

Fig. 9. Summary of *HrzicN* function in three cell lineages. (A) The a-line neural tube precursors, (B) A6.2 and A6.4 lineages and (C) the B6.2 lineage. See Discussion for details.

Since the neural marker genes we used here are not expressed in the b-line neural tube precursors, it was not determined whether this is also the case with the b-line precursors.

In *Xenopus*, overexpression of any of the *Zic* family genes caused transformation of the epidermis cells into neural and/or neural crest-derived tissues (Mizuseki et al., 1998; Nakata et al., 1998). Therefore, it has been thought that they are involved in fate choice between epidermal and neural/neural crest fates. This seems to be different from the function of the ascidian *Zic* suggested above. The reason for this discrepancy is unknown but may be simply because *HrzicN* and *Xenopus Zic* family genes play different roles during neural fate specification, or it may be due to the difference in methodologies or experimental systems. Further analysis of function of vertebrate *Zic* genes and *HrzicN* may resolve this problem.

HrzicN is necessary for specification of the neural tube fate in the A-line precursors

The A-line neural tube cells are derived from A6.2 and A6.4 blastomeres of the 32-cell stage embryo (Fig. 9B). In this lineage, unlike the a-line neural tube precursors, *HrzicN* seems to be involved in the initial specification of the neural fate, since *HrETR-1* was not expressed in A-line precursors of *HrzicN* knockdown embryos. Previous experiments showed that A6.2 and A6.4 descendants adopt the neural tube fate autonomously without any cellular interactions (Minokawa et al., 2001). We have shown that vegetal expression of *HrzicN* is independent of FGF-like signaling, a crucial and multifunctional regulator, occurring around the onset of *HrzicN* expression. Therefore, a possible model is that *HrzicN* expression is activated in the A6.2 and A6.4 blastomeres autonomously and this in turn promotes initial specification of the neural tube fate through activation of early neural genes like *HrETR-1* (Fig. 9B).

It has been shown that removal of the A-line neural tube precursors at the 64-cell stage leads to failure in pigment cell differentiation but not in early neural fate specification in the a-line neural tube precursors (Darras and Nishida, 2001b). Therefore, it is possible that disturbance of the specification of the A-line neural tube precursors may be the cause of defects in the development of the a-line neural tube precursors in *HrzicN* knockdown embryos. However, this cannot fully explain the a-line defects, because our preliminary experiments showed that injection of HrzicNMO into a4.2 blastomeres of the 8-cell stage embryos leads to a failure in the sensory vesicle differentiation similar to that shown in *HrzicN* knockdown embryos. Defects in development of the a-line neural tube precursors found in *HrzicN* knockdown embryos may occur as combined consequences of loss of *HrzicN* function in both a- and A-line precursors.

HrzicN plays a novel role in notochord formation

We have shown that *HrzicN* is required for both of the primary and secondary notochord cell formation. It has been shown that *HrBra* expression in the notochord precursors and the notochord formation depend on a cellular interaction with the endoderm precursors, which can be mimicked by FGF (Darras and Nishida, 2001a; Kim and Nishida, 2001; Nakatani and Nishida, 1994; Nakatani et al., 1996; Shimauchi et al., 2001). *HrBMPb* (*Halocynthia roretzi BMP2/4*) is also involved in this process (Darras and Nishida, 2001a). Together with the result that *HrzicN* expression is independent of FGF-like signaling, we suggest that *HrzicN* is required for A6.2, A6.4 and B6.2 blastomeres to respond to FGF-like molecules (and/or HrBMPb) emanating from the endoderm precursors, and to activate *HrBra*, which in turn promotes notochord development after the next cleavage (Fig. 9B,C).

Previous reports showed that all descendants of the A6.2 and A6.4 blastomeres assume the neural fate when they are in isolation and, conversely, they adopt the notochord fate after treatment with human recombinant basic FGF (Minokawa et al., 2001; Nakatani and Nishida, 1994; Nakatani et al., 1996).

Since *HrzicN* is required for the A6.2 and A6.4 blastomeres to develop into both the neural tube and the notochord, it is possible that *HrzicN* prompts these blastomeres to pursue the neural fate in the absence of FGF-like signaling while it allows them to follow the notochord fate in the presence of the FGF-like signaling.

Developmental fate of the A6.2 and A6.4 blastomeres in *HrzicN* knockdown embryos is unclear. It is unlikely that they adopt the epidermis fate because expression of *HrEpiG* was excluded from them. It is also unlikely that they assume the endoderm fate because endoderm-specific alkaline phosphatase activity was restricted to the original endoderm cells in *HrzicN* knockdown embryos (data not shown). One possibility is that they remain undifferentiated, although expression of other markers must be examined to verify this possibility.

HrzicN is required for specification of the anterior mesenchyme but not for the primary muscle

It has been shown that the B6.2 blastomere requires FGF-like signaling from the endoderm precursors to form not only the notochord but also the mesenchyme (Kim and Nishida, 1999; Kim and Nishida, 2001; Kim et al., 2000). In the absence of the signaling, all of B6.2 descendants pursue muscle fate possibly because of the action of muscle determinants they inherit (Kim and Nishida, 1999; Kim and Nishida, 2001). We found that *HrzicN* knockdown led to failure in specification of B8.5 blastomeres into mesenchyme. In this case, the blastomeres did not exhibit a muscle character either. Together with a role of *HrzicN* in the notochord specification in B8.6 blastomeres discussed above, we suggest that *HrzicN* is required for the B6.2 blastomeres to respond to FGF-like molecules to facilitate the notochord and the mesenchyme fates. Suppression of the muscle fate probably occurs independently of *HrzicN* (Fig. 9C).

Expression of the actin gene and *Hrsna* was normal in two primary muscle precursor pairs, the B8.8 and B8.7 pairs, irrespective of *HrzicN* expression there. This indicates that *HrzicN* is unnecessary for these cells to adopt muscle fate. It is well known that the primary muscle of ascidian embryos develops autonomously owing to the action of maternally provided cytoplasmic determinants. Recently, *macho-1*, a muscle determinant has been isolated from *Halocynthia roretzi* (Nishida and Sawada, 2001). Depletion of the transcripts results in loss of *all* of the primary muscle cells and overexpression of *macho-1* caused ectopic muscle formation in non-muscle-lineage such as endoderm and epidermis. Therefore, it is highly likely that *HrzicN* is not required for primary muscle development: it is dependent on *macho-1* (Fig. 9C). However, since both *macho-1* and *HrzicN* belong to the *Zic* family, they may interact to provide the B8.8 and B8.7 pairs with unknown specific characters. Such a possibility should be tested in future study.

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