REVIEW

Pattern and Process During Sea Urchin Gut Morphogenesis: The Regulatory Landscape

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Summary: The development of the endoderm is a multistage process. From the initial specification of the endodermal domain in the embryo to the final regionalization of the gut, there are multiple stages that require the involvement of complex gene regulatory networks. In one concrete case, the sea urchin embryo, some of these stages and their genetic control are (relatively) well understood. Several studies have underscored the relevance of individual transcription factor activities in the process, but very few have focused the attention on gene interactions within specific gene regulatory networks (GRNs). Sea urchins offer an ideal system to study the different factors involved in the morphogenesis of the gut. Here we review the knowledge gained over the last 10 years on the process and its regulation, from the early specification of endodermal lineages to the late events linked to the patterning of functional domains in the gut. A lesson of remarkable importance has been learnt from comparison of the mechanisms involved in gut formation in different bilaterian animals; some of these genetic mechanisms are particularly well conserved. Patterning the gut seems to involve common molecular players and shared interactions, whether we look at mammals or echinoderms. This astounding degree of conservation reveals some key aspects of deep homology that are most probably shared by all bilaterian guts. genesis 52:251–268.   © 2013 Wiley Periodicals, Inc.

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INTRODUCTION

During metazoan evolution, one of the first crucial innovations to arise was the development of an internalized gastrointestinal system. Cells specialized to perform extracellular digestion, thus releasing multicellular organisms from body size constraints, allowing the further evolution of highly specialized internal structures. Thus it can be argued that one of the first developmental patterning systems to evolve was the molecular network that orchestrates the formation of the digestive system, involving networks of genes that were then available to be co-opted for the development and patterning of new body parts (Nielsen, 2008; Roberts, 2000; Wolpert, 1994). The development of the gut has been the subject of extensive studies in both protostome and deuterostome models (McGhee, 2007;

Sheaffer and Kaestner; Stainier, 2002, 2005; Zorn and Wells, 2009).

Gastrulation is the first complex morphogenetic process that reorganizes the embryo from a spherical layer of cells into a multi-layered organism. This process results in the separation of the three germ layers in triploblast Bilaterians: the outer ectoderm, the middle mesoderm, and the inner-most endoderm. The primitive gut, or archenteron, is formed from a subpopulation of endodermal plus—in some cases—mesodermal cells, which eventually differentiate into the digestive apparatus. In most animals, gastrulation results in the formation of a tube (the archenteron) with no apparent morphological differentiation. However, even in the absence of overt morphological differences, several distinct or partially overlapping expression territories of signaling molecules and transcription factors can be detected along the axis of the archenteron (Jacobs *et al*., 2012; Lengyel and Iwaki, 2002; McGhee, 2007; Sherwood *et al*., 2011; van den Brink, 2007; Zorn and Wells, 2009). Interaction between these types of regulatory molecules leads to the establishment of molecularly distinct domains that are defined by precise boundaries of gene expression, indicating the precursors of various digestive organs. Unraveling the mechanisms underlying the generation of these molecularly distinct domains during development is critical for our understanding of gut formation and the acquisition of its diverse physiological functions in various lineages throughout evolutionary time.

One class of genes that have been shown to play an integral role in the patterning of many animal body parts, from all three germ layers, are the homeodomain containing HOX genes (Beck *et al*., 2000). Mutations of Hox genes result in only minor defects in the vertebrate gut (Aubin *et al*., 1997; Boulet and Capecchi, 1996; Manley and Capecchi, 1995; Warot *et al*., 1997; Zacchetti *et al*., 2007), suggesting that anterior–posterior (A–P) patterning of the archenteron is controlled by genes other than members of the Hox-class.

A central role in the gut development of several animals has been proposed for two ParaHox genes, Xlox and Cdx (Brooke *et al*., 1998; Holland, 2013). Xlox is involved in the differentiation of mid-gut compartments, including accessory organs such as the vertebrate pancreas, whereas Cdx is required for proper formation of the posterior part of the gut, or hind-gut, in all animals that have been examined. A large number of signaling molecules such as Wnt, Fgf, Bmp, Shh, and RA, have also been shown to regulate different aspects of vertebrate gut patterning and differentiation (Feng *et al*., 2012; Jacobs *et al*., 2012; Spence *et al*., 2011). Here we review the status of our knowledge on the molecular mechanisms that control gut development in the sea urchin, and highlight the various levels of conservation that can be identified when we analyze homologous processes in other deuterostomes.

THE SEA URCHIN LARVAL GUT: FROM MORPHOLOGY TO ULTRASTRUCTURE

Most sea urchins are indirect developers and pass through a bilaterally symmetrical swimming (and feeding) pelagic larval stage (the pluteus), before metamorphosis leads to the formation of the adult form with its characteristic pentameric body plan. The larvae of many echinoids can, in fact, be considered as dispersal vessels within which adult structures develop: the entire repertoire of larval behavior seems to be dedicated to feeding (Strathmann, 1971, 1974, 1975).

The larval digestive system is tripartite, composed of a muscular esophagus, a large spherical stomach, and a short tubular intestine. The muscle fibers associated with the digestive system in feeding larval stages can be clearly visualized using Myosin heavy chain immunolocalization (Fig. 1). The microhistology (Burke, 1981) and feeding physiology (Pearse, 1991; Strathmann, 1971) of sea urchin digestive systems have been well described for a number of species, and the major features are outlined below.

