Two ParaHox genes, SpLox and SpCdx, interact to partition the posterior endoderm in the formation of a functional gut

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We report the characterization of the ortholog of the Xenopus XLHox8 ParaHox gene from the sea urchin Strongylocentrotus purpuratus, SpLox. It is expressed during embryogenesis, first appearing at late gastrulation in the posterior-most region of the endodermal tube, becoming progressively restricted to the constriction between the mid- and hindgut. The physiological effects of the absence of the activity of this gene have been analyzed through knockdown experiments using gene-specific morpholino antisense oligonucleotides. We show that blocking the translation of the SpLox mRNA reduces the capacity of the digestive tract to process food, as well as eliminating the morphological constriction normally present between the mid- and hindgut. Genetic interactions of the SpLox gene are revealed by the analysis of the expression of a set of genes involved in endoderm specification. Two such interactions have been analyzed in more detail: one involving the midgut marker gene Endo16, and another involving the other endodermally expressed ParaHox gene, SpCdx. We find that SpLox is able to bind Endo16 cis-regulatory DNA, suggesting direct repression of Endo16 expression in presumptive hindgut territories. More significantly, we provide the first evidence of interaction between ParaHox genes in establishing hindgut identity, and present a model of gene regulation involving a negative-feedback loop.

KEY WORDS: Strongylocentrotus purpuratus, QPCR, Endomesoderm, Sea urchin larval development, Hindgut specification, GRN, Triple in situ hybridization

INTRODUCTION

The sea urchin embryo is a powerful developmental system that has been used to study embryological processes at many levels, from mechanisms of fertilization (e.g. Briggs and Wessel, 2006; Parrington et al., 2007) to gastrulation (e.g. Ettensohn, 1984; Hardin, 1996) and cell specification (e.g. pigment cells) (Calestani et al., 2003). In the recent era of molecular biology, the purple sea urchin Strongylocentrotus purpuratus is the first non-chordate deuterostome to have its genome sequenced (Sea Urchin Sequencing Consortium, 2006), and is the taxon used in building of one of the first and most fully resolved gene regulatory networks that describes the genetic basis behind the separation of the germ layers from fertilization through gastrulation (Davidson et al., 2002).

One of these germ layers is the endoderm, which eventually gives rise to a three-part gut consisting of an esophagus, stomach and hindgut or intestine. Evidence of patterning is obvious in the hindgut or intestine. Evidence of patterning is obvious in the posterior endoderm in the formation of a functional gut. Here, we describe our detailed analysis of the functional tripartite gut. Throughout development with the exception of the mouse homolog Pdx1, which plays an important role in pancreas formation (for a review, see Al-Qubaili and Montenarh, 2008).

SpLox, the purple urchin xLox homolog, is expressed in the mid- and hindguts of gastrula stage embryos, and is restricted to the posterior sphincter separating these two gut regions in the pluteus larva (Arnone et al., 2006). This expression pattern is conserved also in sea stars (R. Annunziata, unpublished) (Hwang et al., 2003). Given the relative simplicity of the sea urchin digestive system and the wealth of data available concerning the gene regulatory network (GRN) specifying the endodermal precursors, the purple urchin represents an optimal developmental system with which to investigate the role of ParaHox genes in endodermal partitioning to create a functional tripartite gut. Here, we describe our detailed analysis of the expression and function of the sea urchin SpLox gene and its genetic interaction with a second endodermally expressed ParaHox gene, SpCdx.

MATERIALS AND METHODS

Animals

Adult Strongylocentrotus purpuratus were obtained from the Kerchoff Marine Laboratory, Corona del Mar USA, and housed in circulating sea water aquaria at the Stazione Zoologica Anton Dohrn, Naples, Italy. Spawning was induced by intraocoecomic injection of 0.5 M KCl and embryos were kept in a temperature-controlled incubator (15°C), cultured in filtered seawater diluted 9:1 with de-ionized water.

