Evolutionary origins of blastoporal expression and organizer activity of the vertebrate gastrula organizer gene \textit{lhx1} and its ancient metazoan paralog \textit{lhx3}

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Expression of the LIM homeobox gene \textit{lhx1} (\textit{lim1}) is specific to the vertebrate gastrula organizer. Lhx1 functions as a transcriptional regulatory core protein to exert ‘organizer’ activity in \textit{Xenopus} embryos. Its ancient paralog, \textit{lhx3} (\textit{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 \textit{lhx1}, but possibly in the ancestral chordate for \textit{lhx3}, and that in the ascidian lineage, blastoporal expression of \textit{lhx1} ceased, whereas endodermal expression of \textit{lhx3} has persisted. Analysis of organizer activity using \textit{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 cnidian embryos suggests that Lhx1 is required for \textit{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, \textit{Nematostella}, Spemann-Mangold organizer, \textit{Xenopus}

INTRODUCTION

The mechanisms of eumetazoan embryogenesis have evolved tremendously during the $\sim$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 (\textit{lim1}), Lhx3 (\textit{lim3}), Lmx, Islet, Lhx2 and Lhx6 (Hobert and Westphal, 2000). Importantly, all are encoded in eumetazoan genomes ranging from humans to the sea anemone \textit{Nematostella vectensis} (Putnam et al., 2007), suggesting that LIM homeodomain proteins diverged at a very early stage of eumetazoan evolution. Among the family, \textit{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 \textit{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 \textit{lhx1}, blastoporal expression of organizer genes (Kawasaki et al., 1999; Martindale, 2005). Recently, it has been reported that some organizer genes, including \textit{nog}, \textit{chd} and \textit{gsc}, are expressed in the \textit{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 \textit{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 \textit{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 (CCR)s 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 (Taia 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; Moreillo 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 hlx1 from the ascidian *Halocynthia roretzi* (Hr_hlx1) were isolated by 5’ and 3’ RACE using RNA from tailbud stage embryos after a homebox fragment was PCR-cloned from genomic DNA using degenerate primers. A full-length Hr_hlx1 cDNA was obtained by RT-PCR using RNA from tailbud stage embryos. The coding sequence of hlx3 from the sea urchin *Hemicentrotus pulcherrimus* (Hp_hlx3) was isolated by RT-PCR using RNA from hatched blastula stage embryos with specific primers designed from hlx3 of another sea urchin, *Strongylocentrotus purpuratus*. The coding sequences of hlx1 and ldb from the sea anemone *Nematostella vectensis* (Nv_hlx1 and Nv_ldb) were isolated by RT-PCR using RNA from polysp 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).

**RESULTS**

Identification of Nv_Lhx1, Nv_Lhx3, Hp_Lhx3 and Hr_Lhx1

To explore the ancestral expression patterns of hlx1 and hlx3 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 hlx1 and hlx3 from the sea anemone *Nematostella vectensis* (Nv_hlx1 and Nv_hlx3), hlx3 from the sea urchin *Hemicentrotus pulcherrimus* (Hp_hlx3) and hlx1 from the ascidian *Halocynthia roretzi* (Hr_hlx1). 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).
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 motif and truncation of its coding sequence (see Fig. S1 in the supplementary material), 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 disected 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 endoderm 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., 2007), suggest the possibility that the blastoporal expression of lhx1 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 lhx1.

Evolutionarily conserved function of Nv_Ldb to activate XI_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 XI_Ldb1 (Fig. 2A; see Fig. S4A
Bilaterian 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 naïve 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 paped 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;
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) of all 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.
Deletion analysis of Lhx3 Ct regions using chimeric constructs revealed that SAI activity resides in the two conserved regions of Xl_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 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).

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_CtA1), a region which might have a negative regulatory role, as previously shown for XI_Lhx1 (Hiratani et al., 2001). Thus, although the transactivation
activity of Vcw\textsubscript{Ct}1\textsubscript{T} was much weaker than that of Xl\textsubscript{Ct}1\textsubscript{T} and Hr\textsubscript{Ct}1\textsubscript{T} (Fig. 5), Vcw\textsubscript{Lhx1} might still act as a transcriptional activator.

The AHAD of Hr\textsubscript{Lhx3} showed a stronger transactivation activity than that of Xl\textsubscript{Lhx1} CCR2\textsuperscript{+}, which includes CCR2 and its flanking regions (see Fig. S6 in the supplementary material). This activity was lost in the AHAD10\textsubscript{A} construct, as well as in CCR2\textsuperscript{+}(5YA), in which five tyrosines were substituted with alanines (Fig. 5). However, CCR2\textsuperscript{+} and the AHAD were much weaker than powerful transactivation domains such as VP16 and GAL4\textsubscript{AD}, suggesting that both CCR2\textsuperscript{+} and the AHAD might function in a context-dependent manner, as discussed below.

**Nv\textsubscript{Lhx1} is required for Nv\textsubscript{chd} expression in the Nematostella embryo**

Although Vcw\textsubscript{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 Vcw\textsubscript{Lhx1} in Nematostella embryos by microinjection of a morpholino (Vcw\textsubscript{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 Vcw\textsubscript{Lhx1} MO-injected embryos, but not in control morpholino (ctr MO)-injected embryos. Under these conditions, qRT-PCR analysis revealed that Vcw\textsubscript{chd} was specifically downregulated in Vcw\textsubscript{Lhx1} MO-injected, but not ctr MO-injected, embryos, in contrast to another organizer gene, Vcw\textsubscript{otx4} (blastoporal), and positional marker genes such as Vcw\textsubscript{wnt2} (central) and Vcw\textsubscript{FGFa1} (aboral); the downregulation of Vcw\textsubscript{chd} was rescued by co-injection with Vcw\textsubscript{Lhx1} mRNA (Fig. 6C). WISH analysis also confirmed that blastoporal expression of Vcw\textsubscript{chd} was specifically downregulated by Vcw\textsubscript{Lhx1} MO (Fig. 6D). These results suggest that Vcw\textsubscript{Lhx1} regulates Vcw\textsubscript{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) A new Clade 3 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\textsubscript{Lhx3}* upregulates some Xl\textsubscript{Lhx1} target genes (Fig. 3B) and that Xl\textsubscript{Lhx3} activates the gsc promoter with Ldb1 as efficiently as does Xl\textsubscript{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, Xl\textsubscript{Lhx3} does not activate the cor promoter with Siamois
and Mix.1, in contrast to Xl_Lhx1 (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://sugp.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.

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Supplementary material
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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 Nemastolella 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.