Develop. Growth Differ. (2006) **48**, 463–472 doi: 10.1111/j.1440-169x.2006.00883.x

Hindgut specification and cell-adhesion functions of *Sphox11/13b* in the endoderm of the sea urchin embryo

César Arenas-Mena,[1,*] R. Andrew Cameron[2] and Eric H. Davidson[2]

[1]*Department of Biology, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182-4614, USA and* [2]*156–29, Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA*

Sphox11/13b is one of the two *hox* genes of *Strongylocentrotus purpuratus* expressed in the embryo. Its dynamic pattern of expression begins during gastrulation, when the transcripts are transiently located in a ring of cells at the edge of the blastopore. After gastrulation, expression is restricted to the anus–hindgut region at the boundary between the ectoderm and the endoderm. The phenotype that results when translation of *Sphox11/13b* mRNA is knocked down by treatment with morpholino antisense oligonucleotides (MASO) suggests that this gene may be indirectly involved in cell adhesion functions as well as in the proper differentiation of the midgut–hindgut and midgut–foregut sphincters. The MASO experiments also reveal that *Sphox11/13b* negatively regulates several downstream endomesoderm genes. For some of these genes, *Sphox11/13b* function is required to restrict expression to the midgut by preventing ectopic expression in the hindgut. The evolutionary conservation of these functions indicates the general roles of posterior *Hox* genes in regulating cell-adhesion, as well as in spatial control of gene regulatory network subcircuits in the regionalizing gut.

Key words: cell adhesion, endoderm, hindgut, Hox, sea urchin.

Introduction

Sphox11/13b is one of several posterior-group *hox* genes of the sequenced *Strongylocentrotus purpuratus hox* gene cluster (Cameron *et al*. 2005). Both qualitative (Dobias *et al*. 1996) and quantitative (Arenas-Mena *et al*. 1998) measurements detect *Sphox11/13b* transcripts as early as 8 h after fertilization (late cleavage), and its expression continues during later embryonic and larval stages (Arenas-Mena *et al*. 1998; Arenas-Mena *et al*. 2000). *Hox* genes have been implicated in the specification of vertebrate (Yokouchi *et al*. 1995; Van der Hoeven *et al*. 1996; Beck *et al*. 2000), *Drosophila* (LeMotte *et al*. 1989; Tremml & Bienz 1989), and *Caenorhabditis elegans* digestive tracts (Chisholm 1991; Ferreira *et al*. 1999). In particular, the chicken Hoxa-13 is expressed in the large intestine (Grapin-Botton & Melton 2000) and mutations in mouse Hoxa-13 and Hoxd-13 cause hindgut malformation (Warot *et al*. 1997). These studies suggest conservation of function in the hindgut and anus of *hox* genes of the paralogue group with which we are here concerned.

Embryonic *hox* gene expression in indirectly developing marine invertebrates requires special consideration. In contrast to the conserved expression in direct developing embryos as divergent as those of vertebrates and arthropods (McGinnis & Krumlauf 1992), indirectly developing bilaterians lack colinear *Hox* cluster gene expression during the embryonic specifications resulting in the formation of the larva (Arenas-Mena *et al*. 1998; Peterson *et al*. 2000). However, these animals do display a panbilaterian pattern of spatially colinear *hox* gene deployment later, during the postembryonic formation of their adult body (Arenas-Mena *et al*. 2000; Irvine & Martindale 2000). Two individual Hox genes display embryonic expression with no hint of colinearity (Angerer *et al*. 1989; Dobias *et al*. 1996). We have decided to explore the expression of one, *Sphox11/13b*, which has been described to have embryonic expression in this (Dobias *et al*. 1996) and other echinoid species (Ishii *et al*. 1999; Wilson *et al*. 2005).

*Author to whom all correspondence should be addressed.
Email: arenas@sciences.sdsu.edu
Author contributions: César Arenas-Mena, 75%; R. Andrew Cameron, 15%; Eric H. Davidson, 10%.
Received 17 May 2006; revised 3 July 2006; accepted 12 July 2006.

In this report, we describe the expression pattern of the sea urchin posterior-group *hox* gene *Sphox11/13b* during embryogenesis, and we then examine the phenotypic effects of its suppression with regards to morphology and downstream gene expression.

Materials and methods

Embryo procurement and culture, and whole-mount in situ *hybridization*

The protocol used for embryos is a version of that previously described for postembryonic stages (Arenas-Mena *et al*. 2000), with the following minor alterations. Embryos were fixed in 0.1 M MOPS pH 7, 0.5 M NaCl, and 4% formaldehyde for 3 h at room temperature. For the 12 h stage the fertilization membrane was removed by passing fertilized eggs through a 60 μm nylon membrane in the presence of *p*-aminobenzoic acid (PABA). A 1:2000 dilution of the alkaline phosphatase conjugated antibody was used instead of a 1:1500 dilution.

Treatment with sphox11/13b *morpholino antisense oligonucleotide*

Translation of *Sphox11/13b* mRNA was reduced by microinjection of a morpholino oligonucleotide (Sp*Hox* 11/13b MASO) with the sequence 5′-AGCCTGTTCCATGCCGATCTGCAT-3′, synthesized by Gene Tools (Philomath, OR, USA). The initial methionine codon is underlined. This sequence was checked against the sequence of the whole genome to preclude shared specificity with other genes. The control morpholinio antisense oligonucleotide (MASO) had the arbitrary sequence 5′-CCTCTTACCTCAGTTACAATTTATA-3′. Embryos were microinjected as previously described (McMahon *et al*. 1985) with 10 or 4.5 pL of 500 μM MASO solution. Microinjected embryos were collected at different stages and fixed for whole-mount *in situ* hybridization (WMISH), as described above, or for quantitative polymerase chain reaction analysis (QPCR) as described previously (Davidson *et al*. 2002).

The specificity of the *Sphox11/13b* MASO oligonucleotide was tested by its ability to block the expression of a *sphox11/13b*::GFP fusion construct driven by the hatching enzyme transcriptional regulatory sequence (Wei *et al*. 1995). To produce this construct, the oligonucleotides 5′-CATCGGTCGACGGATCCTTAT-3′ and 5′-GCCAGAATGCAGATCGGCATGGAACAGGCTAGCAAGGCGAGGAACT-3′ were used in a PCR with GreenLantern2 plasmid (Gibco BRL, Gaithersburg, MD, USA) as template. This product was then cloned into pGem-T vector (Promega, Madison, WI, USA), and the hatching enzyme promoter *Sac*I fragment (–1225, +20) cloned into the *Sac*I site of the same recombinant plasmid.