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Whole-mount in situ hybridization
Embryos and larvae were collected as needed and fixed for 2 hours to overnight in 4% paraformaldehyde in filtered seawater, washed in Tris-buffered saline (TBS) and stored in 70% ethanol until use. For detailed expression analysis of the onset of SpLox and SpCdx expression, a series of embryos was fixed every 2 hours between 48 and 72 hours post fertilization (hpf). Labeled probes were transcribed from linearized DNA using digoxigenin-11-UTP or fluorescein-12-UTP (Roche), or labeled with DNP (Mirus Cat # MIR 3800) following kit instructions. In situ RNA probe sequences are as previously published (SpCdx, SpLox) (Arnone et al., 2006) Endo16 (Ransick et al., 1993). For single gene expression, the protocol outlined by Minokawa et al. (Minokawa et al., 2004) was followed. For multi-gene fluorescent in situ (up to three genes contemporaneously), fixed embryos were washed in Tris-buffered saline containing 0.1% Tween-20 (TBST), pre-hybridized for 1 hour at 65°C in fresh hybridization buffer (50% formamide, 5× SSC, 0.1% Tween-20, 50 μg/ml heparin, and 50 μg/ml yeast tRNA) and incubated overnight at 65°C with antisense labeled probes. Embryos were washed in a descending gradient of SSC (2×, 0.2×, 0.1×) at 65°C followed by TBST washes at room temperature. Embryos were then blocked in 3% (v/v) goat serum in fresh 0.5% Perkin Elmer Blocking Reagent (PEBR) in TBST, and incubated overnight at 4°C with peroxidase conjugated antibodies (Roche: 1 μl in 100 μl of 0.5% PEBR in TBST). Antibodies were removed with washes in TBST, and signal was developed with fluorophore-conjugated tyramide (1 μl/50 μl reagent dilutant: Perkin Elmer). Residual enzyme activity was inhibited via a 20-minute incubation in 0.1% hydrogen peroxide, followed by TBST washes prior to addition and development of the second and third antibody. Embryos were imaged with a Zeiss Axio Imager.M1. Triple in situs were imaged with a Zeiss 510Meta confocal microscope.

Morpholino antisense oligonucleotide (MASO) injections
We followed the method indicated by Oliveri et al. (Oliveri et al., 2003). All morpholino antisense oligonucleotides (MADOS) were used in a concentration of 150 μM. To assess the effect of morpholino injections, fertilized eggs were injected with the injection solution (1% rhodamine dextran in 0.1 M KCl) without morpholino oligonucleotides, or with one of the following control morpholinos: GeneTools standard control oligonucleotide, SpLox mutated morpholino sequence (mismatch control, see below), or a GCM specific morpholino that blocks pigment formation (Ransick and Davidson, 2006). We see no phenotypic effect upon injection of control morpholinos, and endoderm development is unaffected in the presence of GCM-MASO (see Fig. S1F in the supplementary material). Two different SpLox specific MASOs were used in combination with a mismatch control morpholino:

mLox1, AGTACcCgGATcTTCcCTCcGAT (mismatch control);
Lox1, AGTACgCGGgATGTTcCCTCCTCCTC;
Lox2, AGGACATGGATATTCAGACGCCAT.

The mismatch control morpholino produced no morphological phenotype (see Fig. S1H in the supplementary material), and was unable to block in vitro synthesis of SpLox protein (performed as described below under EMSA), which was completely inhibited by the Lox1 MASO (see Fig. S1J in supplementary material). No significant difference in phenotype was observed between Lox1 and Lox2 MASOs, and all data presented herein derives from the MASO directed against the 5′ start codon (Lox1). After injection, fertilized eggs were washed with fresh filtered seawater (FSW) and incubated overnight. The following day, rhodamine-positive embryos were transferred to new plates and incubated at 15°C in fresh FSW.

Assay on digestive function
One-week-old larvae were cultured for 6 days in the presence of the micro alga Isochrysis galbana (2106 cell/ml). At different time points, larvae were selected and observed under a fluorescent microscope using a FITC filter set. The presence of chlorophyll in this phytoplankton species allows direct observation of the micro alga under fluorescent light, and allows for differential detection between degraded and intact algal cells. To assess levels of alkaline phosphatase in the guts of injected versus control larvae, alkaline phosphatase staining was performed as detailed by Livingston and Wilt (Livingston and Wilt, 1989).

