

Evolutionary origins of blastoporal expression and organizer activity of the vertebrate gastrula organizer gene *lhx1* and its ancient metazoan paralog *lhx3*

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Expression of the LIM homeobox gene *lhx1* (*lim1*) is specific to the vertebrate gastrula organizer. Lhx1 functions as a transcriptional regulatory core protein to exert 'organizer' activity in *Xenopus* embryos. Its ancient paralog, *lhx3* (*lim3*), is expressed around the blastopore in amphioxus and ascidian, but not vertebrate, gastrulae. These two genes are thus implicated in organizer evolution, and we addressed the evolutionary origins of their blastoporal expression and organizer activity. Gene expression analysis of organisms ranging from cnidarians to chordates suggests that blastoporal expression has its evolutionary root in or before the ancestral eumetazoan for *lhx1*, but possibly in the ancestral chordate for *lhx3*, and that in the ascidian lineage, blastoporal expression of *lhx1* ceased, whereas endodermal expression of *lhx3* has persisted. Analysis of organizer activity using *Xenopus* embryos suggests that a co-factor of LIM homeodomain proteins, Ldb, has a conserved function in eumetazoans to activate Lhx1, but that Lhx1 acquired organizer activity in the bilaterian lineage, Lhx3 acquired organizer activity in the deuterostome lineage and ascidian Lhx3 acquired a specific transactivation domain to confer organizer activity on this molecule. Knockdown analysis using cnidarian embryos suggests that Lhx1 is required for *chordin* expression in the blastoporal region. These data suggest that Lhx1 has been playing fundamental roles in the blastoporal region since the ancestral eumetazoan arose, that it contributed as an 'original organizer gene' to the evolution of the vertebrate gastrula organizer, and that Lhx3 could be involved in the establishment of organizer gene networks.

KEY WORDS: Evolution, Lhx1, Lhx3, *Nematostella*, Spemann-Mangold organizer, *Xenopus*

INTRODUCTION

The mechanisms of eumetazoan embryogenesis have evolved tremendously during the ~700 million years since an ancestral eumetazoan emerged. During eumetazoan evolution, the gene repertoires of developmental regulatory proteins have been well conserved, as demonstrated by recent genome analyses of various living organisms ranging from sea anemones to humans (Putnam et al., 2007). This implies that the evolution of intricate developmental systems must have resulted from repeated reconstructions of developmental gene regulatory networks through changes in expression domains and in the timing of action of regulatory genes, and by changing parts of protein sequences to create new protein-protein interactions.

In developmental gene regulatory systems, the LIM domain-containing homeodomain protein family was one of the evolutionary innovations of the Metazoa (Putnam et al., 2007). This family of proteins comprises six subfamilies distinguished by their homeodomain sequences: Lhx1 (Lim1), Lhx3 (Lim3), Lmx, Islet, Lhx2 and Lhx6 (Hobert and Westphal, 2000). Importantly, all are encoded in eumetazoan genomes ranging from humans to the sea anemone *Nematostella vectensis* (Putnam et al., 2007), suggesting that LIM homeodomain proteins diverged at a very early stage of

eumetazoan evolution. Among the family, *lhx1* is expressed in the blastoporal region during gastrulation in vertebrates and other bilaterians (Kawasaki et al., 1999; Langeland et al., 2006; Lilly et al., 1999; Taira et al., 1992).

Gastrulation, which generates germ layers following blastopore formation, is one of the fundamental developmental features of Eumetazoa. One of major characteristics of vertebrates is the dorsal blastopore lip, which functions as the gastrula organizer, playing a crucial role in establishing the basic body plan. The molecular basis of organizer function has been extensively studied in *Xenopus*, leading to the identification of organizer-specific transcription factors such as Goosecoid (Gsc) and Lhx1 and secreted factors such as Noggin (Nog) and Chordin (Chd) (Gerhart, 2001; Lemaire and Kodjabachian, 1996). The expression patterns of these genes in the gastrula are well conserved in vertebrates and amphioxus (Langeland et al., 2006; Yu et al., 2007). Compared with these chordates, other deuterostome groups among the Echinodermata, such as sea urchins, have reportedly neither the gastrula organizer nor, with the exception of *lhx1*, blastoporal expression of organizer genes (Kawasaki et al., 1999; Martindale, 2005). Recently, it has been reported that some organizer genes, including *nog*, *chd* and *gsc*, are expressed in the *Nematostella* gastrula, but their spatiotemporal expression patterns are not like those of their vertebrate counterparts (Matus et al., 2006). In addition, the expression pattern of *Nematostella lhx1* has not been examined. Thus, important questions still remain as to how the gastrula organizer evolved in the eumetazoan lineage leading to the vertebrates, both in terms of a gene regulatory network and protein function.

To address this question, we first focused on Lhx1 because *Xenopus laevis* Lhx1 (Xlim-1) is thought to play a central role in the transcriptional regulatory network in the gastrula organizer, also

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known as the Spemann-Mangold organizer. *Xenopus laevis* Lhx1 has four outstanding features. First, it has secondary axis-inducing (SAI) activity in *Xenopus* embryos when two LIM domains are mutated, or when the LIM domain-binding protein Ldb1, a co-factor of LIM homeodomain proteins, is coexpressed (Agulnick et al., 1996; Taira et al., 1994). Second, it is a transcriptional activator that upregulates other organizer genes, including *gsc*, *chd*, *otx2*, *cerberus* (*cer*) and *paraxial protocadherin* (*papc*) (Collart et al., 2005; Hiratani et al., 2003; Hukriede et al., 2003; Mochizuki et al., 2000; Taira et al., 1994; Yamamoto et al., 2003). Third, *Xenopus* Lhx1 has additional conserved subregions, called the C-terminal conserved regions (CCRs) 1 to 5, among which only CCR2, a transactivation domain with five crucial tyrosines, is required for SAI activity (Hiratani et al., 2001). Fourth, it is one of only a few organizer-specific transcription factors that exhibit conserved organizer functions between *Xenopus* and mouse (Hukriede et al., 2003; Lemaire and Kodjabachian, 1996). Thus, *lhx1* is one of the best genes to analyze if we are to understand the evolution of the organizer.

We also focused on *lhx3*, the ancient paralog of *lhx1*. It has been shown that *lhx3* exhibits blastoporal expression in an amphioxus, *Branchiostoma belcheri* (Wang et al., 2002), and in the ascidians *Halocynthia roretzi* (Wada et al., 1995) and *Ciona savignyi* (Satou et al., 2001), contrasting with the absence of blastoporal expression of vertebrate *lhx3* (Taira et al., 1993). In this paper, we show that in contrast to *lhx3*, *lhx1* is not expressed in the blastula or gastrula in ascidians. Furthermore, deuterostome Lhx3 has organizer activity in *Xenopus* embryos. These findings imply that the acquisition of a new expression domain of *lhx3* in the blastoporal region might have occurred during the process of organizer evolution. To further investigate this, we carried out systematic analyses of blastoporal expression and organizer activities of *lhx1* and *lhx3* for various organisms among the Eumetazoa. We also examined the functional conservation of *Nematostella* Ldb because the Ldb protein is known to be a key co-factor of LIM homeodomain proteins (Agulnick et al., 1996; Morcillo et al., 1997). Finally, we carried out functional analysis of Lhx1 in *Nematostella* embryos. Whereas, as mentioned above, most previous studies have focused on the expression patterns of organizer genes, especially those encoding secreted proteins such as *chd* and *nog*, this is the first analysis of the evolution of the gastrula organizer from the perspective of organizer-specific transcriptional regulatory networks based on both protein evolution and gene expression.