Quantitative measurement of transcript abundance

In order to measure the changes in transcript abundance for a suite of genes involved in the endomesoderm specification gene regulatory network when Sp*Hox* 11/13b expression was reduced, a now routine QPCR protocol was used (Rast *et al*. 2000; Davidson *et al*. 2002; Oliveri *et al*. 2002). RNA was isolated from groups of injected or normal embryos by extraction with RNAzol (Leedo Medical Laboratories, Houston, TX, USA) and data was collected on a GeneAmp 5700 Sequence Detection System, using SYBR Green chemistry (PE Biosystems, Foster City, CA, USA). Each measurement is the average of at least three QPCR reactions from cDNA derived from the same microinjection experiment at the indicated time point, and microinjection of different embryo batches were usually repeated several times. These independent experiments are represented by the different numbers in our data tables, which can be found online at http://sugp.caltech.edu/endomes/qpcr.html. In order to calculate the absolute number of transcripts for the gene of interest, ubiquitin mRNA was used as an internal reference standard because it is relatively constant during embryogenesis (Nemer *et al*. 1991; Ransick *et al*. 2002). The QPCR data are posted on the Gene Network web page (2006a) and the PCR primers are posted on the resources page (Gene Network 2006b).

Results

Expression of Sphox11/13b *during embryogenesis*

Previous reports indicated that *Sphox11/13b* mRNA molecules are barely present in unfertilized eggs and that they attain an abundance of about 1600 copies per individual in 12 h embryos, as measured by probe excess mRNA titration (Arenas-Mena *et al*. 1998). In agreement with these results, the expression of *Sphox11/13b* is undetectable by WMISH in 6 h embryos and becomes visible in all 12 h embryo veg2 blastomere descendants, which contribute to both endoderm and mesoderm (Fig. 1A–D; for a veg2 fate map see (Cameron *et al*. 1991). The micromere descendants in 12 h embryos lack detectable expression (Fig. 1C). Thus, the initial *Sphox11/13b* expression encompasses the entire endomesoderm progenitor field, excluding the centrally arranged micromeres. This general veg2 lineage restriction continues in 20 h embryos (Fig. 1E–F) and by 24 h,

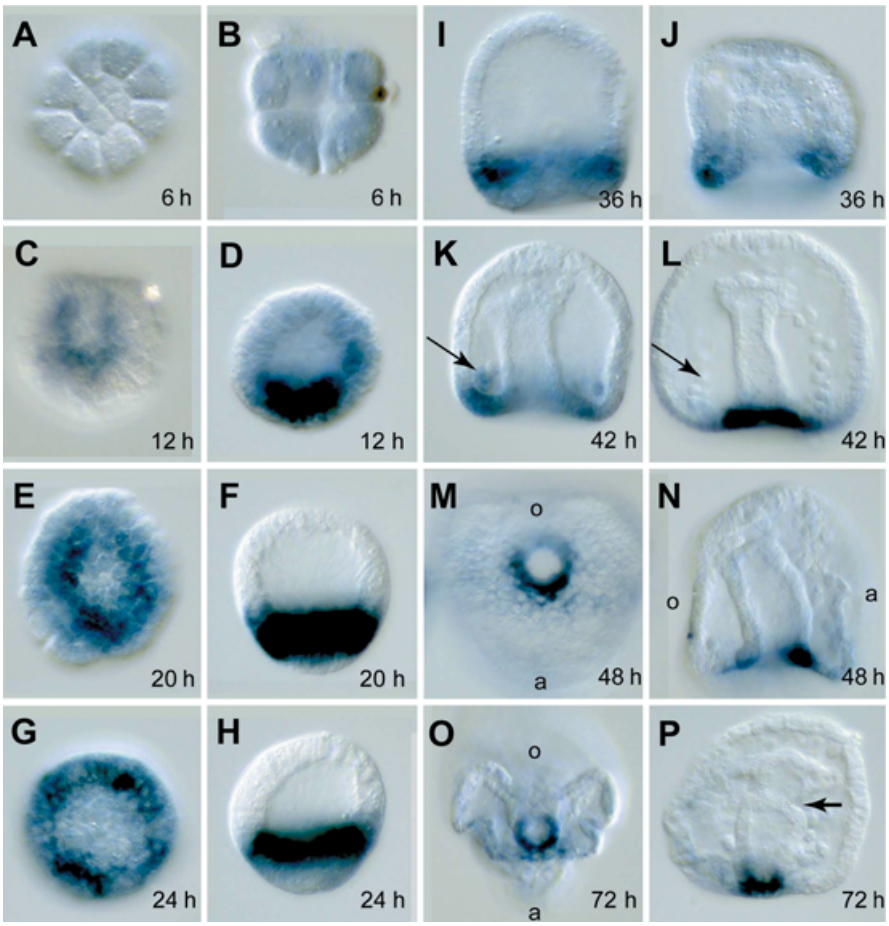

Fig. 1. *Sphox11/13b* mRNA expression during embryogenesis. Whole-mount *in situ* hybridization of embryos fixed at the time after fertilization indicated in hours (h). All but the 36 h stage are from the same batch. (A,C,E,G) are vegetal views. (B,D,F,H–L,N,P) are side views with the blastopore to the bottom. *Sphox11/13b* maintains a transient pattern of expression during gastrulation in the blastopore region in Veg2 descendant cells that will contribute to the endoderm (C–N), and a definitive asymmetric pattern of expression along the oral–aboral axis at the ectoderm–endoderm boundary, which extends into the hindgut (O,P). o, oral side; a, aboral side. Arrows in (K,L) signal primary mesenchyme cells. Arrow in (P) signals the midgut–hindgut sphincter.

after the primary mesenchyme cells (PMC) have ingressed into the blastocoele, expression becomes restricted to the endodermal veg2 blastomere descendants excluding the centrally located disc of mesoderm precursors. Simultaneously, expression expands to Veg1 descendants (Fig. 1G,H). New expression occurs in veg1 cells as they enter the blastopore region during gastrulation, during later stages (Fig. 1I–L). By 42 h postfertilization, expression is restricted to the blastopore area (Fig. 1K,L) and no expression is detectable in fore- and midgut archenteron cell precursors that were expressing this gene during earlier stages (Fig. 1C–F). Note that PMC appear to express *Sphox11/13b* transiently after their ingression, at the time in which they form a ring at the base of the archenteron (Fig. 1I–K), but this expression declines as skeletogenesis begins shortly thereafter (compare PMC expression in Fig. 1K, arrows, with the slightly more advanced stage in Fig. 1L). Near the end of gastrulation, expression remains restricted to the blastopore, but now the stain intensity seems to be stronger in the aboral portion along the oral–aboral axis (Fig. 1M,N), and by 72 h postfertilization expression is undetectable in the most oral cells of the developing anus (Fig. 1O). The terminal portion of the hindgut also shows staining, but it does not expand to the midgut (Fig. 1P). This hindgut–anus area expression continues into later feeding larval stages (Arenas-Mena *et al.* 2000).