Phalloidin and Endo1 immunostaining of embryos
Larvae were freshly fixed in 4% PFA in FSW for 2 hours at room temperature, washed multiple times in phosphate-buffered saline with 0.1% Tween-20 (PBST), and incubated overnight at 4°C with anti-Endo1 primary antibody (Wessel and McClay, 1985), diluted at working concentration (1:5) in 5% goat serum in PBST. Following primary antibody incubation, larvae were washed three times with PBST, and incubated for 1 hour at room temperature with the secondary antibody Alexa Fluor 555 goat anti-mouse IgG (Molecular Probes) diluted 1/100 in 5% goat serum in PBST. After removal of secondary antibodies, larvae were incubated in 1 μl phalloidin-488 in 100 μl PBST for 1 hour (Roche). Larvae were washed in PBST and mounted for imaging with a confocal microscope (Zeiss 510Meta).

Quantitative PCR (qPCR)
Total RNA was collected from a minimum of 400 larvae per experimental trial using a RNeasy mini-kit (Qiagen) following the manufacturer’s instructions. cDNA was synthesized with Sprint PowerScript (Clontech). qPCR was performed according to Rast et al. (Rast et al., 2002), using an ABI prism 7000 sequence detection system and SYBR green chemistry (PE Biosystems). For all qPCR experiments, the data from each cDNA sample were normalized against ubiquitin mRNA levels, which are known to remain relatively constant during embryogenesis (Nemer et al., 1991). Details of primer sets can be provided on request.

Electrophoretic mobility shift assay (EMSA)
Putative binding sites were identified by alignment of the Endo16 regulatory sequence (Yuh et al., 1994) with the Pdx1-binding sequence A1 from the insulin promoter (Liberson et al., 2004). Of the putative sites found, we chose to analyze the most proximal one, which contains two core homeobox DNA-binding sites (site n. 28) (Yuh et al., 1994). Mobility shift assays were performed as previously described (Martin et al., 2001). In vitro translated SpLox protein was synthesized using the TNT coupled in vitro transcription/translation system (Promega) from the cDNA clone p1611ES under the control of a T7 promoter. DNA-protein complexes were resolved by gel electrophoresis and imaged on a Typhoon Trio (Amersham).

RESULTS
Endodermal expression of SpLox
The spatial pattern of SpLox expression at 72 and 96 hours has been previously reported (Arnone et al., 2006). We have further characterized the detailed spatial expression pattern from pre-gastrula through 72 hours by whole-mount in situ hybridization, as illustrated in Fig. 1.

At the mesenchyme blastula stage (Fig. 1A), SpLox transcripts are not detectable, consistent with the limited number of transcripts per embryo detected by quantitative PCR (Arnone et al., 2006). The first developmental stage at which the signal is clearly visible is the late mid-gastrula, when the archenteron is more than three-quarters invaginated. At this stage, expression is first seen within single cells at the edge of the blastopore (Fig. 1B). The onset of this expression pattern is consistently asymmetrical; however, within 2 hours, all cells surrounding the inner edge of the blastopore exhibit high levels of expression (Fig. 1C). The original asymmetric pattern of expression appears to be retained, with an expanded signal localized in the aboral side as the archenteron elongates (Fig. 1D,E). When gastrulation is completed, the domain of SpLox-expressing cells encompasses the entire posterior region of the developing gut (Fig. 1F). As differentiation of the tripartite gut continues, expression within the hindgut is reduced and expression becomes restricted to the constrictions between the mid- and hindgut (Fig. 1G,H). This endodermal pattern is stable, and persists at least into the pluteus stage, although at lower levels (Fig. 1I).
and posterior sphincter separating the midgut from the foregut and hindgut, respectively, whereas SpLox-MASO injected embryos have an anterior cardiac sphincter (gray arrows in Fig. 2D,E) but show only a mild posterior constriction (white arrows). We further investigated the lack of this posterior sphincter using FITC-labeled phalloidin to visualize actin fibers, and analyzed the larvae with a confocal microscope to ensure accurate interpretation of the staining patterns. Control animals have a well-delineated border between the gut compartments, including a muscular sphincter (Fig. 2G-J). SpLox-MASO-treated embryos lack this sphincter, possessing solely a mild posterior constriction (Fig. 2K-N). In addition, the entire mid and hindgut region exhibits staining of short processes within the lumen (arrowheads in Fig. 2N) that are not seen in control larvae (Fig. 2J). Surprisingly, we find that morpholino-injected embryos also exhibit a reduction in the organization of the muscle fibers surrounding the foregut. In order to investigate whether these effects are species specific, we also tested the morphological effects of this morpholino in another distantly related sea urchin species, *Paracentrotus lividus*. We find that these morpholino-injected embryos also lack the posterior sphincter and exhibit a disorganized foregut musculature (data not shown), suggesting a conserved gene function within the echinoids.