MATERIALS AND METHODS

cDNA cloning of *Hr_lhx1*, *Hp_lhx3*, *Nv_lhx1* and *Nv_ldb*

cDNA fragments of *lhx1* from the ascidian *Halocynthia roretzi* (*Hr_lhx1*) were isolated by 5' and 3' RACE using RNA from tailbud stage embryos after a homeobox fragment was PCR-cloned from genomic DNA using degenerate primers. A full-length *Hr_lhx1* cDNA was obtained by RT-PCR using RNA from tailbud stage embryos. The coding sequence of *lhx3* from the sea urchin *Hemicentrotus pulcherrimus* (*Hp_lhx3*) was isolated by RT-PCR using RNA from hatched blastula stage embryos with specific primers designed from *lhx3* of another sea urchin, *Strongylocentrotus purpuratus*. The coding sequences of *lhx1* and *ldb* from the sea anemone *Nematostella vectensis* (*Nv_lhx1* and *Nv_ldb*) were isolated by RT-PCR using RNA from polyps with specific primers designed from its genome sequence (<http://genome.jgi-psf.org/Nemve1/Nemve1.home.html>). Abbreviations of species names and accession numbers of proteins are shown in Tables S1 and S2, respectively, in the supplementary material.

Whole-mount in situ hybridization (WISH)

WISH was performed with digoxigenin-labeled antisense or sense (negative control) RNA probes (1–2 kb) as described for *H. roretzi* (Wada et al., 1995), *H. pulcherrimus* (Minokawa et al., 2004) and *N. vectensis* (Matus et al., 2006).

Plasmid constructs and mRNA synthesis

Plasmid constructs for mRNA synthesis were constructed with the pCS2- and pCS107-derived vectors pCSd107X, pCSd107mT and pCSf107mT (constructed by Y. Mii, T. Shibano and M.T.). For point-mutated, deleted or chimeric constructs, two-round PCR-based mutagenesis was used. LIM domain mutant constructs were prepared as described (Taira et al., 1994). Synthetic capped RNAs were generated by in vitro transcription as described (Hiratani et al., 2001).

Secondary axis assay

mRNAs, together with nuclear *lacZ* (*nlacZ*) mRNA (62.5 or 125 pg/embryo) as a tracer, were injected into one blastomere in the ventral equatorial region of 2- to 16-cell stage *Xenopus* embryos. Injected embryos were fixed at tailbud stages (stages 32–36) for β -galactosidase staining and whole-mount immunostaining of somites with the mouse monoclonal antibody 12/101 as described (Hiratani et al., 2001), but using TBS instead of PBS. mRNAs were injected in two different amounts that were molar-equivalent to either 750 or 375 pg of *lhx1* mRNA from *Xenopus laevis* (*Xl_lhx1*). These two doses of each construct gave almost the same results, which were therefore combined for presentation in the figures. Each experiment included *Xenopus* β -globin mRNA-injected and uninjected embryos as negative controls, in which secondary axis formation was less than 3% (data not shown).

Animal cap assay and RT-PCR

mRNAs, molar-equivalent to 250 pg of *Xl_lhx1* mRNA, were injected into both blastomeres in the animal pole region of 2-cell stage embryos. Animal caps were dissected from the blastulae (stages 8.5–9) and collected at the stage equivalent to the gastrula for analyzing gene expression by RT-PCR as described (Hiratani et al., 2003). Primer sets for RT-PCR are listed in Table 1.

Luciferase reporter assay in *Xenopus* embryos

The GAL4-UAS reporter assay was performed as described (Hiratani et al., 2001), except that whole embryos instead of animal caps were used for the preparation of cell extracts.

Knockdown experiments in the *Nematostella* embryo

Nematostella fertilized eggs were dejellied and injected with Vivo-Morpholinos (Gene Tools) and mRNAs. The splice-blocking Vivo-Morpholino for *Nv_lhx1* (*Nv_lhx1* MO) was designed from the splice donor site of the first intron (ATTCATTGACAGTACTCACCTGTAG; first exon underlined). The control Vivo-Morpholino (ctr MO) was as described previously (Rentzsch et al., 2008). *Nv_lhx1* MO solution was colored with 0.5 mg/ml Fast Green FCF in 0.1 mM Tris-HCl (pH 7.5), 0.01 mM EDTA. ctr MO solution was colored with 0.25 mg/ml Phenol Red in 5 mM HEPES buffer (pH 7.6) because the ctr MO was precipitated by Fast Green FCF. The diameter of an injected drop was about one fifth of the cell diameter. Injected embryos were incubated at 22°C, allowed to develop to early gastrulae stage (30 hours post-fertilization) and frozen or fixed for RT-PCR or WISH, respectively. Quantitative RT-PCR (qRT-PCR) was performed on the ABI 7000 sequence detection system using SYBR Green chemistry. Expression levels of genes were measured in triplicate and normalized to those of *ef1 α* . Primer sets for RT-PCR are described in Table 1.

RESULTS

Identification of *Nv_lhx1*, *Nv_lhx3*, *Hp_lhx3* and *Hr_lhx1*

To explore the ancestral expression patterns of *lhx1* and *lhx3* it is essential to examine the existing expression patterns of these genes in the Eumetazoa. We have chosen animals that occupy phylogenetically important positions, analyzing the newly isolated *lhx1* and *lhx3* from the sea anemone *Nematostella vectensis* (*Nv_lhx1* and *Nv_lhx3*), *lhx3* from the sea urchin *Hemicentrotus pulcherrimus* (*Hp_lhx3*) and *lhx1* from the ascidian *Halocynthia roretzi* (*Hr_lhx1*). These genes were identified by phylogenetic analysis using deduced amino acid sequences of LIM domains and the homeodomain (see Fig. S1 in the supplementary material).

Table 1. Primer sets (5' to 3') for RT-PCR

Gene	Forward	Reverse	Product (bp)	Reference
<i>Xl_gsc</i>	ACAACCTGGAAGCACTGGA	TCTTATTCCAGAGGAACC	278	Xenbase*
<i>Xl_cer</i>	GCTGAACTATTTGATTCCACC	ATGGCTTGATTCTGTGGGGC	255	Bouwmeester et al., 1996
<i>Xl_chd</i>	CCTCCAATCCAAGACTCCAGCAG	GGAGGAGGAGGAGCTTTGGGACAAG	267	Bouwmeester et al., 1996
<i>Xl_otx2</i>	GGATGGATTTGTTGCCACAGTC	CACTCTCCGAGCTCACTTCTC	315	Sasai et al., 1995
<i>Xl_papc</i>	CACCTGGACAATTGTGC	ATGGTTCGGTATGTGCAGG	357	Medina et al., 2004
<i>Xl_h4</i>	CGGGATAACATTACGGGTATCACT	ATCCATGGCGGTAAGTCTTCTCCT	188	Niehrs et al., 1994
<i>Nv_lhx1</i> (Fig. 1)	GCCATAGATGAGCCAAAGGA	TCCGTAGTGCTAGGTGGAG	414	This study
<i>Nv_lhx1</i> (Fig. 6)	TCCAATGTAGCGACTGCAAG	GGCTCATCTATGGCATCATCAA	313	This study
<i>Nv_actin</i>	ACGGGATCGTCACTAACTGG	AGGAAGGAAGGCTGGAACAT	583	This study
<i>Hp_lhx1</i>	GTCCTTTAAGAGGCCCGTTT	ATGGTCCATAGGATGGTTGC	239	This study
<i>Hp_lhx3</i>	ACCGGTGACGAATTCTTC	GTCGTTCTGAAATGTGATTCC	492	This study
<i>MitCO1</i>	AGGCACAGCTATGAGTGT	TCATCCAGTCCCTGCTC	292	Yamazaki et al., 2005
<i>Nv_ef1α</i>	ACAAGCGAACCATCGAGAAG	AACTCCACAGGGCAATGTC	136	This study (qRT-PCR)
<i>Nv_chd</i>	TAGACTGCCAAAGCCATTC	GTGCGTTGTTGGGATATGTG	135	This study (qRT-PCR)
<i>Nv_otxA</i>	GATGAATCCCACGCTATGG	CGTAGGGTGAGAGCATGAAG	85	This study (qRT-PCR)
<i>Nv_wnt2</i>	GACTCCAGAGAGGTGAAAAAG	CCAACGACATGTTGGTCTTG	94	This study (qRT-PCR)
<i>Nv_FGFa1</i>	TAGTGCAGGAACATTGGAC	TGCACCGTCTCTTTCATTG	128	This study (qRT-PCR)

*<http://www.xenbase.org/other/static/methods/RT-PCR.jsp>.