The esophagus is divided in two regions: the proximal zone (nearest to the mouth) is narrow and densely ciliated, and surrounded by circumferential muscle fibers (Fig. 1a–c) whereas the distal half of the esophagus is bulbous, only sparsely ciliated and surrounded by muscle fibers that run parallel to its central axis. The contraction of muscle fibers starting from cells at the posterior end of the coelom and inserting on the larval skeleton, pulls posteriorly the lower lip of the larval mouth and the esophagus (Burke and Alvarez, 1988).

Separating the esophagus and the stomach is the cardiac sphincter, a constriction made from a simple striated myoephithelium whose myofibrils encircle the perimeter of the sphincter giving the structure an "hourglass-like" shape. The larval stomach is made of a simple columnar epithelium that includes at least two ultrastructurally distinct cell types (Burke, 1981): type I cells represent the major component of the epithelium and are distributed throughout the organ; type II cells form a small fraction and are restricted to the anterior portion of the stomach. Type I stomach cells form the luminal surface and contain numerous irregular microvilli. This cell type is characterized by the presence of a single cilium, and includes several different types of cytoplasmic vesicles. Spindle-shaped type II cells in contrast do not have cilia and typically contain residual algal cells in different stages of digestion. Based on their morphology, type I stomach cells are likely involved in secretory functions while type II stomach cells are apparently devoted to phagocytosis and digestion of the algal cells. Pinocytic activity has been observed in both
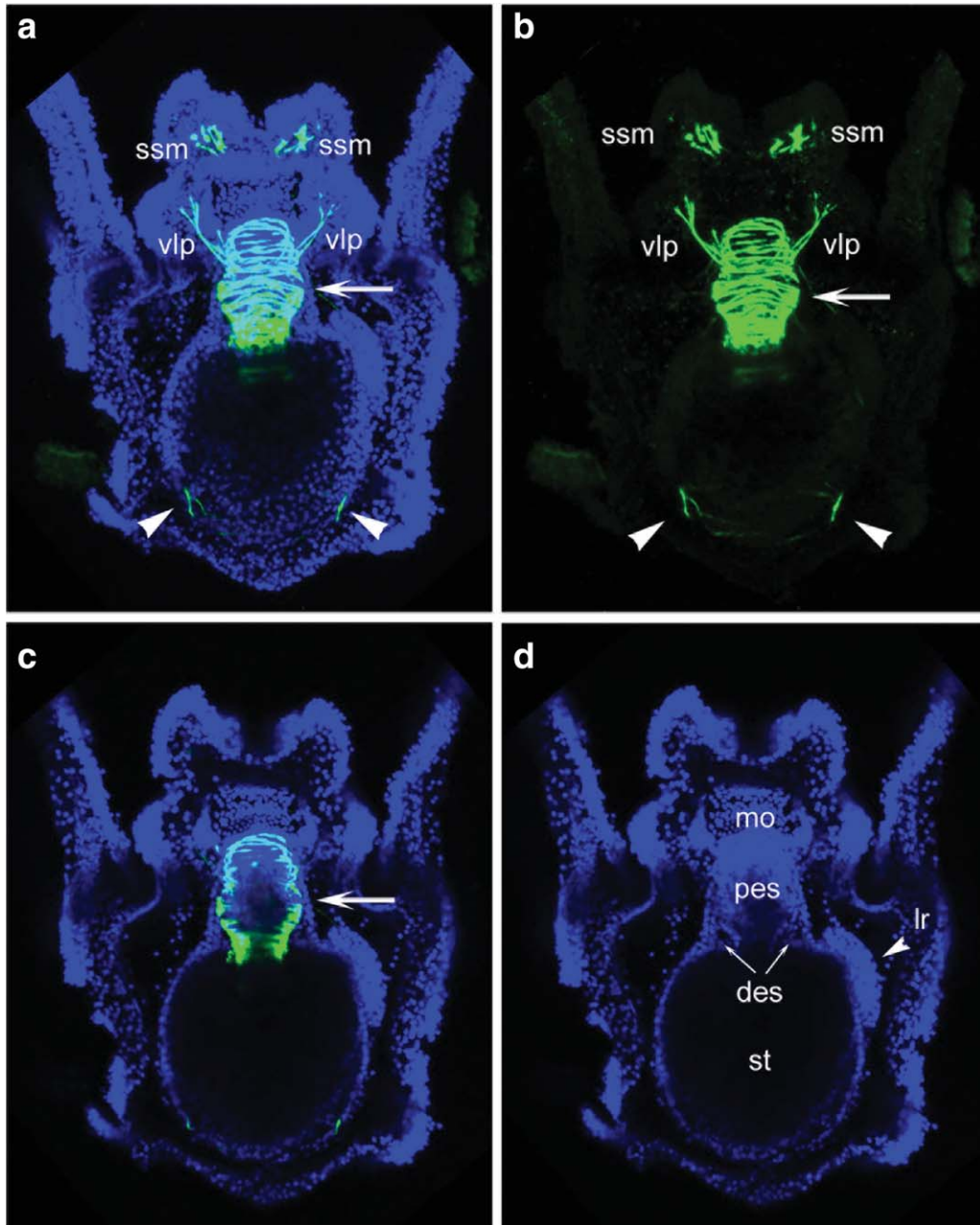
FIG. 1. The digestive system of the 8-arm sea urchin pluteus larva. (**a–d**) Immunohistochemistry of a whole mount *Strongylocentrotus purpuratus* 3-week-old larva, viewed from the anal side, obtained with an antibody against an *S. purpuratus* class II Myosin heavy chain (MHC) (Andrikou *et al.*, 2013) coupled with nuclear staining (DAPI, blue). Full (a, b) or partial (c, d) projections of confocal z-series are shown for single (b, d) and superimposed (a, c) channels. The anti-MHC antibody (green) stains the circumesophageal muscle fibers (arrows in a–c), the ventrolateral processes (vlp in a, b), the recently identified star-shaped muscles (Dyachuk and Odintsova, 2013) (ssm; in the oral hood at the base of the protruding preoral arms (a, b), and in addition, to our knowledge so far un-described, a pair of muscle fibers attached to the lower stomach (arrowheads in a, b). (d) The distal esophagus (des; arrow), the mouth (mo), the proximal esophagus (pes), the left rudiment (lr; arrowhead) and the stomach (st) are indicated.