### The digestive functions of SpLox-MASO injected embryos are inhibited

In addition to the morphological effect, the absence of SpLox function alters the digestive properties of the embryonic gut. We analyzed food ingestion in mutant and control larvae by regularly feeding animals with a culture of single-celled alga, *Isochrysis galbana*, starting at 72 hours of development. The passage of food along the gut cavity is easily detected owing to the transparency of the embryonic wall and to the fluorescent signal of the microalga with which the larvae are fed. Whereas in control larvae the algal cells are degraded and ingested by the intestinal cells, in the SpLox-MASO injected larvae the algal cells remain intact and are excreted without further degradation (Fig. 3A,B,D,E). Further evidence for loss of digestive capacity within the gut of MASO-injected larvae is the reduction of the activity of a gut-specific enzyme, alkaline phosphatase (Livingston and Wilt, 1989), as demonstrated by a reduction in phosphatase activity within the gut (Fig. 3C,F).

### Levels of endoderm marker genes are altered in the absence of SpLox function

To address the genetic mechanism by which *SpLox* exerts its effect, we evaluated changes in the levels of transcription in morpholino-injected larvae 72 and 96 hours post-fertilization using quantitative PCR. Transcript levels were evaluated for some endoderm differentiation genes (Fig. 4). Only those genes whose transcript levels differ by a factor of ±1.8 are considered to be significant (see bold text in Fig. 4). Genes from the endomesoderm GRN, which are expressed much earlier in development than *SpLox*, show no significant changes in transcript levels, with the exception of brachyury (*brac*), the expression of which increases by a moderate twofold, in three out of five replicate experiments (Fig. 4). By contrast, significant changes in transcript levels were identified for both *SpLox* itself, as well as for the other endodermally expressed ParaHox gene *SpCdx*. *SpLox* transcript levels increase by 2-7 times
over the levels in control embryos at both time points, whereas SpCdx transcript levels decrease 2-5 times at both time points when compared with control embryos. Two genes identified as being part of the murine Pdx1 pathway, the islet factor 1 gene (Isl1) (Kojima et al., 2002) and myelin transcription factor 1 gene (Myt1) (Gu et al., 2004), are the only other transcription factors examined that demonstrate altered expression in response to silencing the SpLox gene.

Terminal differentiation genes displayed much greater responses to the silencing of SpLox. Both endodermal markers examined demonstrate significant changes in transcriptional expression. Consistent with the decrease in enzyme activity in injected larvae (see Fig. 3C,F), alkaline phosphatase (ap) transcripts decrease, whereas Endo16 levels increased. By contrast, a mesodermal terminal differentiation marker, capk (Rast et al., 2002), showed little change in transcript prevalence. Also consistent with the morphological phenotype, wherein there is a disruption of the organization of the musculature associated with the gut (see Fig. 2), a second mesodermal differentiation marker, actin M (actM) (Cox et al., 1986), consistently decreased in expression (two- to fivefold decrease), as did a sea urchin muscle-specific transcription factor (sum1, a MyoD-like gene) (Beach et al., 1999).