From this analysis, two notable points emerged: (1) *Hr_Lhx1* has deviated substantially from other chordate *Lhx1* proteins and is inconsistent with the phylogenetic tree of species as commonly accepted (Dunn et al., 2008) (see Table S3, Fig. S1 and Fig. S2A in the supplementary material); and (2) curiously, an *Nv_lhx3* genomic fragment encoding the first LIM domain has a 4 bp deletion, the same as in the *Nematostella vectensis* genomic sequence, causing deletion of the conserved histidine in the LIM motif and truncation of its coding sequence (see Fig. S1 in the supplementary material). Furthermore, *Nv_lhx3* transcripts could not be detected by RT-PCR in *Nematostella* embryos or adults (data not shown), and are also absent from the available nucleotide and EST databases, suggesting that the laboratory line of *Nematostella* might be an *lhx3* mutant.

Expression patterns of *Nv_lhx1*, *Hp_lhx1*, *Hr_lhx1*, *Hp_lhx3* and *Hr_lhx3*

Nv_lhx1 expression was detected by RT-PCR from the blastula stage onwards (Fig. 1A). Whole-mount in situ hybridization (WISH) analysis revealed that the expression was limited to one side of the blastula (Fig. 1B). This was most likely to be on the blastopore side because blastoporal expression of this gene was seen at the gastrula stage (Fig. 1C,D). After gastrulation, the expression of *Nv_lhx1* was detected in an oral region of the larvae (data not shown) and was maintained there until the adult stage, as examined by RT-PCR with dissected samples (Fig. 1A). By comparison with the reported expression patterns of organizer genes in the *Nematostella* embryo (Fritzenwanker et al., 2004; Matus et al., 2006; Mazza et al., 2007), *chd*, *otx* and *foxa* appear to be coexpressed with *lhx1* in the blastoporal region, whereas *gsc*, *nog* and *follistatin* only start to be expressed at later stages in the endoderm.

Re-examination of *Hp_lhx1* expression using an improved WISH protocol for sea urchins (Minokawa et al., 2004) revealed that *Hp_lhx1* started to be expressed in the equatorial region at the hatched blastula stage (Fig. 1E), which differs from previous data (Kawasaki et al., 1999). At the early gastrula stage, *Hp_lhx1* was expressed in the vegetal region and later in the oral ectoderm of the late gastrula (Fig. 1F,G). These results, together with the reported expression patterns of chordate and *Drosophila melanogaster lhx1* (*Dm_lhx1*) (Langeland et al., 2006; Lilly et al., 1999; Taira et al., 1992), led to the important conclusion that the

blastoporal expression of *lhx1* is very ancient among the Eumetazoa. Therefore, we consider *lhx1* to be an ‘original’ organizer gene.

However, expression analysis of *Hr_lhx1* in the ascidian showed that whereas late expression of *Hr_lhx1* was detected in the brain from the tailbud stage onward, early expression of *Hr_lhx1* was not detectable at the blastula to gastrula stages by WISH (Fig. 1H,I; data not shown). Because *Hr_lhx3* is expressed at the 32-cell to gastrula stages (Fig. 1J,K), the temporal expression patterns of *Hr_lhx1* and *Hr_lhx3* are completely opposite to those of vertebrates.

Next, we examined which of the other organisms show blastoporal expression of *lhx3*. In the sea urchin, *Hp_lhx3* was expressed maternally and zygotically until the gastrula stages as assayed by RT-PCR (see Fig. S3 in the supplementary material). However, its expression was much weaker than that of *Hp_lhx1*, and localized expression of *Hp_lhx3* was not detected by WISH (data not shown), consistent with published data from another sea urchin, *Strongylocentrotus purpuratus* (*Sp_lhx3*) (Howard-Ashby et al., 2006). In the ascidian, we reconfirmed that *Hr_lhx3* is expressed in the endoderm lineage at the 32-cell to early gastrula stages and in the CNS at tailbud stages (Fig. 1J-L) as reported (Wada et al., 1995); this is similar to the expression pattern reported in another ascidian, *Ciona savignyi* (*Cs_lhx3*), in which strong evidence for its role in endoderm differentiation was reported (Satou et al., 2001). Thus, our present analysis and previous reports on *lhx3* expression in the amphioxus *Branchiostoma belcheri* (*Bb_lhx3*) and *Drosophila melanogaster* (*Dm_lhx3*) (Thor et al., 1999; Wang et al., 2002) suggest the possibility that the blastoporal expression of *lhx3* was acquired in a common ancestor of chordates and was lost in the vertebrate lineage. However, to determine the evolutionary root more precisely, it is necessary to analyze hemichordate and other echinoderm gastrula embryos for the expression of *lhx3*.

Evolutionarily conserved function of *Nv_Ldb* to activate *Xl_Lhx1*

To investigate the evolutionary origins of the organizer activity of *Lhx1* in vertebrate gastrulae, we first addressed whether the interaction between *Lhx1* and its co-factor *Ldb* is conserved, for which purpose we isolated *ldb* from *Nematostella* (*Nv_ldb*). The functional domains of *Nv_Ldb* were found to be highly conserved with its *Xenopus laevis* counterpart *Xl_Ldb1* (Fig. 2A; see Fig. S4A

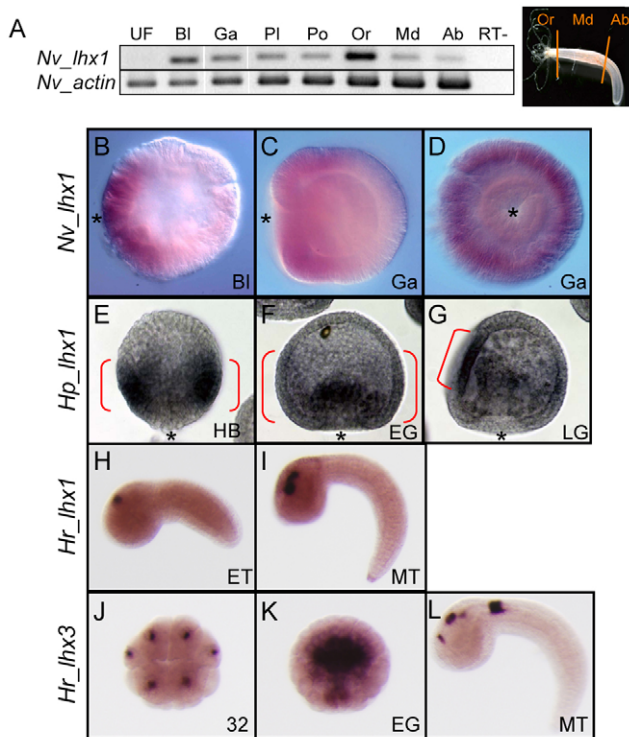


Fig. 1. Developmental expression of cnidarian, sea urchin and ascidian *lhx1* and ascidian *lhx3* genes. (A) RT-PCR analysis of *Nv_lhx1*. Stages of embryos (UF through Po) and dissected regions of adults (Or, Md, Ab, as shown in the right-hand panel), are indicated. UF, unfertilized eggs; BI, blastula; Ga, gastrula; Pl, planula larva; Po, early polyp; Or, oral region; Md, mid region; Ab, aboral region; RT-, negative control; *Nv_actin*, loading control. (B-L) Whole-mount in situ hybridization (WISH) analysis of *Nv_lhx1* (B-D), *Hp_lhx1* (E-G), *Hr_lhx1* (H,I) and *Hr_lhx3* (J-L). Stages are indicated in each panel: HB, hatched blastula; EG, early gastrula; LG, late gastrula; ET, early tailbud; MT, mid tailbud; 32, 32-cell stage. Views are lateral (B,C,E-I,L) with oral (B,C,G) or anterior (H,I,L) to the left and with animal (E-G) or dorsal (H,I,L) to the top, or blastoporal (D,J,K) with anterior to the top (J,K). Asterisks, blastopore side; red brackets, ranges of expression. *Nv*, sea anemone (cnidarian) *Nematostella vectensis*; *Hp*, sea urchin *Hemicentrotus pulcherrimus*; *Hr*, ascidian *Halocynthia roretzi*.