stomach and intestine cells in the sea urchin *Lytechinus pictus* (Huvard, 1986).

The pyloric sphincter separates the stomach and the intestine. This is composed of a ring of type I stomach cells that basally contain a single band of thick and thin circumferentially oriented non-striated myofilaments. Whereas the stomach is composed of columnar epithelial cells, the intestine is made up of a squamous epithelium. Similar to the type I stomach cells, the intestinal cells contain numerous vesicles and a single cilium. The

intestine terminates with the anal sphincter that is, as the pyloric sphincter, provided with intracellular basal circumferential filaments (see Fig. 2d,h).

The pluteus feeding behavior has been observed in many echinoderms, included *Dendraster* and *Strogylocentrotus* (Pearse, 1991; Strathmann, 1971). Food particles (algal cells) are passed through the mouth and transported down the esophagus via ciliary beating and peristaltic contractions of the proximal esophagus where they are aggregated into a "bolus." As the esophagus is contracted, the cardiac sphincter is opened and the bolus is transferred to the stomach. The sphincter is then closed and the esophagus returns to its resting position. The bolus is agitated and dispersed in the stomach, through ciliary beating and myoid contraction of the stomach walls (Pearse, 1991). Undigested material collects at the pyloric end of the stomach, is passed to the intestine and defecated. Several lines of evidence show the presence in different echinoids of a neural system associated with the gut, suggesting that these coordinated muscular movements and the ciliary beating are neuronally controlled. In the sea urchin larva, the nervous system is associated with the ciliary band surrounding the larval mouth. The lower lips of the larval mouth are innervated by oral ganglia; additionally, a diffused network of neural processes overlay the esophagus (Burke *et al*., 2006). Moreover, neurons have been also described in the sea urchin larval intestine and in the anus (Nakajima *et al*., 2004). Strikingly, some of the neurons in the pharynx of *Strongylocentrotus purpuratus* derive from the endoderm (Wei *et al*., 2011).

ENDODERM SPECIFICATION IN THE SEA URCHIN EMBRYO

The archenteron is derived from the endoderm, thus endoderm specification is the first molecular event occurring during the formation of the digestive system. The gene regulatory network (GRN) that controls endomesodermal specification has been analyzed in great detail for the sea urchin (Croce *et al.*, 2011; Croce and McClay, 2010; Davidson *et al*., 2002; Peter and Davidson, 2010). These studies describe an important regulatory transition from common endomesodermal precursors to the permanent separation of endodermal from mesodermal fates in descendent cells.

The endomesoderm in sea urchin embryo derives from the vegetal plate, more precisely from the group of skeletogenic micromeres and macromeres that are segregated at the fourth cleavage. The micromeres will develop into the skeletogenic mesenchyme (SM) cells that will give rise to the skeleton of the pluteus larva. The descendants of the macromeres (the veg1 and veg2 lineages) in turn will give rise to the mesoderm and endoderm, as well as a few ectodermal cells. In particu-

lar, lineage tracing experiments (Logan and McClay, 1997; Ransick and Davidson, 1998) have shown that veg2 descendants give rise to non-skeletogenic mesodermal cells plus endoderm (forming the foregut and contributing to the formation of the oral midgut), whereas veg1 descendants form oral and aboral ectoderm plus endoderm (giving rise to the hindgut and contributing to the formation of the aboral midgut).

Using a system-wide perturbation approach, Davidson and his team have provided a causal mechanistic explanation for the segregation of the veg2 and veg1 lineages. This comprehensive analysis of gene activities during endomesoderm formation has been translated into a complex and continuously updated GRN (the latest updated diagram is available in Davidson's laboratory webpage http://sugp.caltech.edu/endomes). In the following paragraphs we summarize the main regulatory mechanisms driving endomesoderm specification in the sea urchin embryo.

Before the separation of endoderm and mesoderm, the veg2 lineage consists of two concentric rings of cells, the inner ring destined to become mesoderm and the outer ring destined to become oral endoderm. The endoderm regulatory state is induced in all veg2 cells through the activity of the β-catenin/TCF signaling pathway acting in synergy with a maternal/early zygotic form of *Otx*, thus activating the endodermal regulatory genes *Blimp1b, Eve* and *Hox11/13b* (Arenas-Mena *et al*., 2006; Smith *et al*., 2008; Yuh *et al*., 2002). This cascade of genes will in turn regulate other transcription factors such as *Brachyury, FoxA*, and *GataE*. A Wnt6 maternal signal has been shown to be necessary to trigger the activation of the endodermal specific genes in the veg2 cells (Croce *et al.*, 2011). The separation of endodermal and mesodermal derivatives within the veg2 lineage depends on the Delta/Notch signaling whose duration has been demonstrated to be crucial for mesoderm specification (Croce and McClay, 2010). The result of the Delta/Notch signaling effect is that the cells that are adjacent to the micromeres will express mesodermal genes, whereas the more distal veg2-descendant cells will express only endodermal genes (Peter and Davidson, 2009; Ransick and Davidson, 2006). A few hours later in development, endodermal genes will be repressed in the mesodermal ring of cells.