Fig. 2. Morphological phenotype resulting from disruption of SpLox in Strongylocentrotus purpuratus. (A,B) Stage I pluteus larvae developed from rhodamine dextran-KCl-injected eggs show a well-defined tripartite gut with anterior (gray arrows) and posterior (white arrows) sphincters separating the midgut (m) from foregut (f) and hindgut (h), respectively. (A) Lateral and (B) oral views. (D,E) SpLox-MASO injected larvae show disorganized structure of the endodermal epithelia and are missing the posterior sphincter between the midgut and hindgut (white arrows), exhibiting only a mild posterior constriction of the gut. (D) Lateral and (E) oral views. Gray arrows indicate anterior sphincter. (C,F) Posterior regions of the developing gut from prism stage larvae, wherein the distinct reduction of the posterior constriction (white arrows) is first apparent in SpLox-MASO-injected embryos (F) compared with control injected embryos (C). (G-I,K-M) Three-dimensional maximal projection of confocal image stacks from phalloidin stained (green) control (G-I) and SpLox-MASO (K-M) injected larvae. (G,K) Lateral view of a stage I pluteus larva showing staining of the midgut and hindgut by an Endo1 antibody (red). Note the muscle fibers that define the foregut anteriorly (*) in the control larva (G) and are less conspicuous in the experimental larva (K). (H,L) Oral views, phalloidin staining (green) with nuclear counterstain (purple). (I,M) Reconstructions of phalloidin staining in endoderm from the same larvae imaged in H and L. Phalloidin fibers collect at the level of the midgut-hindgut sphincter (white arrows) and are present in the control larvae G-I, but are absent from SpLox-MASO injected larvae K-M. (J,N) A single optical section from the same larvae shown in H,I and L,M taken at the level of the midgut-hindgut junction; bright-field above, fluorescence below. The smooth surface of stomach lumen in control (J) when compared with the SpLox-MASO larva (N) where many phalloidin-positive projections are evident (white arrowheads).

Fig. 3. Silencing of SpLox results in disruption of feeding abilities in Strongylocentrotus purpuratus larvae. (A,C) Control MASO-injected (D-F) and SpLox-MASO injected larvae. (A,D) Bright-field images with overlaid fluorescence excited through a 488 wavelength filter showing degraded chlorophyll (green) and intact algal cells (red). (B,E) Higher magnification of the gut of the same larvae shown in A,D imaged under UV excitation to illustrate degraded (blue) and intact (red) chlorophyll. Control injected larvae (A,B) show incorporation of chlorophyll within the midgut, whereas SpLox-MASO injected larvae (D,E) show only undigested algal pellets within the digestive tract. (C,F) Alkaline phosphatase staining. The control injected larva imaged in C shows high levels of alkaline phosphatase activity when compared with the SpLox-MASO-injected larva imaged in F.
SpLox represses Endo16 expression in the hindgut

Endo16 is an extremely well-studied endodermal marker gene, encoding a calcium-binding protein (Soltsyk-Española et al., 1994) that is initially expressed throughout the vegetal plate and invaginating endoderm. However, at the end of gastrulation, its expression is restricted to the midgut (Ransick et al., 1993). The early domain of Splox expression almost completely overlaps with the posterior-most expression domain of Endo16 (Fig. 5A,B). Our qPCR data indicate that the transcript levels of Endo16 are highly elevated in response to silencing Splox. Thus, we investigated whether this increase corresponds to a change in the Endo16 expression domain or simply an upregulation of the gene within the confines of its normal expression area. Expression levels of Endo16 are consistently elevated in injected embryos; Endo16 transcripts are detectable by in situ hybridization in less than half the time it takes for the in situ signal to develop in control embryos processed in parallel (data not shown). Furthermore, we find that the domain of Endo16 expression is expanded to encompass the entire mid- and hindgut territories (Fig. 5E,F), whereas expression of this gene in control larvae is confined to the midgut region (Fig. 5C,D). This expansion of the expression domain remains restricted to the endoderm; at no time is ectopic expression of Endo16 observed. This expression pattern is also retained in 1-week-old stage I pluteus larvae (data not shown).