Targeted morpholino antisense oligonucleotide specifically downregulates SpHox11/13b and interferes with gastrulation

The downstream effects of Sphox11/13b protein downregulation were determined using a MASO complementary to the N-terminal coding region. The specificity of this synthetic inhibitor is demonstrated in Figure 2, where it is shown to block the translation of an mRNA coding for a recombinant fusion protein of 5′SpHox11/13b and green fluorescent protein (5′SpHox 11/13b::GFP). This fusion protein is expressed in embryos under control of the hatching enzyme enhancer, thus providing broad and lasting embryonic expression. The same MASO does not block the expression of GFP mRNA lacking any

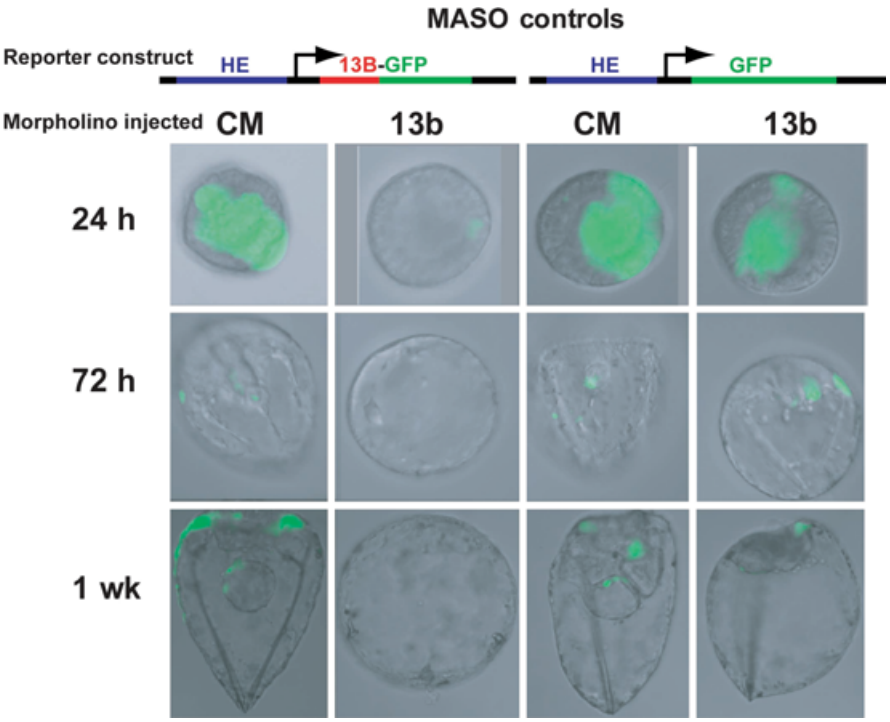

Fig. 2. Morpholino antisense oligonucleotide (MASO) controls for late phenotypic effects. To control for effective morpholino translational repression during late stages, sea urchin embryos were injected with MASO targeted against the 5′-coding region of the *Sphox11/13b* gene (13b) or with a control morpholino oligonucleotide (CM) at the same concentration (see methods for details), in combination with reporter green fluorescent protein (GFP) constructs driven by the broadly expressed hatching enzyme enhancer (HE). In one of the constructs (13B-GFP), diagramed at top left, the HE promoter was fused with the 5′*Sphox11/13b* coding region matching the *Sphox11/13b* morpholino sequence (13B), while in the other (GFP), the GFP sequence was left intact, diagram top right. Fluorescent and optical overlays show the expression of 13B-GFP (left pair of columns), and GFP (right pair of columns) proteins at the time points indicated. The results indicate that the 13b morpholino specifically blocks 13b-GFP mRNA translation at least up to 1 week after fertilization, and that the control morpholino does not block GFP nor 13b-GFP translation.

5′Sphox11/13b sequence. The sequence-specificity of the inhibition is further demonstrated by injection of a random sequence control MASO, which fails to inhibit either *5′Sphox11/13b::GFP* or *GFP* mRNA translation (Fig. 2, first and third columns). It is interesting to note that the effects of the *Sphox11/13b*-MASO last well beyond the temporal scope of this study. Because of the continuous transcription provided by the proximal hatching enzyme promoter, both the *5′Sphox11/13b::GFP* mRNA and the GFP mRNA continue to be produced in larvae of up to 1 week of development (Fig. 2, 1 week, bottom row), and the translation of *5′Sphox11/13b::GFP* mRNA is still inhibited by the *5′Sphox11/13b*-MASO. In summary, these controls confirm the target specificity of the *5′Sphox11/13b*-MASO and gauge its long-extending translational effects.

Figure 3 shows a sample of the phenotypic effects of *Sphox11/13b* downregulation by means of the *Sphox11/13b*-MASO. The morphology of 24 injected embryos was individually observed repeatedly through early development. In addition, groups of more than 200 injected embryos where observed en masse at specific time points (not shown). This protocol was repeated four times with different batches of embryos. Injection of *Sphox11/13b*-MASO completely blocks gastrulation in most embryos (Fig. 3G–I) while the injection of the control morpholino oligonucleotide at the highest experimental concentration does not cause any major developmental abnormalities (Fig. 3A–C,J). Differentiation of pigment cells ensues in *Sphox11/13b*-MASO treated embryos, and the red cells accumulate to one side (top in the embryo in Figure 3I), which is perhaps consistent with their normal placement in the aboral ectoderm. Thus some ectodermal polarity has not been completely lost in these otherwise abnormal embryos (Fig. 3I). Nevertheless, pigment cell differentiation is probably delayed in these embryos because the very same embryo depicted in Figure 3I did not exhibit any pigment cells at the time point in which control embryos did (compare Figure 3H with B). Injection of a lesser amount of *Sphox11/13b*-MASO allows gastrulation to occur in most embryos (Fig. 3E), though there is a substantial delay in the process, and the morphology of the archenteron is abnormal (compare Figure 3E with 3B, and see below).