in the supplementary material). *Nv_ldb* EST sequences and its genomic sequence reveal various splicing variants (see Fig. S4A,B in the supplementary material), which are very similar to those of vertebrate *ldb* genes (Agulnick et al., 1996; Enkhmandakh et al., 2006; Tran et al., 2006). Among them, we used a variant that is most similar to *Ldb1a* of vertebrates. To evaluate the interaction between *Lhx1* and *Ldb*, we coexpressed them in the *Xenopus* embryo and analyzed secondary axis-inducing (SAI) activity. This is a measure of the ability to convert the ventral mesoderm to dorsal mesoderm (the organizer) (see Fig. S5A,B in the supplementary material). We found that *Nv_Ldb* was as capable as *Xl_Ldb1* of activating *Xl_Lhx1* to induce a secondary axis (Fig. 2A), suggesting that the interaction between LIM domains and *Ldb* is evolutionarily highly conserved among the Eumetazoa. By contrast, *Nv_Lhx1* did not induce a secondary axis when coexpressed with either *Xl_Ldb1* or *Nv_Ldb* (Fig. 2A). These data indicate that the evolution of the *Lhx1* protein in regions other than the LIM domains was crucial to acquire organizer activity in *Xenopus* embryos.

Bilateria *Lhx1* exhibits organizer activity

To further investigate the evolutionary origins of the organizer activity of *Lhx1*, we compared the function of *Lhx1* proteins from various organisms using an 'activated' form (*Lhx1**) that has point-mutated LIM domains. We assayed for SAI and organizer gene-inducing (OGI) activities with the *Xenopus* embryo. OGI activity was assayed by RT-PCR with the *Xenopus* animal cap system to examine the ability of each protein to upregulate organizer genes in the pluripotent naive ectoderm, which shows detailed gene regulatory properties to postulated target genes of *Xl_Lhx1*. High activity in both assays was considered to indicate organizer activity. *Lhx1** constructs were used instead of wild-type constructs for the following two reasons. First, *Xl_Lhx1** can activate all postulated target genes, but combinations of *Lhx1* and co-regulators, including *Ldb*, activate only a subset of target genes. For example, *Xl_Lhx1* with *Ldb1* activates *gsc* but not *cer* expression, whereas a combination of *Xl_Lhx1* with *Siamois/Mix.1* activates *cer* expression (Mochizuki et al., 2000; Yamamoto et al., 2003). Second, because the interaction between *Ldb* and LIM domains is evolutionarily highly conserved, as shown above, we expect that evolution of LIM homeodomain proteins must have occurred in the linker sequence (the LH linker) between the second LIM domain and the homeodomain, and in the C-terminal (Ct) region, both of which are less well conserved than the LIM domains and homeodomain (see Table S3 in the supplementary material). Thus, this LIM domain mutant is suitable for analyzing the organizer activity of LIM homeodomain proteins from various organisms.

Using LIM domain mutants, we compared organizer activity among the eumetazoan *Lhx1* proteins *Xl_Lhx1*, *Hr_Lhx1*, *Bf_Lhx1* (from the amphioxus *Branchiostoma floridae*), *Hp_Lhx1*, *Dm_Lhx1* and *Nv_Lhx1*. As shown in Fig. 2B,C, all bilaterian *Lhx1** proteins we examined showed high levels of both SAI and OGI activities. By contrast, and consistent with the data for wild-type *Nv_Lhx1* (Fig. 2A), *Nv_Lhx1** had neither SAI nor OGI activity, except that it weakly upregulated *gsc* and *papc* in the animal cap. In most cases, including this result (Fig. 2B,C), there was a good correlation between the SAI and OGI activity levels. In the case of *Nv_Lhx1**, the weak OGI activity of this protein turned out to be insufficient for SAI activity, possibly owing to its poor induction of *chd*.

To determine the structural basis for the lack of SAI activity of *Nv_Lhx1**, we performed chimeric protein analysis with *Xl_Lhx1* (Fig. 2D). The results showed that the Ct region was responsible for the lack of SAI activity of *Nv_Lhx1** (Fig. 2D; construct 3), whereas the LIM domains (point-mutated) and the homeodomain of *Nv_Lhx1** were functionally equivalent to those of *Xl_Lhx1** (Fig. 2D; construct 1). The lack of SAI activity of *Nv_Lhx1* might be due to less conservation of CCR2, a region that is crucial for SAI activity in *Xl_Lhx1* (Hiratani et al., 2001) and that is well conserved among bilaterians (see Fig. S6 in the supplementary material). We assessed the stability of the *Nv_Lhx1** protein by western blotting with HA-tagged proteins, and excluded the possibility that *Nv_Lhx1** is unstable in *Xenopus* embryos (data not shown). In addition to the Ct region, the LH linker was also responsible for the lack of activity of *Nv_Lhx1** (Fig. 2D; constructs 4 and 5), possibly because the LH linker of *Nv_Lhx1* is the shortest of all *Lhx1* proteins examined (32 versus the 59-148 amino acids in the others).

We next examined the importance of the length of the LH linker. When the LH linker of *Xl_Lhx1** was replaced with an unrelated 12 amino acid sequence, which is the same length as that of vertebrate *Lhx3* (12 versus the 12-20 amino acids for other *Lhx3* proteins), the SAI and OGI activities were greatly reduced (Fig. 2D; construct 6;