The Endo16 gene possesses one of the most thoroughly studied cis-regulatory regions (Yuh et al., 1998). Owing to the strong effect of the Splox morpholino injections on Endo16 expression, we investigated its cis-regulatory sequence for possible Splox-binding sites. We analyzed the proximal-most putative binding sequence (Fig. 6A) by competitive binding assay, using in vitro synthesized Splox and oligonucleotides containing this sequence. We find that Splox specifically binds this oligonucleotide (Fig. 6B, lane 1), as assessed by competition with increasing concentrations of unlabeled oligonucleotide (Fig. 6B, lanes 2,3). By contrast, addition of unlabeled oligonucleotides containing a mutation within the ATTA core binding sequence (Fig. 6A) does not interfere with the binding of Splox to the site. Additionally, we show that in vitro synthesized Splox also binds with high efficiency to oligonucleotides containing the Pdx1-binding site A1 from the insulin promoter (Liberzon et al., 2004) (Fig. 6B, lanes 6,7).

Posterior endodermal patterning results from interaction between ParaHox genes

One of the most intriguing results from the qPCR analysis is the decrease in expression of a second ParaHox gene expressed in the gut: the caudal homolog SpCdx. In order to address the interactions between these two genes, we re-evaluated the expression pattern of the SpCdx gene at the same level of detail as that of Splox. SpCdx expression appears slightly later than Splox expression (Fig. 7A,B), but similarly appears as cells enter the blastopore near the end of gastrulation (Fig. 7C,D). SpCdx expression is retained throughout the developing hindgut region (Fig. 7E,F), and persists at high levels in the hindguts of 72-hour pluteus larvae (Fig. 7G-I). To investigate the extent of overlapping expression between these two genes, we performed double-fluorescent in situ hybridization. We find that at the onset of Cdx expression, the expression domains of these two genes largely overlap (Fig. 8A-C). As development progresses, Splox progressively clears from the hindgut region (Fig. 8D-F) so that the fully developed larva retains SpCdx expression throughout the hindgut, whereas Splox is restricted to the posterior sphincter in a non-overlapping domain of expression (Fig. 8G-I).
By contrast, SpLox-MASO injected larvae show a distinct absence of SpCdx expression within the hindgut (Fig. 8L), in accordance with the decrease in the transcripts of this gene indicated by qPCR. The absence of SpCdx expression suggests that SpLox is involved in SpCdx activation in the zone of overlapping expression.

We also find that SpLox expression is no longer restricted to the posterior sphincter, as in control larvae, but rather maintained within the entire posterior-most region of the developing gut in the SpLox-MASO-injected animals (Fig. 8J,K). This result is consistent with the elevated SpLox transcript levels revealed by qPCR (see Fig. 4), and together illustrate that translation of SpLox is required for inhibiting further transcription of this gene within the region of the hindgut.

