Hindgut specification and cell-adhesion functions of \textit{Sphox11/13b} in the endoderm of the sea urchin embryo

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\textit{Sphox11/13b} is one of the two \textit{hox} genes of \textit{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 \textit{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 \textit{Sphox11/13b} negatively regulates several downstream endomesoderm genes. For some of these genes, \textit{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 \textit{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, \textit{Hox}, sea urchin.

Introduction

\textit{Sphox11/13b} is one of several posterior-group \textit{hox} genes of the sequenced \textit{Strongylocentrotus purpuratus} \textit{hox} gene cluster (Cameron et al. 2005). Both qualitative (Dobias et al. 1996) and quantitative (Arenas-Mena et al. 1998) measurements detect \textit{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). \textit{Hox} genes have been implicated in the specification of vertebrate (Yokouchi et al. 1995; Van der Hoeven et al. 1996; Beck et al. 2000), \textit{Drosophila} (LeMotte et al. 1989; Tremml & Bienz 1989), and \textit{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 \textit{hox} genes of the paralogue group with which we are here concerned.

Embryonic \textit{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 \textit{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 \textit{hox} gene deployment later, during the postembryonic formation of their adult body (Arenas-Mena et al. 2000; Irvine & Martindale 2000). Two individual \textit{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, \textit{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).
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 (Sphox 11/13b MASO) with the sequence 5′-AGCCT-GTTCCATGCGATCTGCA-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 morpholino antisense oligonucleotide (MASO) had the arbitrary sequence 5′-CCTTACTCATTCAAATT-TATA-3′. Embryos were microinjected as previously described (McMahon et al. 1985) with 10 or 4.5 µL 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′-CATCGGTGACAG- GATCCTTAT-3′ and 5′-GCCAGATGCGATCGGCA- TGGACAGCTAGCAAGGCGAGAAGTTTTACT-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 SacI fragment (−1225, +20) cloned into the SacI 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 SpHox 11/13b expression was reduced, a new 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 vegetative blastomere descendants, which contribute to both endoderm and mesoderm (Fig. 1A–D; for a vegetative 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 vegetative lineage restriction continues in 20 h embryos (Fig. 1E–F) and by 24 h,
after the primary mesenchyme cells (PMC) have ingressed into the blastocoel, 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...
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.
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, Endo16, Apobec and SpHox11/13b 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.
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
pattern of expression is similar to that described by the \textit{wnt8} and \textit{blimp1/krox} genes (Livi & Davidson 2006; Minokawa \textit{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 \textit{et al.} 2000).

The final expression pattern of \textit{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 \textit{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 \textit{et al.} 2004). It will be interesting to explore regulatory relationships between \textit{SpfoxB} and \textit{Sphox11/13b}. A previous report shows that oral \textit{veg1} cell descendants contribute more and extend deeper into the archenteron than do aboral \textit{veg1} cells (Ransick & Davidson 1998). Nonetheless, there is some invagination of \textit{veg1} even on the aboral side, and the pattern of expression of \textit{Sphox11/13b} by 48 h, when the invagination is long complete, clearly lies within the domain of \textit{veg1} descendants as this includes the ectoderm surrounding the anus on the aboral side.

The pattern of \textit{Sphox11/13b} expression reported here does not agree with that reported previously (Dobias \textit{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 \textit{Sphox11/13b} probe used here is the same as that used by Dobias \textit{et al.} (1996), the hybridization conditions in this study (Arenas-Mena \textit{et al.} 2000) are 8.5$^\circ$C more stringent, and the wash conditions about 12.5$^\circ$C more stringent, according to the hybridization melting point formula that derives from corrections for histological conditions (Cox \textit{et al.} 1984). A very long hybridization incubation of 1 week was also introduced to compensate

\begin{figure}
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\caption{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 \textit{endo16}, \textit{gataE} and \textit{apobec} are shown, as indicated. Injections were of approximately 4.2 pl. of 500 $\mu$M control MASO (A2,B2,C2,D2,E2,F2,J2) or \textit{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.

\(\text{Experimental}\) & \(\text{Control}\) & \(\text{Experimental}\) & \(\text{Control}\)
\hline
A1 & A2 & Endo16 & D1
B1 & B2 & GataE & E1
C1 & C2 & Apobec & F1
J1 & SpHox11/13b & J2
\end{tabular}
\end{figure}
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 SpHox11/13b.

**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 Brn11/2/4; 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 posttranscriptionally. 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 pregastrula phase of Sphox11/13b expression, but in the veg2 cells, which ultimately form the anterior gut.

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