Later in development, pre-feeding larvae with reduced expression of *Sphox11/13b* protein fail to differentiate a proper tripartite gut (Fig. 3F). By 72 h after fertilization, control larvae have well differentiated midgut–hindgut and midgut–foregut constrictions (Fig. 3C), while experimental ones have a thin, cylindrical gut with no signs of regional differentiation

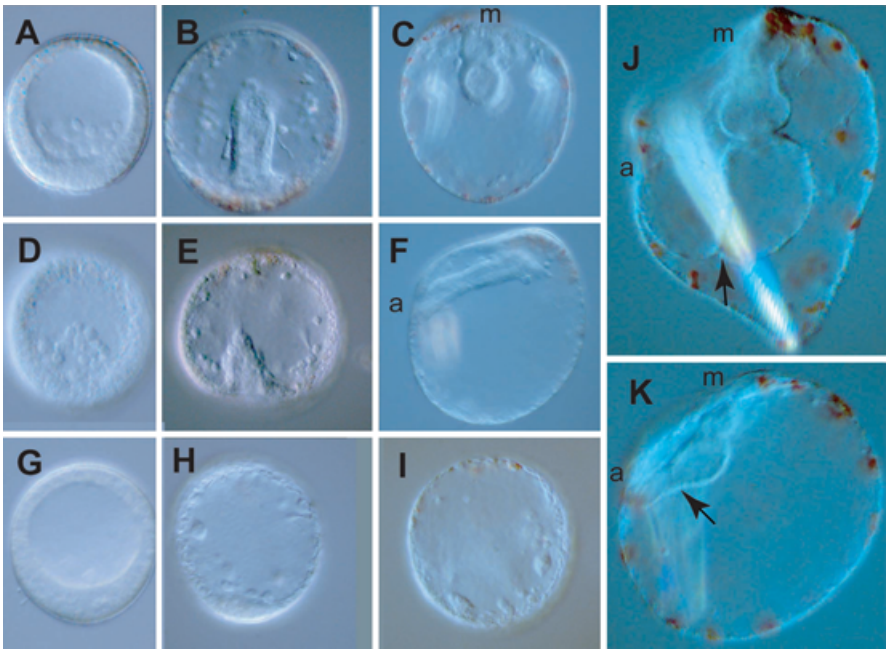

Fig. 3. Morphological study of Sphox11/13b morpholino antisense oligonucleotide (MASO) phenotype. Reduction of *Sphox11/13b* function affects gastrulation and the morphological differentiation of the digestive tract. Living embryos were photographed at the indicated times during their development after injection with approximately 10 pL of a 500 μM control MASO solution (A–C; different embryos in each plate), about 4.2 pL of a 500 μM *Sphox11/13b*-MASO solution (D–F,K; the same embryo at different stages), or 10 pL of a 500 μM *Sphox11/13b*-MASO solution (G–I; the same embryo at different stages). Embryos were photographed at 24 h (A,D,G), 48 h (B,E,H), and 72 h (C,F,I). Embryos (J) and (K) were raised for one week without feeding; (K) is the larva derived from the embryo embryo in (D–F). High doses of *SpHox11/13b*-MASO prevent gastrulation (H,I), and low doses disrupt proper morphological differentiation of the tripartite gut (compare C with F, and J with K). The arrows in (J) and (K) point to the midgut–hindgut boundary. a, anus; m, mouth.

(Fig. 3F). Eventually, these larvae may differentiate a tripartite gut (Fig. 3K); however, it is smaller and has an abnormally indistinct midgut–foregut transition (compare Figure 3K and J). That abnormal gastrulation and gut differentiation of treated embryos occurs in parallel with the relatively normal development of other ectodermal and mesodermal structures such as skeletal rods, pigment cells and oral ectoderm is illustrated in observations of a single individual at different time points (Figure 3D–F, later becoming the larva shown in K). This further demonstrates that the MASO effects are specific, that is, only tissues where *Sphox11/13b* is expressed show phenotypes. Even so, some polarity within the aboral ectoderm, where *Sphox11/13b* is partially expressed (Fig. 1O), may have been lost given the round shape without a vertex that these late embryos and larvae usually adopt (Fig. 3F,K). The abnormal larvae fail to feed properly, and algae accumulate in the hindgut. Detachment of the hindgut from the ectoderm was observed in some 2 week old larvae (not shown). The study of this later phenotype was not pursued further. However, disconnection of the hindgut from the ectoderm never occurred in control MASO-injected animals.

Downstream targets of Sphox11/13b

The effect of the downregulation SpHox11/13b protein on the expression of 23 embryonic genes at 12, 24 and 72 h of development was studied by quantitative PCR (QPCR; see Gene Network 2006c). All the genes studied are normally expressed in the endomesoderm domain and are located in the regulatory network underlying its specification (Davidson *et al.* 2002). At 24 h five of these endomesoderm specification genes (*Bra*, *Krox*, *GataE*, *OrCt*, and *Endo16*) show an increase in abundance of more than threefold between embryos injected with *Sphox11/13b*-MASO and the control (Gene Network 2006c). This suggests that, directly or indirectly, *Sphox11/13b* normally downregulates the expression of these genes.

To further explore the phenotypic changes resulting from SpHox11/13b depletion, WMISH of *GataE*, *Endo 16*, *Apobec* and S*phox11/13b* was performed in *Sphox11/13b*-MASO and control MASO embryos at 24, 48, and 72 h of development (Figs 4,5). *Endo16* is an endomesoderm marker the normal expression of which is restricted to vegetal plate cells (Ransick A *et al.* 1993). It remains so in both experimental and control embryos of this study (Fig. 4A1,2). However, the cells expressing *endo16* in SpHox11/13b depleted embryos do not appear to maintain the tight intercellular contacts seen in control embryos. They thus lose the normal ring-shape domain of gene expression, and endoderm marker expressing cells lie dispersed among nonexpressing cells (Fig. 4A1,2). In treated embryos *endo16* expressing cells do not invaginate, but rather ingress into the blastocoele