**DISCUSSION**

**SpLox is necessary for establishing the boundary between the stomach and intestine**

During organogenesis, multiple cell types are generated and organized into elaborate structures. This process depends on the exquisite coordinated activity of genes and gene networks, to ensure that the final pattern and functionality of the organ is properly achieved. Using gene knockdown, in situ hybridization and quantitative PCR analyses, we have provided data supporting the hypothesis that ParaHox genes are essential in the regional endodermal patterning leading to a functional gut. In the absence of SpLox protein translation, mutant embryos demonstrate an anterior-posterior identity shift, wherein the midgut marker Endo16 is expanded posteriorly, SpLox expression is maintained posteriorly throughout the presumptive hindgut region, and the hindgut marker SpCdx is extremely reduced or absent. A similar phenotype in a distantly related urchin species, *Paracentrotus lividus* (data not shown), supports the notion that the results presented here hold not only for the sea urchin *S. purpuratus*, but also for echinoid echinoderms as a group. The SpLox phenotype described here is similar to that observed in mice lacking the murine homolog Pdx1. In these mice, the rostral duodenum is malformed with a misshapen pyloric sphincter, leading to deficiencies in gastric emptying, and the anterior duodenum resembling a posterior extension of the stomach (Jonsson et al., 1994; Offield et al., 1996). Thus, consistent with our data from echinoid echinoderms, the boundary between expression domains of murine gastric and duodenal markers appear shifted posteriorly. Pdx1 also acts as a potent repressor of posterior gut enzymes, including those normally mediated by Cdx homologs (Heller et al., 1998; Wang et al., 2004). Taken altogether, these data clearly indicate the importance of SpLox in establishing the mid-hindgut boundary within the deuterostome lineage.
Hox genes are known for their coordinated control of patterning of body regions, for example rhombomere patterning under the control of Hoxb genes (Maconochie et al., 1997). Little is known, however, about whether ParaHox genes also function in a coordinated manner to establish boundaries between adjacent territories. Our results reveal that the silencing of an anteriorly expressed ParaHox gene ($SpLox$) leads to the loss of a posterior identity (hindgut), accompanied by the downregulation of a second ParaHox gene ($SpCdx$) that is normally expressed in the missing territory. These data suggest coordination between these two ParaHox genes at a gene regulatory level. Here, we present a proposal for a gene regulatory network involved in hindgut specification (Fig. 9), derived from the data described in this article.

We suggest a model of regulatory interactions with the following components:

1. $SpLox$ protein represses the expression of the midgut marker gene $Endo16$ in the hindgut territory (see Figs 4 and 5). We suggest that this interaction may be direct, owing to the amplitude of $Endo16$ response as assayed by qPCR (see Fig. 4), and to the fact that $SpLox$ protein binds efficiently to a putative binding site within the $Endo16$ promoter (see Fig. 5). We tested only the proximal-most putative binding site, leaving open the possibility that $SpLox$ may bind also other more distal sites.

2. $SpLox$ protein activates the expression of $SpCdx$ (see Fig. 4 and Fig. 8L). This activation occurs necessarily in concert with other hindgut specific regulatory genes, because $Cdx$ is not activated throughout the entire $SpLox$ domain. $Hox 11-13b$ is a good candidate for one such gene, as it appears to be expressed in the same domain as $Cdx$. However, its expression is initiated much earlier in development. Knockdown of $Hox 11-13b$ results in embryos that show a posterior expansion of $Endo16$ into the hindgut (Arenas-Mena et al., 2006), similar to the phenotype of $SpLox$ morpholino

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**Fig. 7. Expression pattern of the $SpCdx$ gene from blastula through pluteus larvae in *Strongylocentrotus purpuratus*.**

Embryos correspond to the same batch as those shown in Fig. 1 for $SpLox$ expression. (A-C) Mesenchyme blastulae through mid-late gastrulae show no $SpCdx$ expression. (D) Late gastrula. First detectable expression appears in cells surrounding the blastopore (*). (E) Late gastrula. Expression is detected in all cells surrounding the blastopore and the posterior-most endodermal tube. (F) End of gastrulation: prism stage larva shown in oral view. The posterior-most endodermal tube exhibits $SpCdx$ expression. (G,H) Early pluteus larvae collected 4 hours after that shown in F show continued high levels of expression throughout the hindgut region. (G) Oral view; (H) lateral view. (I) 72-hour pluteus larva in lateral view showing retention of the high expression levels throughout the hindgut.