Much less is known about the specification of the veg1 progeny. The gene *Eve* is the only known regulatory gene expressed in veg1 at the early blastula stage, and a model involving its recruitment has been proposed for veg1 specification (Peter and Davidson, 2010). In this model *Eve* is responsible for the spatial definition of the veg1 regulatory state through the activation of *Hox11/13b*. An unknown signal predicted to be under the control of *Hox11/13b* diffuses from veg2 to the veg1 cells, inducing the expression of Hox11/
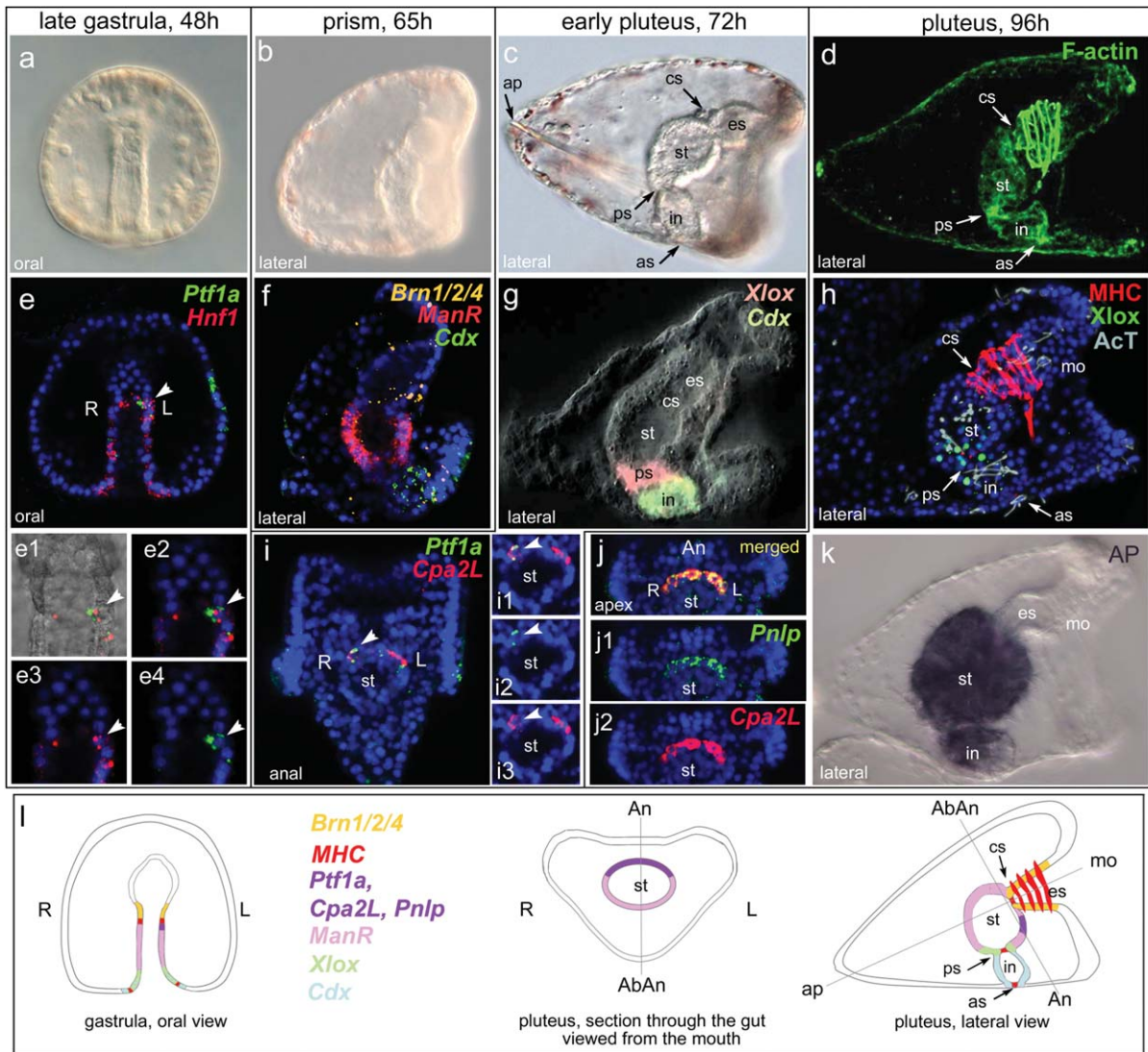
FIG. 2. Sea urchin digestive system formation. (**a–c**) Morphogenesis of the sea urchin developing gut. Images of living embryos viewed with differential interference contrast optics. (a) At the late gastrula stage, the gut appears as a straight tube with two openings, the mouth and the anus. (b) At the prism stage, a mild constriction between foregut and stomach is already visible. (c) At the pluteus stage, the gut is subdivided in three compartments (esophagus, stomach, and intestine) separated by the cardiac (cs) and pyloric sphincters (ps). (**d**) F-actin immunostaining with phalloidin (green) highlights the muscle fibers associated with the digestive tube and shows the digestive tube internally covered by cilia from the mouth to the anus. (**e–g**) Transcription factors and terminal differentiation genes are differentially expressed along the AP axis from the late gastrula stage. Full projections of confocal z-series of (e) *Ptf1a* and *Hnf1* double fluorescent in situ hybridization (FISH); (f) *Brn1/2/4, ManR* and *Cdx* triple FISH whole mount embryos; (g) epifluorescence image superimposed to brightfield image of a *Xlox* and *Cdx* double FISH of an early pluteus embryo demonstrate precise boundaries of gene expression. (e1–e4) A higher magnification of the coexpression domain between *Ptf1a* (green) and *Hnf1* (red). (**h–k**) The gut of the sea urchin pluteus larva is divided into three compartments and display differentiated cell types. (h) Three color immunohistochemistry highlights the circumesophageal muscle fibers (MHC: red), sphincteric muscles (acetylated tubulin: grey) and cells with specific gene signature (Xlox: green); compare with f-actin immunostaining in (d). Full projections of confocal z-series of (i) *Ptf1a* (green) and *Cpa2L* (red) and (j) *Cpa2L* (red) and *Pnlp* (green) double FISH in plutei larvae show a unique cell type localized in the upper, oral part of the stomach. Magnification of the coexpression domains indicated by arrowheads are shown in separate channels of single slices (i1–i3) or full projections (j1–j2). (k) Alkaline phosphatase staining shows active digestive functions more prominently in the stomach in the pluteus larva. (**l**) Schematic of a late gastrula (left drawing) and a pluteus larva (central and right drawing). Endodermal domains are depicted with different colors according to gene expression. Overlapping domains of expression are represented with colored lines. Orientation of embryos and larvae are indicated at the left bottom of each panel. An, anal; AbAn, abanal; ap, apex; as, anal sphincter; cs, cardiac sphincter; es, esophagus; in, intestine; L, left; mo, mouth; ps, pyloric sphincter; R, right; st, stomach.