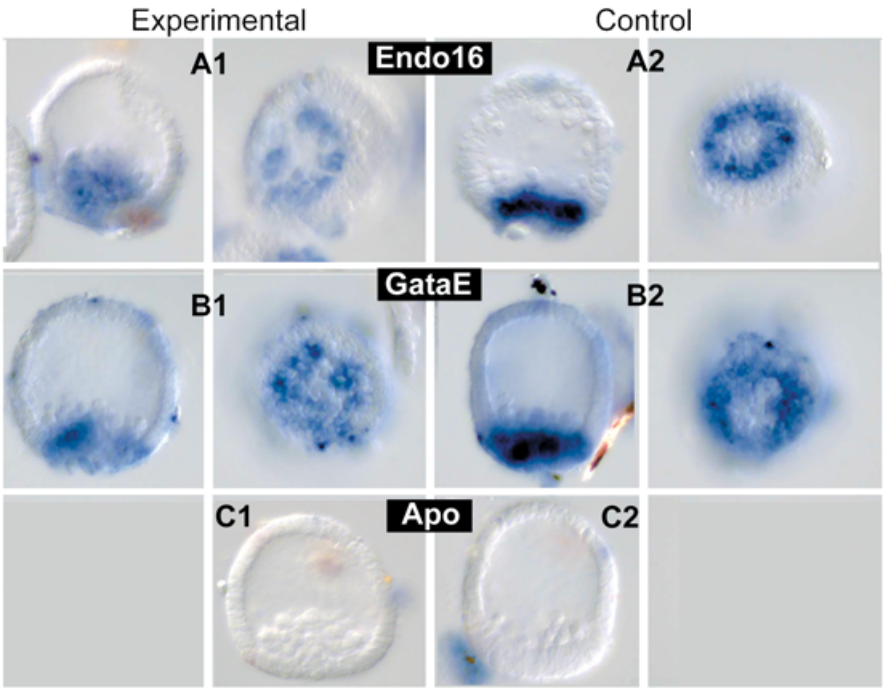

Fig. 4. Cell adhesion phenotype of Sphox11/13b downregulation in the vegetal plate. The vegetal plate looses its integrity in embryos with lowered levels of SpHox11/13b protein. The position of endoderm cells was detected by the expression pattern of endoderm markers in 24 h embryos. Whole-mount *in situ* hybridizations (WMISH) were performed for *Endo16* (A1,2), and *GataE* (B1,2) in embryos microinjected with *Sphox11/13b*-MASO (A1,B1) and control morpholino antisense oligonucleotide (MASO) (A2,B2). Injections were of approximately 4.2 pL of 500 μM MASO solutions. In MASO-injected embryos, endoderm precursors loose their typical ring-shape organization and intermingle with nonendoderm precursors. All the embryos shown are from the same batch. The right image of each pair is a vegetal view, the left a lateral view. (C1,2) Unstained experimental and control embryos, to display abnormal accumulation of cells within the blastocoele caused by MASO treatment. These embryos were treated the same as those in (A) and (B), that is, subjected to the WMISH protocol, but the probe used targets *apobec* mRNA (Rast *et al.* 2000) which is not expressed at this stage, although it seems to be regulated by *SpHox11/13b* (Gene Network 2006c; Fig. 5).

where they form a disorganized clump (Fig. 4A1, left). This abnormal ingression phenotype is clearly seen in the unstained embryos of Figure 4C1 and 2. The expression patterns obtained for *gatae* in experimental and control embryos are very similar to those of *endo16* (Fig. 4B1,B2). Considering that these embryos display extra cells in the blastocoele when compared to controls (Fig. 4C1,2), the overall phenotype suggests that the vegetal plate is losing integrity in embryos with reduced levels of SpHox11/13b protein.

The abnormal gastrulation phenotype continues into late gastrula stages. At 48 h, the treated embryos express *endo16*, *gataE* and *apobec* (Fig. 5A1,B1,C1), but the expressing cells in these embryos still fail to form the compact epithelium seen in the control embryos (Fig. 5A2,B2,C2). The treated embryos that do invaginate show a very loose archenteron from which cells detach, especially at the tip, adopting an abnormal 'open chimney' shape (Fig. 5B1,C1).

In 72 h control embryos the expression of endo16, gatae and apobec is restricted or stronger in the midgut (Fig. 5D2,E2,F2). However, in Sphox11/13b-MASO-treated embryos the expression of these three genes extends or increases in the hindgut–anus area (Fig. 5D1,E1,F1). The homogeneous expression of these three midgut genes in the midgut and hindgut of experimental embryos correlates with the lack of morphological differentiation of the midgut from the hindgut region (Figs 5D1,E1,F1,3F). The expression of Sphox11/13b, a hindgut marker, remains restricted to the hindgut–anus area in both control and MASO-treated embryos (Fig. 5J1,2).

Discussion

Spatial expression of Sphox11/13b *during development*

Pregastrular *Sphox11/13b* embryonic expression in the vegetal plate (Fig. 1C,D) is in agreement with its inferred position as an early downstream target of the *wnt8-blimp1/krox-otx* positive feedback subcircuit that drives endomesoderm specification (Davidson *et al.* 2002). Considering the dynamic expression of *Sphox11/13b* within the *veg2* blastomere lineage, all cells that express *Sphox11/13b* transcripts at some time before or during gastrulation will become endoderm (Fig. 1). Prospective *veg2* mesoderm precursors lose *Sphox11/13b* expression first, then expression decreases in the more central endoderm cells that will form foregut and midgut upon invagination. This