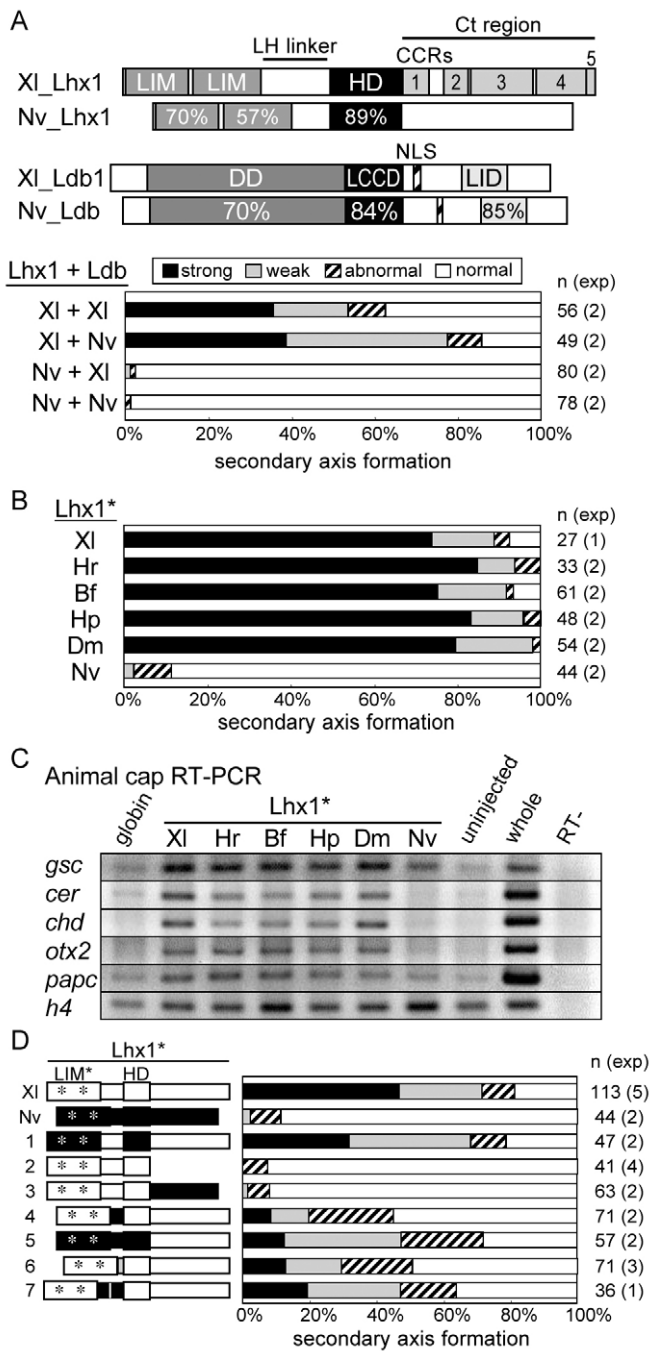


Fig. 2. Comparison of organizer activities between Lhx1 proteins. (A) Activity of sea anemone Nv_Lhx1 and Nv_Ldb. (Above) Schematics of Nv_Lhx1 and Nv_Ldb together with those of *Xenopus laevis* (XI_Lhx1 and XI_Ldb1) for comparison. Percentage amino acid identities with XI_Lhx1 and XI_Ldb1 are shown. (Below) Analysis of secondary axis-inducing (SAI) activity. Combinations of Lhx1 and Ldb are indicated by their species abbreviations. SAI activity was classified as strong or weak according to the amount of somitic muscle in the secondary axis. n, total number of injected embryos; exp, number of independent experiments. Results of negative controls are omitted (see Materials and methods). LIM, LIM domain; LH linker, LIM domain and homeodomain linker; HD, homeodomain; CCRs, C-terminal conserved regions, CCR1-CCR5; DD, dimerization domain; LCCD, Ldb1-Chip conservative domain (interacting with Ssbp); NLS, nuclear localization sequence; LID, LIM-interaction domain. (B) SAI activity of activated Lhx1 (Lhx1*) proteins from different organisms: Bf, amphioxus; Dm, fruit fly; Hp, sea urchin; Hr, ascidian; Nv, sea anemone; XI, frog (for full species names, see Table S1 in the supplementary material). (C) Organizer gene-inducing (OGI) activity of Lhx1* proteins. Injected mRNAs are indicated above the panels; the genes examined by RT-PCR are indicated to the left. uninjected, uninjected animal cap; whole, whole sibling embryos at stages 11-12; *h4*, histone H4 loading control. (D) Chimeric protein analysis. (Left) Structures of the chimeric proteins with XI_Lhx1* sequences in white and Nv_Lhx1* sequences in black. HD, homeodomain; LIM, LIM domains; asterisks, mutated LIM domains. (Right) The SAI activity of the various chimeric proteins.

data not shown). Tandemly repeated LH linkers (76 amino acids) of Nv_Lhx1 did not increase SAI activity very much (compare constructs 4 and 7). These data indicate that both the length and amino acid sequence of LH linkers are important for the organizer activity of Lhx1 in *Xenopus* embryos. In conclusion, we suggest that an ancestral bilaterian Lhx1 acquired organizer activity by evolution of its LH linker and Ct, rather than LIM domain and homeodomain, regions.

Lhx3 exhibits organizer activity

We addressed whether Hr_Lhx3 and Bb_Lhx3, which are expressed at the blastula to gastrula stages, have organizer activity in *Xenopus* embryos. For comparison, we also examined XI_Lhx3, Hp_Lhx3,

Sk_Lhx3 (from the hemichordate *Saccoglossus kowalevskii*) and Dm_Lhx3 using LIM domain mutants (Lhx3*). As shown in Fig. 3A,B, Dm_Lhx3* exhibited almost no activities, whereas deuterostome Lhx3* exhibited SAI and OGI activities. This is the first demonstration that Lhx3 can exhibit organizer activity, implying that Lhx3 functions as an organizer gene in amphioxus and ancestral chordates, similar to Lhx1. The absence of SAI activity for Hp_Lhx3* is possibly due to its inability to induce *chd* (Fig. 3B) and seems to be a secondary reduction in the sea urchin lineage (see Fig. 7H) because Sk_Lhx3* of the Hemichordata, a sister group of the Echinodermata, has significant SAI activity. Notably, among the Lhx3 proteins, Hr_Lhx3* exhibited the strongest SAI and OGI activities, equivalent to those of XI_Lhx1*.

Among other subclasses of LIM homeodomain proteins, deuterostome Lhx3* showed relatively high organizer activity (see Fig. S5C,D in the supplementary material). This is somewhat paradoxical because Lhx3 subfamily members have the shortest LH linker (12-20 amino acids) among the LIM homeodomain family, which normally implies a weak organizer activity as demonstrated with Lhx1 (Fig. 2D). Indeed, substitution of the LH linker of XI_Lhx1* with that of Hr_Lhx3 greatly reduced SAI activity (Fig. 3C; construct 6). Therefore, we examined the Ct regions of Lhx3 by chimeric analysis with XI_Lhx1*. The Ct regions of deuterostome Lhx3, including Hp_Lhx3, but not of Dm_Lhx3 exhibited significant SAI activities (Fig. 3C; constructs 1 to 5). These data suggest that the SAI activity of the Ct region of Lhx3 was gained in the deuterostome ancestor. By contrast, a chimeric construct, in which the Ct region of Hr_Lhx3* was replaced with that of XI_Lhx1, had little SAI activity (construct 7). This activity was partially recovered by further replacing the LH linker with that of XI_Lhx1 (construct 8), indicating that the activity of the Ct region of XI_Lhx1 depends on the presence of its own LH linker.

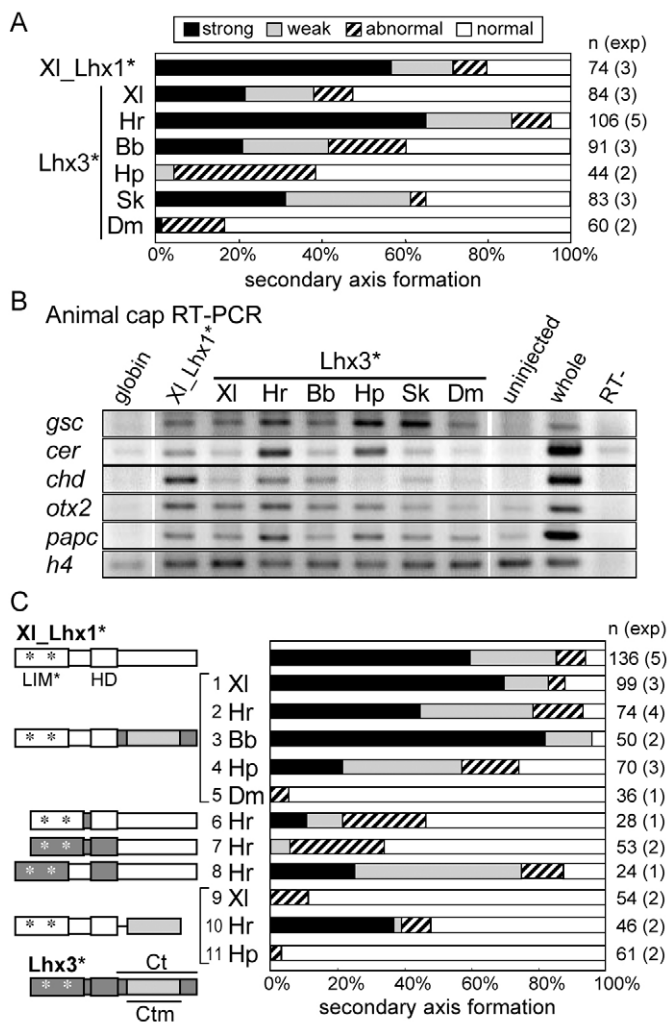


Fig. 3. Comparison of organizer activities between bilaterian Lhx3 proteins. (A) SAI activity of Lhx3* proteins from the different organisms. (B) OGI activity of the Lhx3* proteins. (C) Chimeric protein analysis. (Left) Structures of the chimeric proteins with XI_Lhx1* in white and Lhx3 proteins in gray (shown at the bottom). Species abbreviations indicate the origin of the Lhx3 regions. Dark gray in the Ct region, conserved regions including the Lhx3-specific domain at the C-terminus (Thor et al., 1999); light gray, less conserved region; line, deleted region. Bb, amphioxus; Dm, fruit fly; Hp, sea urchin; Hr, ascidian; Sk, hemichordate; XI, frog (for full species names, see Table S1 in the supplementary material).

Deletion analysis of Lhx3 Ct regions using chimeric constructs revealed that SAI activity resides in the two conserved regions of XI_Lhx3 and Hp_Lhx3 (Fig. 3C; constructs 9 and 11). However, the middle part of the Ct region (Ctm) of Hr_Lhx3 (residues 403-577) had SAI activity (construct 10), suggesting that the ascidian Lhx3 acquired a specific transactivation domain in the Ctm region.