13b, and as a consequence, of the endoderm regulatory state. Hox11/13b acts as an auto-repressor in the veg2 cells, clearing the cells of both its own transcripts and those of the *Bra* gene (Peter and Davidson, 2010, 2011)

The regulatory mechanisms described above control the specification of the endoderm, which will shortly thereafter initiates the different regulatory programs underlying the formation of the fore-, mid- and hind-gut compartments. A detailed description of the relative domains of expression of regulatory genes and terminal differentiation genes involved in patterning the sea urchin gut is provided in the following section.

SEA URCHIN GUT FORMATION: FROM A STRAIGHT TUBE TO THREE FUNCTIONAL COMPARTMENTS

At the end of gastrulation (around 48hpf in *Strongylocentrotus purpuratus*) the sea urchin gut is a straight tube formed by a single cell epithelial layer (Fig. 2a,l). A superficial look at the morphology of the late gastrula gut would suggest no obvious distinction of cell types and uniformity of the structure. However, markers of terminal differentiation are already active in mutually exclusive domains prior to the differentiation of a tripartite larval gut. Examples of gene expression in specific territories are: stomach specific markers already expressed in future midgut cells like *Macrophage mannose receptor* (*ManrC1A*), *Chaperonin precursor* (*ChP*), and *Endo16* (Annunziata and Arnone, unpublished data; Cole *et al.*, 2009); the muscle specific terminal differentiation gene *Myosin Heavy Chain* (*MHC*,), already expressed in the cells that will form the cardiac and anal sphincters (Andrikou *et al.*, 2013) (see Fig. 2l) or the *Carboxypeptidase 2L* (*Cpa2L*, the homologue of the human carboxypeptidase A2, a pancreatic digestive enzyme), which is unilaterally expressed in a few cells posterior to where the cardiac sphincter will form, in a domain that is restricted to the left side of the gut (Fig. 2l).

Around 60-65hpf the future larval skeleton is almost complete and the embryo acquires a prismatic shape (Fig. 2b). At this time the gut undergoes a series of morphological changes that lead to the formation of three clearly separate compartments: a first subdivision between foregut and midgut domains is visible (Fig. 2b) in the form of the cardiac sphincter (Andrikou *et al.*, 2013). The last endodermal muscular structure, the pyloric sphincter, is formed in the pluteus larvae (around 72hpf). The gut is now definitively tripartite, subdivided into the esophagus, stomach and intestine (Fig. 2c,d,h), and is fully functional, complete with esophageal muscles (of mesodermal origin), sphincteric muscles (of endodermal origin) and cilia (Fig. 2d,h,k).

An astonishing degree of differential cellular specialization in the gut of the larvae can be revealed now through the study of cell-type specific markers. These data are providing us with a high-resolution description of the molecular fingerprint for the diverse gut cell types, information that contributes to a better understanding of the molecular mechanisms controlling the gut patterning process. For example, differentiation of the midgut compartment, already described by ultrastructural methods (Burke, 1981), is now confirmed by the identification of specific cell types that express secretory digestive enzymes or hormone-like molecules, a fact that reminds us the well-known physiology of the vertebrate guts in which digestive and endocrine functions are shared by the same organ. In order to illustrate the degree of conservation in patterning mechanisms and physiological functions of the echinoderm and vertebrate guts, we show in Table 1, a comprehensive list of genes whose activities are shared (broadly) between both taxa (see, also, further details in a later section). From upstream regulatory factors such as the HNFs or the NKx to the downstream effectors Cpa2L, Pancreatic lipase (Pnlp, the homolog of the human pancreatic lipase-related protein 3) or the Insulin-related protein Igf1, the degree of conservation in their expression domains is striking. Understanding the extent of this degree of conservation in gene activities and its physiological significance should prove a matter of exciting research in the next few years.