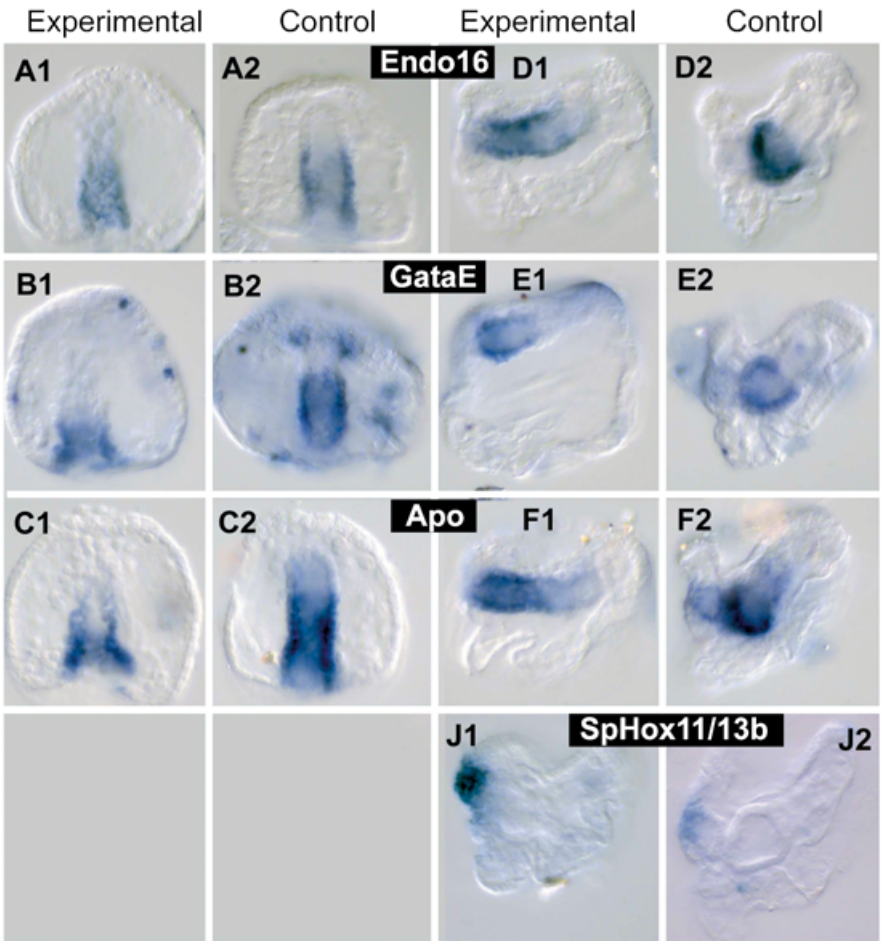

Fig. 5. Effects of Sphox11/13b-MASO treatment on gene expression at late gastrula and pluteus stages. Reduced levels of SpHox11/13b protein cause expansion of expression into the hindgut of genes normally expressed only in midgut. Embryos with reduced SpHox11/13b levels gastrulate abnormally, and at pluteus stage the 'hindgut' region is not morphologically distinct as in controls (see text). Expression patterns of *endo16*, *gataE* and *apobec* are shown, as indicated. Injections were of approximately 4.2 pL of 500 μM control MASO (A2,B2,C2,D2,E2,F2,J2) or *Sphox11/13b*-MASO (A1,B1,C1, D1,E1,F1,J1) solutions. (A–C) display late gastrula stage embryos at 48 h; (D–F) are 72 h pluteus stage larvae.

pattern of expression is similar to that described by the *wnt8* and *blimp1/krox* genes (Livi & Davidson 2006; Minokawa *et al*. 2005). Later expression is maintained in the hindgut–anus region and the immediately surrounding ectoderm, where it continues well beyond embryogenesis (Fig. 1M–P; Arenas-Mena *et al*. 2000).

The final expression pattern of *Sphox11/13b* in the hindgut–anus is asymmetric along the larval plane of bilateral symmetry, in that expression is absent on the oral side in the pluteus stage (Fig. 1O). The late expression domain is complementary to the expression pattern of *SpfoxB*, which is transcribed on the oral side of the anus, as revealed by WMISH carried out on the same batch of embryos (data not shown). This confirms the existence of a previously undescribed gene expression domain along the plane of bilateral symmetry involving ectodermal and endodermal layers (Minokawa *et al*. 2004). It will be interesting to explore regulatory relationships between *SpfoxB* and *Sphox11/13b*. A previous report shows that oral *veg1* cell descendants contribute more and extend deeper into the archenteron than do aboral *veg1* cells (Ransick & Davidson 1998). Nonetheless, there is some invagination of veg1 even on the aboral side, and the pattern of expression of *Sphox11/13b* by 48 h, when the invagination is long complete, clearly lies within the domain of veg1 descendants as this includes the ectoderm surrounding the anus on the aboral side.

The pattern of *Sphox11/13b* expression reported here does not agree with that reported previously (Dobias *et al*. 1996). This previous study described a wide distribution of transcripts and protein during blastula stages, with strong oral ectoderm staining and general expression in the archenteron during later stages. Although the *Sphox11/13b* probe used here is the same as that used by Dobias *et al*. (1996), the hybridization conditions in this study (Arenas-Mena *et al*. 2000) are 8.5°C more stringent, and the wash conditions about 12.5°C more stringent, according to the hybridization melting point formula that derives from corrections for histological conditions (Cox *et al*. 1984). A very long hybridization incubation of 1 week was also introduced to compensate

for the strong decline in hybridization rates reported under histological conditions (Cox *et al*. 1984). This long hybridization much enhances the detection of low prevalence transcripts, and other additional modifications also increased the specificity of this method (Arenas-Mena *et al*. 2000). The more stringent conditions used in the present WMISH protocol may have eliminated non-specific staining in the samples described earlier (Dobias *et al.* 1996), which may have derived from general retention of probes and antibodies by the ciliary band cells. Furthermore, our results are confirmed by the late hindgut expression in another echinoid species (Wilson *et al*. 2005). The application of this protocol has also provided improved characterization of *Spfoxb*, and *Spkrl* mRNA expression patterns (Minokawa *et al*. 2004).

Functions of Sphox11/13b in the vegetal plate

The expression pattern of the *Sphox11/13b* gene illuminates the morphological alterations caused by depletion of SpHox11/13b protein (Figs 3,4C). The morphological phenotype of embryos with reduced levels of SpHox11/13b protein ranges in a dose-dependent fashion from total inhibition of gastrulation to abnormal differentiation of the gut. The cause of the abnormal gastrulation is indicated by the pattern of expression of endoderm marker genes at 24 h (Fig. 4). Embryos with lowered SpHox11/13b protein display *gatae* and *endo16* expressing cells that fail to maintain normal contiguity within the vegetal plate. Instead, these cells have lost contact with one another, intercalated with cells lacking endoderm gene expression, and prematurely ingressed into the blastocoele without any epithelial cohesion. Thus, one of the roles of *Sphox11/13b* seems to be maintenance of physical contiguity within the endoderm territory, rather than establishment or maintenance of transcriptional identity and subsequent differentiation. The implication is that among the early targets of the SpHox11/13b transcription factor might be genes encoding cell adhesion proteins. Cell contacts are likely needed to maintain any cell–cell signaling events required to reinforce endomesoderm identity, to mediate the morphogenetic events of gastrulation, and to maintain fixed cell-territory boundaries. Of course, this is only a possibility and additional experiments would be required to show unequivocally that the defect is a lesion in cell adhesion directly.