Identification of the AHAD of Hr_Lhx3

Using chimeric and deletion analyses, the Ctm region in Hr_Lhx3 was narrowed further to residues 485-577 as being necessary and sufficient for SAI activity (Fig. 4A; constructs 1, 2 and 11). Furthermore, in this region of Hr_Lhx3 we identified ten essential aromatic (F and Y) and hydrophobic (V and M) amino acids using

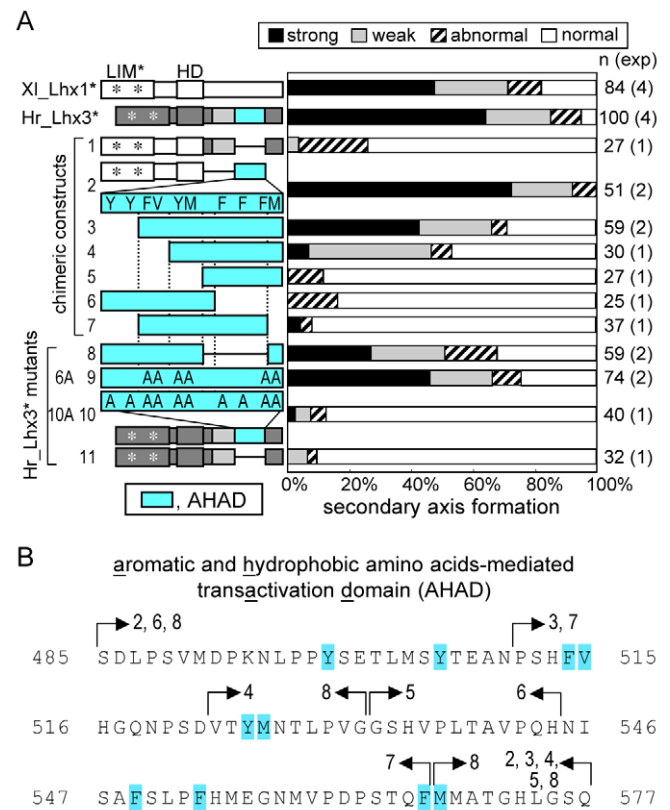


Fig. 4. Identification of the aromatic and hydrophobic amino acid-mediated transactivation domain (AHAD) in ascidian Lhx3. (A) Deletion and point-mutation analysis to define the domain of SAI activity. In the AHAD of Hr_Lhx3 (cyan), ten point-mutated amino acids are indicated. (B) Sequence of the AHAD. Ten point-mutated aromatic and hydrophobic amino acids are highlighted (cyan). Arrows indicate the limits of the deletions in the constructs (2-8) examined.

various deletion and point-mutation constructs (constructs 3 to 10); six or ten mutations of these amino acids into alanines [constructs 9 (6A) or 10 (10A)] resulted in the partial reduction or almost complete loss of SAI activity, respectively. We therefore named this region the aromatic and hydrophobic amino acid-mediated transactivation domain (AHAD). Interestingly, the dependency of the AHAD on aromatic residues is reminiscent of the dependency of CCR2 on five tyrosines (Hiratani et al., 2001). This implies that the AHAD has evolved to be functionally equivalent to CCR2 by sharing coactivator(s) with CCR2. However, it should be noted that the Ct region containing the AHAD but not CCR2 is effective in Hr_Lhx3*-based constructs with the short LH linker (see Fig. 3C).

Analysis of the transactivation domains of Nv_Lhx1 and Hr_Lhx3

To further characterize the transactivation activity of the Ct region of Nv_Lhx1 and the AHAD in a heterologous system, GAL4-UAS reporter analysis was carried out in *Xenopus* embryos (Fig. 5). Despite no SAI activity (Fig. 2A,B,D), the Ct region of Nv_Lhx1, when connected to the GAL4 DNA-binding domain, showed some transactivation activity in the reporter assay. This activity was further enhanced by deletion of CCR1 (Nv_CtΔ1), a region which might have a negative regulatory role, as previously shown for XI_Lhx1 (Hiratani et al., 2001). Thus, although the transactivation

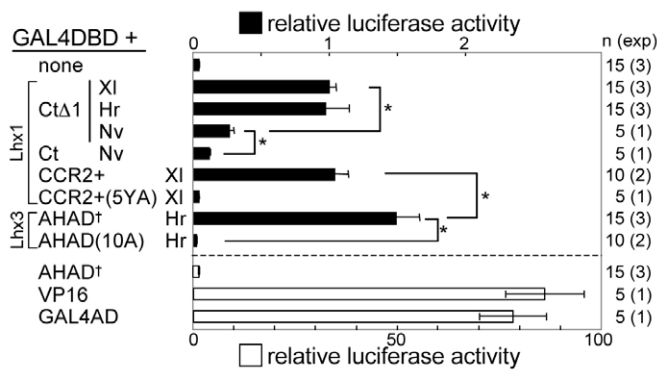


Fig. 5. Comparison of transactivation activity by the GAL4-UAS reporter assay in *Xenopus* embryos. The GAL4 DNA-binding domain (DBD) was fused to a region of Lhx1 or Lhx3 derived from frog (XI), ascidian (Hr) or sea anemone (Nv) as indicated. Bars represent the mean \pm s.e.m. relative to the mean from XI_Lhx1CtΔ1 in each experiment. CtΔ1, the C-terminal region with a deletion of the C-terminal conserved region 1 (CCR1); CCR2+, the C-terminal conserved region 2 with its flanking regions; 5YA, a point-mutated construct of CCR2+; AHAD†, the aromatic and hydrophobic amino acid-mediated transactivation domain; 10A, a point-mutated construct of AHAD; GAL4AD, the GAL4 activation domain (positive control); †, the same result with AHAD but presented on a different scale; **P*<0.01 (*t*-test); n, total number of samples; exp, number of independent experiments.

activity of Nv_CtΔ1 was much weaker than that of XI_CtΔ1 and Hr_CtΔ1 (Fig. 5), Nv_Lhx1 might still act as a transcriptional activator.

The AHAD of Hr_Lhx3 showed a stronger transactivation activity than that of XI_Lhx1 CCR2+, which includes CCR2 and its flanking regions (see Fig. S6 in the supplementary material). This activity was lost in the AHAD(10A) construct, as well as in CCR2+(5YA), in which five tyrosines were substituted with alanines (Fig. 5). However, CCR2+ and the AHAD were much weaker than powerful transactivation domains such as VP16 and GAL4AD, suggesting that both CCR2+ and the AHAD might function in a context-dependent manner, as discussed below.

Nv_Lhx1 is required for Nv_ *chd* expression in the *Nematostella* embryo

Although Nv_Lhx1 did not exhibit organizer activity in *Xenopus* embryos, it might have a role in the regulation of organizer genes such as *chd*, which is coexpressed in the blastoporal region in the *Nematostella* embryo. We performed knockdown analysis of Nv_ *lhx1* in *Nematostella* embryos by microinjection of a morpholino (Nv_ *lhx1* MO) targeting the splice donor site of the first intron (Fig. 6A). As shown in Fig. 6B, correct splicing of the first intron was almost completely blocked in Nv_ *lhx1* MO-injected embryos, but not in control morpholino (ctr MO)-injected embryos. Under these conditions, qRT-PCR analysis revealed that Nv_ *chd* was specifically downregulated in Nv_ *lhx1* MO-injected, but not ctr MO-injected, embryos, in contrast to another organizer gene, Nv_ *otxA* (blastoporal), and positional marker genes such as Nv_ *wnt2* (central) and Nv_ *FGFa1* (aboral); the downregulation of Nv_ *chd* was rescued by co-injection with Nv_ *lhx1* mRNA (Fig. 6C). WISH analysis also confirmed that blastoporal expression of Nv_ *chd* was specifically downregulated by Nv_ *lhx1* MO (Fig. 6D). These results suggest that Nv_Lhx1 regulates Nv_ *chd* in the

blastoporal region at the early gastrula stage, raising the possibility that Lhx1 functions as an ancestral core regulatory gene for organizer evolution.