DYNAMIC OF GENE INTERACTIONS ALONG THE AP AXIS DURING SEA URCHIN GUT DIFFERENTIATION

Although the early events of endoderm specification have been thoroughly studied at the gene expression and regulatory levels (reviewed above), the later events of gut formation and patterning have been the object of more recent investigation (Andrikou *et al.*, 2013; Arnone *et al.*, 2006; Cole *et al.*, 2009; Annunziata and Arnone, unpublished data). These studies have revealed that sea urchin gut development is a highly dynamic process involving the integration of both autonomous and conditional mechanisms of cell specification.

In Figure 3 we provide a schematic view of endoderm gene expression from the postgastrular embryo to the pluteus larval stage, including active transcription factors and terminal differentiation genes. It is clear that most of the regulatory and terminal differentiation genes that show a precise pattern of expression in the pluteus gut are already active at the late gastrula stage in what will be their definitive expression domains. For example, foregut cells express *FoxA, Brn1/2/4, FoxP,* and *Isl1*, an expression that is retained in the esophageal cells of the prism and larval stages. Moreover, midgut cells express *Blimp1a, GataE, Hnf1, Endo-16, ManrC1A*, and *ChP* and maintain this expression as differentiated stomach cells in the pluteus larva.

Table 1
Sea Urchin Homologs of Vertebrate Organ Specific Genes Along the Digestive Tract

| Gene name (synonymous)	Description (gene family)	Domain of expression in mouse	Sea urchin ortholog (SPU#)	Domain of expression in sea urchin	References
Pancreas and liver					
Pdx1 (ipf1, IDX1, xlox)	Pancreatic and duodenum homeobox 1 (Parahox HD)	Early pancreatic progenitors and endocrine cells	Sp-Lox (026099)	Gastrula: hindgut; Pluteus: pyloric sphincter	Arnone et al., 2006; Offield et al., 1996
Cdx2/3	Caudal type homeobox 2 (Parahox-HD)	Pancreatic endocrine cells and gut	Sp-Cdx (024715)	Gastrula and pluteus: hindgut	Arnone et al., 2006; Jin and Drucker, 1996
Brn4	Brain-specific homeobox/POU domain protein 4 (Pou-HD)	Pancreas α-cells	Sp-Brn1/2/4 (016443)	Gastrula and pluteus: foregut and CB	Cole and Arnone, 2009; Hussain et al., 2002
Hnf1β (Tcf2)	Hepatic nuclear factor 1 β (Pou-HD)	Primitive endoderm	Sp-Hnf1 (008196)	Gastrula: hindgut, midgut; Pluteus: not detectable*	Barbacci et al., 1999; Howard-Ashby et al., 2006
Hnf6	Hepatic nuclear factor 6 (Cut-HD)	Pancreas exocrine cells; pancreatic ductal cells; hepatoblasts.	Sp-Hnf6 (016449)	Gastrula: ciliary band	Jacquemin et al., 2000; Otim et al., 2004
Isl1	Islet1 (LIM-HD)	Pancreatic dorsal bud; pancreas endocrine cells	Sp-Isl (023730)	Gastrula and pluteus: CB, foregut, anus[†]	Thor et al., 1991
Pax6	Paired box 6 (Paired-HD)	Pancreas endocrine α and β-cells	Sp-Pax6 (006786)	Gastrula: tip of the archenteron; Pluteus: left CP	St-Onge et al., 1997; Yankura et al., 2010
Nkx2.2	NK2 Homeobox 2 (NK-HD)	Pancreas endocrine cells	Sp-Nkx2-2 (000756)	Gastrula: oral and aboral ectoderm	Chen et al., 2011; Saudemont et al., 2010; Sussel et al. 1998
Nkx6.1	NK6 Homeobox 1 (NK-HD)	Pancreatic progenitors and endocrine precursors; β-cells	Sp-Nkx6-1 (012699)	n.a.	Nelson et al., 2005
Arx	Aristaless-related homeobox (HD)	Pancreas endocrine cells	Sp-Alx1 (025302)	Blastula: PMC	Collombat et al., 2003; Ettensohn et al., 2003
Prox1	Prospero Homeobox 1 (HD)	Pancreas Endocrine cells; Early hepatic endoderm; liver and pancreatic buds	Sp-Prox1 (015984)	n.a.	Burke and Oliver, 2002
HB9 (Hlxb9, Mnx1)	Homeobox protein 9 (HD)	Early pancreatic epithelium; β-cells	Sp-Hb9 (002816)	Prism and pluteus: anus (*Paracentrotus lividus*)	Di Bernardo et al., 2000; Li et al.,1999
Hex	Hematopoietically expressed homeobox (hox-related HD)	Early pre-pancreatic foregut; pre-hepatic ventral endoderm	Sp-Hex (027215)	Early gastrula: PMC, gut	Howard-Ashby et al., 2006; Thomas et al., 1998
Foxa2 (HNF3β)	Forkhead Box Protein A (Fork head/Winged helix)	Early pancreatic epithelium, pancreatic endocrine cells; liver bile duct	Sp-FoxA (006676)	Gastrula and pluteus: mouth, gut	Oliveri et al., 2006; Sasaki and Hogan,1993
PTF1	Pancreatic transcriptional factor1 (bHLH)	Early pancreatic progenitors; pancreas exocrine cells	Sp-Ptf1a (002677)	Gastrula: ectoderm and midgut; Pluteus: midgut*	Krapp et al., 1996
Ngn3	Neurogenin 3 (bHLH)	Pancreas endocrine progenitor cells	Sp-Ngn (007147)	Gastrula and pluteus: apical organ and CB[†]	Desgraz and Herrera, 2009
NeuroD1	Neuronal differentiation 1 (bHLH)	Pancreas endocrine cells	Sp-NeuroD (024918)	Gastrula: oral ectoderm; Pluteus: CB, foregut, stomach[†]	Naya, 1997