Persistent expression of *Sphox11/13b* at the interface of the ectoderm and the endoderm extending into larval stages (Arenas-Mena *et al*. 2000) might control the special cell-adhesion properties required at this boundary. Loss of cell-adhesion properties at this boundary could explain the occasional detachment of the hindgut from the ectoderm and the broken vegetal plates observed in WMISH of MASO treated embryos mentioned earlier. A morphogenetic role for *hox* genes mediated through control of expression of cell adhesion was suggested early on (Garcia-Bellido 1975; Davidson 1991). Recent searches for downstream targets of *hox* genes have indeed revealed genes encoding extracellular matrix and cell-adhesion proteins (reviewed by Pearson *et al*. 2005). Now that the genome sequence of the sea urchin is complete, it is feasible to test systematically for regulatory links between *Sphox11/13b* and candidate cell-adhesion genes that the depletion phenotypes suggest.

Control of cell adhesion functions at the endoderm–ectoderm boundary of the hindgut seems to be an ancestral function of posterior group *hox* genes in deuterostomes. Thus vertebrate posterior group *hox* genes display hindgut cell adhesion functions (Warot *et al*. 1997) implicated in the formation of sphincters (Zakany & Duboule 1999) as well as hindgut cell identity specification functions (Kondo *et al*. 1996). The phenotypes of gene disruptions reported in other species to some extent mimic those reported here for *SpHox*11/13b.

Downstream Sphox11/13b *target functions and later roles in gut subdivision*

The MASO-WMISH experiments also indicate a hindgut specification role for *Sphox11/13b*, in which this gene helps to define the identity of the hindgut by repressing several midgut specific regulatory genes. This is demonstrated by expansion of expression to hindgut of genes normally transcribed only in midgut, in late embryos and larvae in which the level of SpHox11/13b protein has been reduced (Fig. 5D1–F2). The disorganization of gene expression patterns along the gut occurs in parallel with diminished morphological regionalization of the gut (Figs 3F,5D–J). The negative regulation of *endo16*, *gatae*, and *apobec* transcript abundance by *Sphox11/13b*, also seen by WMISH (Fig. 5), is already effective in 24 h embryos (Gene Network 2006c; note that *blimp1/krox* and *foxa* are similarly upregulated in MASO-treated embryos). Expression of *endo16*, *gatae* and *apobec* throughout the hindgut in embryos depleted of *Sphox11/13b* gene product indicates that the hindgut is expressing the regulatory state of the normal midgut (*endo16* expression is at this stage under control of the midgut specific regulator Brn1/2/4; Yuh *et al*. 2005). Yet the normal expression of *Sphox11/13b* persists in the terminal portion of the gut of

MASO treated embryos (Fig. 5J1) indicating that the regulatory network responsible for the specific identity of the veg1-derived posterior hindgut–anus region remains functional.

Repression of midgut functions throughout the hindgut by *Sphox11/13b* is probably not a direct function in the late gastrula embryo, since *Sphox11/13b* is not expressed throughout the hindgut, but only at the blastoporal terminus. If the protein persists longer than the mRNA observed by WMISH, then the hindgut effect could be autonomous. Another possibility is that the blastoporal region is the source of a signal transcriptionally downstream of *Sphox11/13b*, which is relayed upwards to all hindgut cells, reception of which results in expression of repressors of midgut functions. A third and perhaps less baroque possibility is that the gene(s) encoding the repressor(s) are activated by the SpHox11/13b factor in veg1 cells before or during their gastrular invagination, and though *Sphox11/13b* is turned off following invagination in these cells, the activity of the repressor(s) is maintained, either transcriptionally or postranscriptionally. The midgut–foregut boundary, which is distant from the locus of *Sphox11/13b* expression, is also affected in postgastrular embryos treated with *Sphox11/13b* MASO (Fig. 3F,K). This effect too could be a regulatory specification function imposed during the pregastrular phase of *Sphox11/13b* expression, but in the veg2 cells, which ultimately form the anterior gut.

Acknowledgements

We thank Niñon Le for technical assistance and Angela Cone for thoughtful comments on the manuscript. This work was supported by the following grants: NASA/Ames NAG-1368; NSF IBN 9604454; NIH HD-37105; DOE-DE-FG02–03ER63584.

References

Angerer, L., Dolecki, G. J., Gagnon, M. L. *et al.* 1989. Progressively restricted expression of a homeo box gene within the aboral ectoderm of developing sea urchin embryos. *Genes Dev* **3**, 370–383.

Arenas-Mena, C., Cameron, A. R. & Davidson, E. H. 2000. Spatial expression of Hox cluster genes in the ontogeny of a sea urchin. *Development* **127**, 4631–4643.

Arenas-Mena, C., Martinez, P., Cameron, R. A. & Davidson, E. H. 1998. Expression of the Hox gene complex in the indirect development of a sea urchin. *Proc. Natl Acad. Sci. USA* **95**, 13 062–13 067.

Beck, F., Tata, F. & Chawengsaksophak, K. 2000. Homeobox genes and gut development. *Bioessays* **22**, 431–441.

Cameron, R., Fraser, S., Britten, R. & Davidson, E. 1991. Macromere cell fates during sea urchin development. *Development* **113**, 1085–1091.

Cameron, A. R., Lee, R., Nesbitt, R. *et al.* 2005. Unusual gene order and organization of the sea urchin hox cluster. *J. Exp. Zoolog. B Mol. Dev. Evol.* **2006**, 45–58.

Chisholm, A. 1991. Control of cell fate in the tail region of *C. elegans* by the gene egl-5. *Development* **111**, 921–932.

Cox, K., DeLeon, D., Angerer, L. & Angerer, R. 1984. Detection of mRNAs in sea urchin embryos by in situ hybridization using asymmetric RNA probes. *Dev Biol.* **101**, 485–502.

Davidson, E. H. 1991. Spatial mechanisms of gene-regulation in metazoan embryos. *Development* **113**, 1–&.

Davidson, E. H., Rast, J. P., Oliveri, P. *et al.* 2002. A provisional regulatory gene network for specification of endomesoderm in the sea urchin embryo. *Dev. Biol.* **246**, 162–190.

Dobias, S. L., Zhao, A. Z. J., Tan, H. Y., Bell, J. R. & Maxson, R. 1996. SpHbox7, a new Abd-B class homeobox gene from the sea urchin Strongylocentrotus purpuratus: Insights into the evolution of Hox gene expression and function. *Dev. Dyn.* **207**, 450–460.

Ferreira, H. B., Zhang, Y. H., Zhao, C. N. & Emmons, S. W. 1999. Patterning of *Caenorhabditis elegans* posterior structures by the Abdominal-B homolog, eg1–5. *Dev. Biol.* **207**, 215–228.