DISCUSSION

In this paper, we have shown the following results. (1) Blastoporal expression of *lhx1* is probably conserved in Eumetazoa ranging from sea anemones to vertebrates, with some exceptions including ascidians (Fig. 1). (2) Protein evolution of Lhx1 in its LH linker and Ct region, both of which affect organizer activity, might have occurred independently from the emergence of the vertebrate gastrula organizer (Fig. 2). (3) Lhx3, an ancient paralog of Lhx1, evolved to have protein functions similar to those of Lhx1 in the blastoporal region (Fig. 3). (4) Ascidian Lhx3 acquired a specific transactivation domain, designated the AHAD, that exhibits organizer activity in *Xenopus* embryos (Figs 3-5). (5) In the *Nematostella* embryo, Lhx1 regulates one of the organizer genes, *chd* (Fig. 6). Based on these lines of experimental evidence, we propose an evolutionary scenario for the vertebrate gastrula organizer (Fig. 7).

An evolutionary scenario for the gastrula organizer

In this scenario, the blastoporal expression of *lhx1* is an ancestral feature of Eumetazoa (Fig. 7A) and the organizer activity of Lhx1 was acquired in the bilaterian lineage (Fig. 7B). By contrast, the organizer activity of Lhx3 was acquired in the deuterostome lineage (Fig. 7C) and thereafter the blastoporal expression of *lhx3* was possibly acquired in the ancestral chordate (Fig. 7D). Thus, Lhx1 might have contributed as an original organizer gene to organizer evolution after the ancestral eumetazoan arose. Then, the ancestral chordate, like the modern amphioxus, probably utilized Lhx1 and Lhx3 for organizer function (Fig. 7D,G).

In amphioxus, the expression domains of *lhx1* and *lhx3* overlap only in the dorsal mesendoderm region corresponding to the vertebrate gastrula organizer; *lhx1* expression covers the dorsal ectoderm to the dorsal mesendoderm, whereas *lhx3* expression covers the mesoderm to the endoderm at the early gastrula stage (Langeland et al., 2006; Wang et al., 2002). Possible consequences of the recruitment of *lhx3* into this *lhx1*-expressing region are as follows: (1) an increased gene dosage to reinforce expression levels of Lhx1 target genes; and (2) the recruitment of gene batteries of Lhx3 into the blastoporal region. These possibilities are supported by the observations that Bb_Lhx3* upregulates some XI_Lhx1 target genes (Fig. 3B) and that XI_Lhx3 activates the *gsc* promoter with Ldb1 as efficiently as does XI_Lhx1 (see Fig. S7 in the supplementary material). It has also been reported that Lhx1 and Lhx3 activate the *Hesx/Rpx/Xanf* gene through their common enhancers in mice and *Xenopus* (Chou et al., 2006). Thus, Lhx3 could function in the organizer, similar to Lhx1.

During chordate evolution and divergence, vertebrates have preserved the blastoporal expression of *lhx1* and have ceased that of *lhx3* (Fig. 7E). This situation appears similar to that of Wnt genes, as the blastoporal expression of *wnt1* is seen in amphioxus but has ceased and been taken over by other Wnt genes, such as *wnt3*, in vertebrate gastrulae (Holland et al., 2000). The cessation of the blastoporal expression of *lhx3* was not due to gene duplication in vertebrates because vertebrate paralogs of *lhx1* (*lhx5*) and *lhx3* (*lhx4*) are not expressed in the blastoporal region (Toyama et al., 1995; Sheng et al., 1997). We speculate that Lhx3 might have been less efficient than Lhx1 for activating some Lhx1 target genes. For example, XI_Lhx3 does not activate the *cer* promoter with Siamois

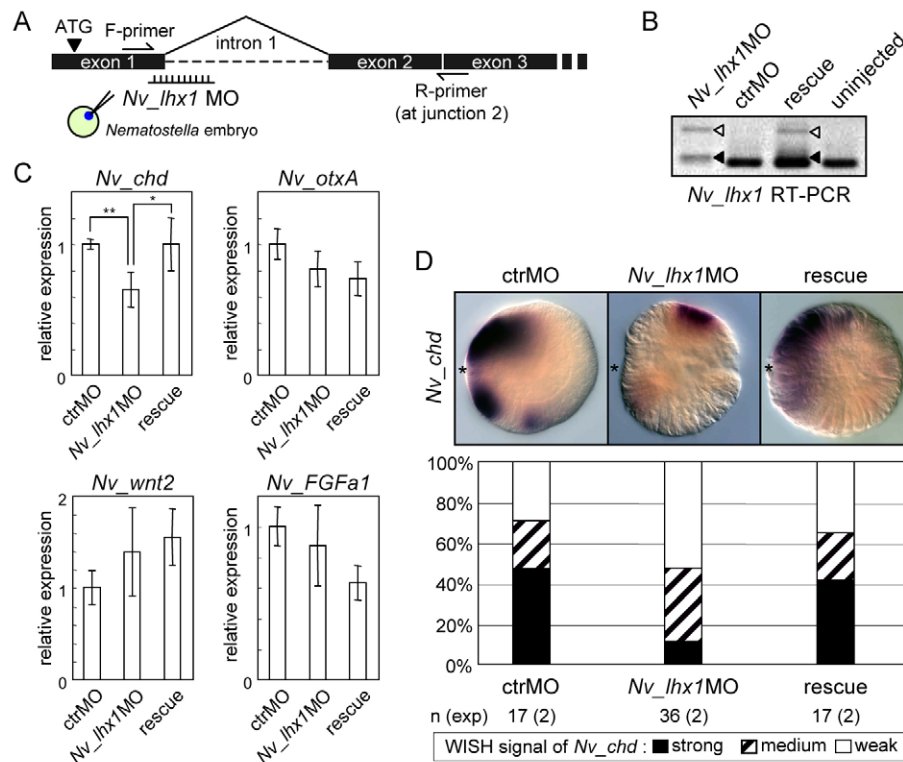


Fig. 6. Knockdown analysis of *lhx1* in *Nematostella* embryos. (A) Schematic representation of the splicing block of the *Nv_lhx1* transcript by the *Nv_lhx1* MO. Locations of the *Nv_lhx1* MO and RT-PCR primers are indicated. (B) RT-PCR detection of the splicing block of the *Nv_lhx1* transcript. A solution containing 450 mM *Nv_lhx1* MO or control MO (ctr MO) was injected with (rescue) or without 0.1 ng/ μ l *Nv_lhx1* mRNA. Open arrowhead, a band from transcripts with intron 1; black arrowhead, a band from aberrant transcripts containing 26 bases of intron 1 by a cryptic donor site (based on the sequence of the band as determined after cloning). (C) Quantitative RT-PCR analysis of several marker genes. Embryos were injected with ctr MO, *Nv_lhx1* MO or *Nv_lhx1* MO plus *lhx1* mRNA (rescue). Because of developmental delay by 3 to 6 hours in MO-injected embryos as compared with uninjected embryos as reported (Rentzsch et al., 2008), the comparison between *Nv_lhx1* MO-injected and ctr MO-injected embryos is shown. Bars represent the mean \pm s.d. relative to ctr MO-injected embryos. * P <0.1, ** P <0.02 (t -test). These statistical differences were reproducible. (D) WISH analysis of injected embryos for *Nv_chd* mRNA. Typical staining patterns are shown. Embryos with different levels of *Nv_chd* signal were classified into three groups (strong, medium and weak) as presented in the graph. Asterisks, blastopore side; n, total number of stained embryos; exp, number of independent experiments.

and Mix.1, in contrast to *Xl_Lhx1* (see Fig. S7 in the supplementary material) (Yamamoto et al., 2003). It is also reported that *Xl_Lhx3* does not have the ability to rescue *Xl_lhx1*-deficient embryos (Hukriede et al., 2003). To further evolve the organizer from amphioxus-type to vertebrate-type, the roles of Lhx3 might have been taken over by Lhx1, which must have created novel gene regulatory networks with other transcription factors, including Otx2, Siamois and Mix.1 (Mochizuki et al., 2000; Yamamoto et al., 2003).