Table 1. *Continued*

Gene name (synonymous)	Description (gene family)	Domain of expression in mouse	Sea urchin ortholog (SPU#)	Domain of expression in sea urchin	References
Mist1	Muscle, Intestine and Stomach BHLH (bHLH)	Pancreas exocrine cells	Sp-Mist1 (027623)	n.a.	Pin *et al.*, 2001
MafB (v-maf)	Musculoaponeurotic fibrosarcoma oncogene homolog B (Blz)	Pancreas α and β-cells	Sp-Maf (025888)	Gastrula: PMCs; Pluteus: CP	Andrikou *et al.*, 2013; Artner *et al.*, 2007
Sox4	Sex determining region Y-box 4 (HMG)	Pancreas endocrine cells and a subset of exocrine cells	Sp-SoxC (002603)	Gastrula: foregut, apical organ and ectoderm; Pluteus: CP, anal and pyloric sphincter, CB	Howard-Ashby *et al.*, 2006; Wilsonn *et al.*, 2005
Sox9	Sex determining region Y-box 9 (HMG)	Pancreatic progenitors	Sp-SoxE (016881)	Gastrula: veg2 derived mesoderm; Pluteus: left CP	Juliano *et al.*, 2006; Luo and Su, 2012; Seymour *et al.*, 2007
Hnf4α	Hepatocyte nuclear factor 4 alpha (Nuclear Receptor)	Developing hepatic diverticulum; hepatoblasts	Sp-Hnf4 (021192)	n.a.	Duncan *et al.*, 1994; Howard-Ashby *et al.*, 2006
Insulin	Peptide hormone	Pancreas β-cells	Sp-Igf1 (007203). Sp-Igf2 (030139)	Late pluteus: stomach, intestine, CP; Gastrula and pluteus: foregut[†]	Burke *et al.*, 2006; Philippe, 1991
Glucagon	Peptide hormone	Pancreas α-cells	Absent[‡]	–	Philippe, 1991
Cpa A2 precursor	Carboxypeptidase metalloCPA	Pancreas exocrine cells	Sp-Cpa2L (015178)	Gastrula and pluteus: stomach*	Pascual *et al.*, 1989
Pnlp1/2/3	Pancreatic lipase related protein (lipase)	Pancreas exocrine cells	Sp-Pnlp (012906)	Pluteus: stomach*	Yang *et al*, 2000
Stomach					
Barx1	BarH-like homeobox 1 (HD)	Stomach mesenchyme	Sp-Barx1 (003920)	n.a.	Kim *et al.*, 2005
Nkx2.5	Natural killer homeobox 2.5 (HD)	Pyloric mesenchyme	Sp-Nkx2.5 (005472)	n.a.	Smith *et al.*, 2005
Nkx3.2 (Bapx1)	Natural killer homeobox 3.2 (HD)	Stomach mesenchyme	Sp-Nkx3.2 (017837)	oral animal pole and foregut	Wei *et al.*, 2011 Verzi *et al.* 2009
Intestine					
Cdx-	Caudal type homeobox 2 (Parahox HD)	Small and large intestine	Sp-Cdx (024715)	Gastrula: hindgut; Pluteus: intestine	Arnone *et al.*, 2006; Beck *et al.*, 2010
Hoxa13	Homeobox a 13 (Hox HD)	Large intestine	Sp-Hox11/13b (002631)[§]	Gastrula: hindgut; Pluteus: posterior intestine	Barbara and Roberts, 2002; De Santa Arenas-Mena *et al.*, 2006;
Isx	Intestine-specific (HD)	Intestine	Sp-Isx (016786)	Gastrula: hindgut; Pluteus: posterior intestine[†]	Choi *et al.*, 2006