Garcia-Bellido, A. 1975. Genetic control of wing disc development in Drosophila. In *Cell Patterning, Ciba Found. Symp.* Vol. 29 (Ed. S. Brenner), pp. 161–182. Associated Scientific Publishers, New York.

Gene Network 2006a. Gene Network web page [Homepage on the internet]. Available from http://sugp.caltech.edu/endomes/.

Gene Network 2006b. Sea Urchin Genome Project resources [Homepage on the internet]. Available from http://sugp.caltech.edu/resources/methods/q-pcr.psp/.

Gene Network 2006c. QPCR data relevant to endomesoderm network [Homepage on the internet]. Available from http://sugp.caltech.edu/endomes/qpcr.html/.

Grapin-Botton, A. & Melton, D. 2000. Endoderm development: from patterning to organogenesis. *Trends Genet.* **16**, 124–130.

Irvine, S. Q. & Martindale, M. Q. 2000. Expression patterns of anterior Hox genes in the polychaete Chaetopterus: Correlation with morphological boundaries. *Dev Biol.* **217**, 333–351.

Ishii, M., Mitsunaga-Nakatsubo, K., Kitajima, T., Kusunoki, S., Shimada, H. & Akasaka, K. 1999. Hbox1 and Hbox7 are involved in pattern formation in sea urchin embryos. *Dev Growth Differ.* **41**, 241–252.

Kondo, T., Dolle, P., Zakany, J. & Duboule, D. 1996. Function of posterior HoxD genes in the morphogenesis of the anal sphincter. *Development* **122**, 2651–2659.

LeMotte, P., Kuroiwa, A., Fessler, L. & Gehring, W. 1989. The homeotic gene Sex Combs Reduced of Drosophila: gene structure and embryonic expression. *EMBO J.* **8**, 219–227.

Livi, C. B. & Davidson, E. H. 2006. Expression and function of blimp1/krox, an alternatively transcribed regulatory gene of the sea urchin endomesoderm network. *Dev. Biol.* **293**, 513–525.

McGinnis, W. & Krumlauf, R. 1992. Homeobox genes and axial patterning. *Cell* **68**, 283–302.

McMahon, A., Flytzanis, C., Hough-Evans, B., Katula, K., Britten, R. & Davidson, E. 1985. Introduction of cloned DNA into sea urchin egg cytoplasm: replication and persistence during embryogenesis. *Dev. Biol.* **108**, 420–430.

Minokawa, T., Rast, J. P., Arenas-Mena, C., Franco, C. B. & Davidson, E. H. 2004. Expression patterns of four different

regulatory genes that function during sea urchin development. *Gene Expr. Patterns* **4**, 449–456.

Minokawa, T., Wikramanayake, A. H. & Davidson, E. H. 2005. cis-Regulatory inputs of the wnt8 gene in the sea urchin endomesoderm network. *Dev Biol.* **288**, 545–558.

Nemer, M., Rondinelli, E., Infante, D. & Infante, A. A. 1991. Polyubiquitin RNA characteristics and conditional induction in sea urchin embryos. *Dev Biol.* **145**, 255–265.

Oliveri, P., Carrick, D. M. & Davidson, E. H. 2002. A regulatory gene network that directs micromere specification in the sea urchin embryo. *Dev Biol.* **246**, 209–228.

Pearson, J., Lemons, D. & McGinnis, W. 2005. Modulating Hox gene functions during animal body patterning. *Nat. Rev. Genet.* **6**, 893–904.

Peterson, K. J., Irvine, S. Q., Cameron, R. A. & Davidson, E. H. 2000. Quantitative assessment of Hox complex expression in the indirect development of the polychaete annelid Chaetopterus sp. *Proc. Natl Acad. Sci. USA* **97**, 4487–4492.

Ransick, A. & Davidson, E. H. 1998. Late specification of veg1 lineages to endodermal fate in the sea urchin embryo. *Dev. Biol.* **195**, 38–48.

Ransick, A., Ernst, S., Britten, R. J. & D. 1993. Whole mount in situ hybridization shows Endo 16 to be a marker for the vegetal plate territory in sea urchin embryos. *Mech Dev* **42**, 117–124.

Ransick, A., Rast, J. P., Minokawa, T., Calestani, C. & Davidson, E. H. 2002. New early zygotic regulators expressed in endomesoderm of sea urchin embryos discovered by differential array hybridization. *Dev Biol.* **246**, 132–147.

Rast, J. P., Amore, G., Calestani, C., Livi, C. B., Ransick, A. & Davidson, E. H. 2000. Recovery of developmentally defined gene sets from high-density cDNA macroarrays. *Dev Biol.* **228**, 270–286.

Tremml, G. & Bienz, M. 1989. Homeotic gene expression in the visceral mesoderm of Drosophila embryos. *EMBO J.* **8**, 2677–2685.

Van der Hoeven, F., Sordino, P., Fraudeau, N., Izpisua-Belmonte, J.-C. & Duboule, D. 1996. Teleost HoxD and HoxA genes: comparison with tetrapods and functional evolution of the HOXD complex. *Mech. Dev.* **54**, 9–21.

Warot, X., Fromental-Ramain, C., Fraulob, V., Chambon, P. & Dolle, P. 1997. Gene dosage-dependent effects of the Hoxa-13 and Hoxd-13 mutations on morphogenesis of the terminal parts of the digestive and urogenital tracts. *Development* **124**, 4781–4791.

Wei, Z., Angerer, L. M., Gagnon, M. L. & Angerer, R. C. 1995. Characterization of the SpHE promoter that is spatially regulated along the animal–vegetal axis of the sea urchin embryo. *Dev. Biol.* **171**, 195–211.

Wilson, K. A., Andrews, M. E. & Raff, R. A. 2005. Dissociation of expression patterns of homeodomain transcription factors in the evolution of developmental mode in the sea urchins Heliocidaris tuberculata and H. *Erythrogramma*. *Evol. Dev.* **7**, 401–415.

Yokouchi, Y., Sakiyama, J. & Kuroiwa, A. 1995. Coordinated Expression of Abd-B subfamily genes of the Hoxa cluster in the developing digestive tract of chick embryo. *Dev. Biol.* **169**, 76–89.

Yuh, C.-H., Dorman, E. R. & Davidson, E. H. 2005. Brn1/2/4, the predicted midgut regulator of the endo16 gene of the sea urchin embryo. *Dev. Biol.* **281**, 286–298.

Zakany, J. & Duboule, D. 1999. Hox genes and the making of sphincters. *Nature* **401**, 761–762.