In the ascidian lineage, opposite to in the vertebrate lineage, early expression of *lhx1* ceased, whereas that of *lhx3* was maintained (Fig. 7F). Why did this happen? It should be noted that ascidians are thought to eliminate the organizer to simplify and accelerate their early embryogenesis (Ikuta and Saiga, 2005; Satoh, 2001). One way to eliminate the organizer would be to cease the blastoporal expression of *lhx1* and then of other Lhx1 target organizer genes in turn. By contrast, *lhx3* expression was maintained in the endoderm, and concomitantly Lhx3 evolved to become more potent by acquiring the AHAD (Figs 3-5).

Is there a common function of Lhx1 in the blastoporal region of Eumetazoa? Knockdown experiments have shown that *Nv_lhx1* is required for expression of the organizer gene *chd* (Fig. 6), the possible Lhx1 target in *Xenopus* (Collart et al., 2005; Taira et al.,

1994). Besides affecting gene expression, *Nv_lhx1* MO-injected embryos have defects in gastrulation movements at late gastrula to early planula stages, compared with ctr MO-injected embryos (data not shown). Although it is not known whether *Nv_Lhx1* directly regulates *chd*, it is likely that the regulatory axis from Lhx1 to *chd* might be conserved in Eumetazoa, a contention that will be investigated in the future, together with the role of *Nv_Lhx1* in cell movements. As regards sea urchin, *lhx1* knockdown reportedly reduces the expression of the organizer gene *gsc* [unpublished data (<http://supg.caltech.edu/endomes/ectoQPCRandNano.html>)], which is coexpressed with *lhx1* in the oral ectoderm (Martindale, 2005), although not in the blastoporal region. Thus, the evidence appears to be accumulating that the prototype of an organizer gene regulatory network involving Lhx1 is conserved in Eumetazoa.

Protein evolution of Lhx1 and Lhx3

In our scenario (Fig. 7), the organizer activities of Lhx1 and Lhx3 are not always associated with blastoporal expression. For example, *Nv_Lhx1* is expressed in the blastoporal region of *Nematostella* embryos but does not exhibit organizer activity in *Xenopus* embryos, whereas the situation for *Hr_Lhx1* is reversed (Figs 1 and 2). How, then, have Lhx1 and Lhx3 acquired organizer activity? A clue might

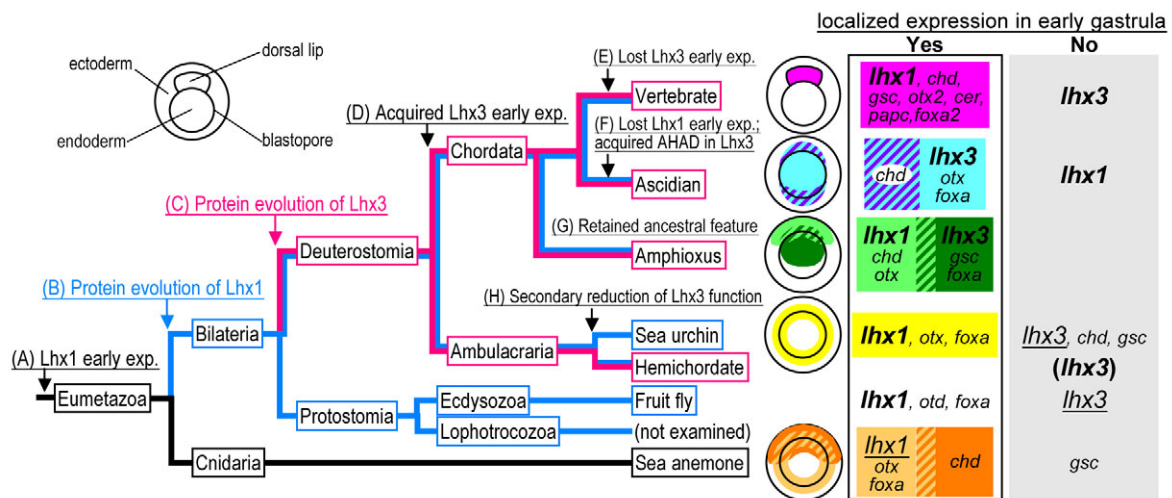


Fig. 7. An evolutionary scenario for the blastoporal expression and protein evolution of Lhx1 and Lhx3. The phylogeny of Eumetazoa (Dunn et al., 2008), with expression and functional data from the present and previous studies. In the phylogenetic tree, colored branches show Lhx1 (blue) and Lhx3 (red) that exhibit organizer activity in *Xenopus* embryos. (A-H) Evolutionary events suggested in this study. To the right are shown schematic representations of gastrulae (blastoporal view) with dorsal (vertebrate and amphioxus), anterior (ascidian) or a pole of the directive axis (sea anemone) to the top. Expression domains are illustrated for those genes with localized expression in the early gastrula (Yes). Hatched regions indicate coexpression of the genes shown in both colored boxes. Genes with organizer activity are in bold, and those with no or little organizer activity in the *Xenopus* embryo are underlined. In the hemichordate, *lhx3* expression in the early gastrula is speculative, as indicated by parentheses.

be found in the fundamental roles of LIM homeodomain proteins in neuronal differentiation in bilaterians (Hobert and Westphal, 2000; Shirasaki and Pfaff, 2002). One may speculate that Lhx1 and Lhx3 might have evolved in association with the creation of more diverged types of neurons, from a simple system in diploblasts to the much more intricate systems of insects and vertebrates. In this situation, structural features of Lhx1 and Lhx3 might have been strictly constrained by their functions in neuronal differentiation. This kind of evolutionary constraint by neuronal function is supported by the fact that *Xl_Lhx1* and its vertebrate paralog *Xl_Lhx5*, which is exclusively expressed in neural tissues (Toyama et al., 1995), are highly conserved throughout the entire region that includes the CCRs (Hiratani et al., 2001).

From these evolutionary constraints, Lhx1 and Lhx3 must have retained distinct structural features, one of which is the length of the LH linker. Lhx1 has a long LH linker of ~60 amino acids, whereas Lhx3 has a short LH linker of 12-20 amino acids. The significance of the length of the LH linker has not been examined before. We revealed that the length, as well as the amino acid sequence, of the LH linker affects Lhx1 activity (Figs 2 and 3). Despite having a short LH linker, deuterostome Lhx3* proteins exhibit organizer activity similar to that of *Xl_Lhx1**, and this is attributed to protein evolution of the Ct regions of Lhx3, including the AHAD of *Hr_Lhx3* (Fig. 3). The AHAD has a stronger transactivation activity than CCR2+, but the transactivation activity of the AHAD is much weaker than that of VP16 and GAL4AD, which are acidic-type transactivation domains that interact with a global coactivator, p300/CBP (Fig. 5). CCR2+ does not interact with CBP (Hiratani et al., 2001) and the AHAD seems not to either. Thus, the relatively weak transactivation activity of Lhx1 and Lhx3 could be suitable for context-dependent combinatorial gene regulation with various co-factors such as Ldb and a putative CCR2- or AHAD-binding protein.

Our scenario presents an outline of organizer evolution from the viewpoint of the transcriptional regulatory core proteins Lhx1 and Lhx3 and provides a working model for further studies. Regarding the question of when organizer genes were incorporated into the ancestral gene regulatory networks around the blastopore, a prototype organizer gene regulatory axis from Lhx1 to *chd* has been suggested (Fig. 6). Notably, the *Nematostella* blastopore lip, in which *lhx1* and *chd* are expressed, reportedly has body axis-inducing activity, whereas aboral ectoderm and invaginating endoderm do not (Kraus et al., 2007). Among organizer genes, only *lhx1* and *chd* continue to be expressed in the blastoporal lip from the diploblast to the vertebrate, also implying the importance of the regulatory axis from Lhx1 to *chd*. Therefore, *lhx1* is a suitable gene for studying the evolution of the organizer at the level of transcriptional regulatory genes. Thus, our evolutionary study of Lhx1, as well as of Lhx3 and Ldb, provides significant insight into the evolution of the vertebrate gastrula organizer and the protein evolution of transcription factors.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/12/2005/DC1>

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