**Fig. 8. ParaHox genes $SpLox$ and $SpCdx$ interact to regulate hindgut formation, as illustrated with double in situ hybridization.** (A,D,G,J) Embryos photographed using DIC imaging, with the fluorescent gene expression patterns overlaid, overlapping expression appears white. (B,E,H,K) The green channel only ($SpLox$ expression). (C,F,I,L) The red channel ($SpCdx$ expression). (A-I) Double in situ of expression domains in normal embryos. (J-L) Double in situ illustrating expression domains in $SpLox$-MASO-injected embryos. (A-C) Late gastrula stage embryo showing largely overlapping expression domains of both genes, although the $SpLox$ domain extends further anteriorly (green), and the $SpCdx$ domain extends further posteriorly (purple). (D-F) Prism stage larva showing a greater region of non-overlapping expression domains. (G-I) Control injected pluteus larva showing mutually exclusive, non-overlapping expression domain for both genes. (J-L) Pluteus larva developed from an egg from the same batch as the larva shown in G-I, injected with the $SpLox$-MASO. $SpLox$ expression is maintained throughout the hindgut (K) when compared with control (G,H), whereas $SpCdx$ is absent in the posterior hindgut in injected larvae (L) compared with control (I).
embryos (see Fig. 5). Unfortunately SpLox was not analyzed in the Hox11-13b knockdown experiments, and, thus, we cannot speculate whether or not this similar phenotype is due to abolition of the SpLox protein.

(3) We propose that transcribed SpCdx protein acts as a repressor of SpLox in its posterior domain of expression. Silencing of SpLox leads to increase in SpLox transcripts (see Fig. 4) and retention of SpLox expression in the hindgut territory (see Fig. 5K), suggesting that SpLox protein initially activates a repressor in the hindgut territory that restricts SpLox expression to the level of the posterior sphincter (see Fig. 1G-I). SpCdx is expressed in this territory and is activated by SpLox protein (see Figs 4 and 7, and Fig. 8L). Furthermore, we have found that silencing SpCdx leads to a similar expansion of SpLox expression into the hindgut (preliminary data; Fig. 9C), supporting the supposition that SpCdx is involved in repressing SpLox in the hindgut.

These data add considerable strength to the hypothesis that a negative-feedback loop exists between these two ParaHox genes, and that this gene regulatory circuit is necessary for the establishment of hindgut territories. An ancestral function of caudal homologs in determining hindgut territories was first proposed based upon work in Drosophila (Wu and Lengyel, 1998). As our data illustrate, the sea urchin provides a precise example of how a developmental field (gut) is subdivided using homeobox genes and how these subfields are subsequently refined by a negative-feedback loop at the gene regulatory level.

Conclusions and perspectives

Our results are consistent with the view that gut morphogenesis in sea urchins has different temporal components: an early specification process (governed by the Wnt pathway) (Wikramanayake et al., 2004) and a late differentiation process in which genes of the ParaHox complex (SpLox and SpCdx) are involved in regionally partitioning the endoderm. The phenotypes generated through the use of gene specific MASOs have shown us that SpLox is a key regulator in the formation of the midgut-hindgut boundary, repressing regions of the midgut differentiation program and activating the hindgut gene battery through a second ParaHox gene, SpCdx.

SpLox endodermal expression conforms to the rule that xLox homologs are endodermal markers, expressed in specific regions within the gut and endodermal derivatives (Wright et al., 1989; Brooke et al., 1998; Stoffers et al., 1999; Fröbius and Seaver, 2006) and supports the speculation that the SpLox orthologs are part of a group of genes dedicated to generate diversity in the gut (Brooke et al., 1998; Fröbius and Seaver, 2006). Although no functional data are available from a protostome, the nested expression pattern of the endodermal ParaHox genes in annelids (Fröbius and Seaver, 2006; Kukalova et al., 2008) suggests a similar system of regionalization. This would imply that ParaHox gut regionalization is shared among all bilaterians, thus representing a pan-bilaterian character. Moreover, our data indicate that at least portions of the gene regulatory network that control pancreas development and maintenance in vertebrates were available for cooption from the gene regulatory network used to partition the gut of the common ancestor of the deuterostomes. It seems clear now that the ParaHox group of genes regionalizes the endoderm along the anteroposterior axis. Thus, given the apparent use of the same system in both deuterostomes and protostomes, we assume that this arose early in evolution, most probably coincidently with the origin of bilaterians.

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ParaHox genes interact to partition endoderm

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**References**


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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/4/S1/DC1

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**RESEARCH ARTICLE**

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**DEVELOPMENT**

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