*This work.
[†]Arnone, unpublished.
[‡]Not found in the *S. purpuratus* genome (Burke *et al.*, 2006).
[§]In sea urchins, three *Hox11/13* genes have been identified (a, b, c); *Hox11/13b* is the only paralogue expressed during embryogenesis.
bHLH, basic Helix-Loop-Helix; Blz, Basic leucine zipper; CB, Ciliary Band; CP, Coelomic Pouches; HD, HomeoDomain; HMG, High Mobility Group; n.a., not available; PMC, Primary Mesenchyme Cells.
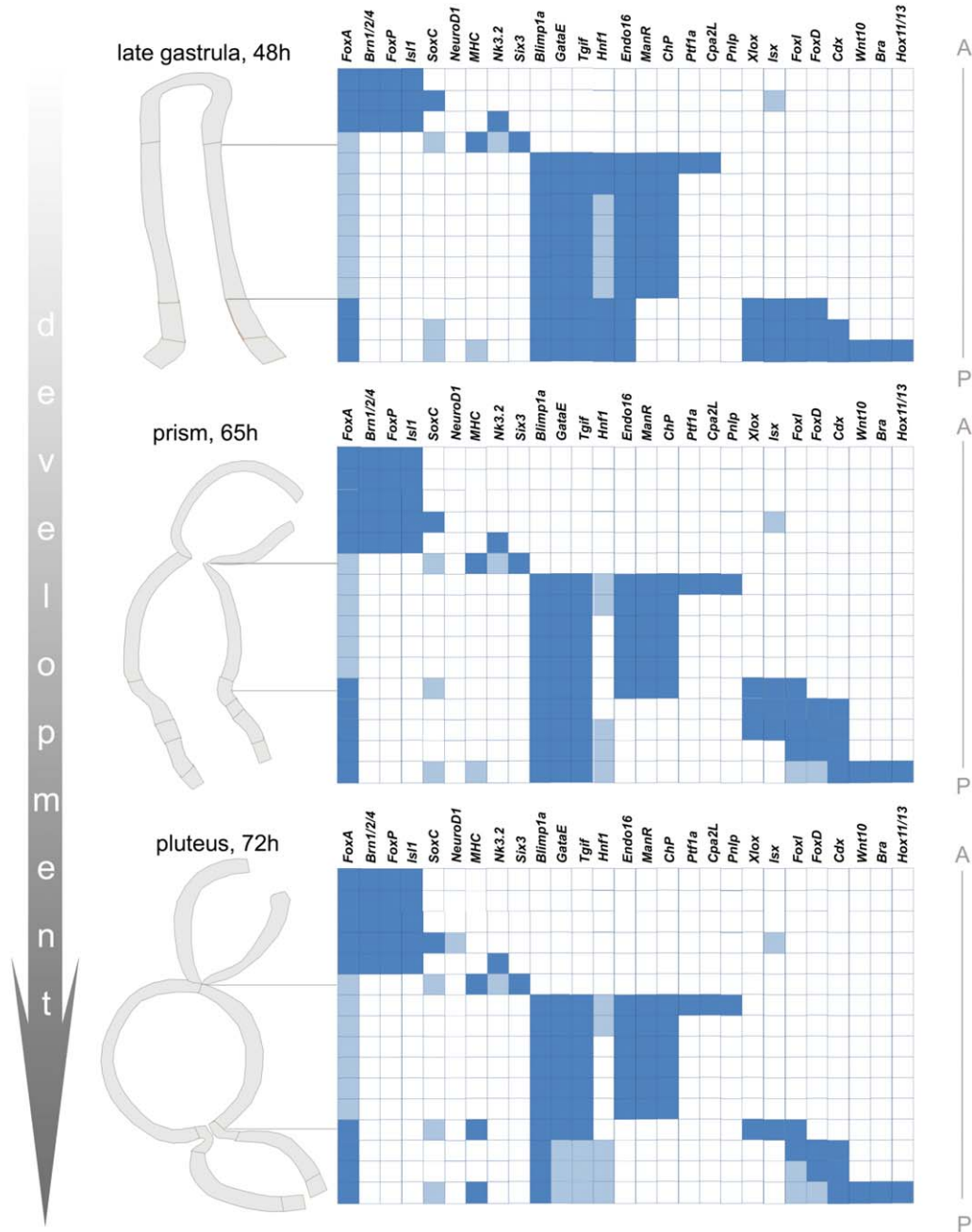
FIG. 3. Summary of known dynamics of gene expression along the AP axis during sea urchin gut differentiation. In the left panels of the figure, three stages of the sea urchin embryonic developing gut are depicted in lateral view. In the right part of the figure, three tables representing the expression of transcription factors, terminal differentiation and signaling molecule genes along the A-P axis of the developing gut show presence of transcripts (blue squares), low transcript numbers (light blue squares), and absence of transcripts (white squares). References for the expression dynamics are: *Blimp1a* (Livi and Davidson, 2006); *Bra* (Rast *et al.*, 2002); *Brn1/2/4* (Cole and Arnone, 2009); *FoxA* (Oliveri *et al.*, 2006); *Endo16* (Ransick *et al.*, 1993); *FoxP, FoxI, FoxC* (Tu *et al.*, 2006); *GataE* (Lee and Davidson, 2004); *Hnf1, Ptf1a, Cpa2L* and *Pnlp* (this study); *Hox11/13b* (Arenas-Mena *et al.*, 2006); *Isl, Isx, NeuroD1* and *NK3.2* (Wei *et al.*, 2011); *MHC* and *SoxC* (Andrikou *et al.*, 2013); *Six3* (Poustka *et al.*, 2007); *Tgif* (Howard-Ashby *et al.*, 2006b); *Wnt10, ManR* and *ChP* (Annunziata and Arnone, unpublished data); *Xlox* and *Cdx* (Arnone *et al.*, 2006; Cole *et al.*, 2009).

While the foregut and midgut domains are molecularly defined from late gastrula on, and undergo mainly morphological changes during the subsequent stages of development (see Fig. 2), the hindgut domain is a hot spot for cell specification throughout the late gastrula and prism stages (Fig. 2). The molecular mechanisms underlying intestine differentiation during this period have been partially unraveled. In particular, the role of

SpXlox and *SpCdx* in intestine development has been documented (Arnone *et al*., 2006; Cole *et al*., 2009): *SpXlox* knockdown embryos show a malformed gut that is missing the pyloric sphincter and shows reduced digestive capacity. Moreover, *Xlox* is required for *Cdx* activation and *Cdx* is in turn responsible for *Xlox* repression in the intestinal cells. Combining the use of functional analysis methodologies with high resolution imaging, we have recently expanded our understanding of ParaHox gene function and regulation (Annunziata and Arnone, unpublished data). Reconstructions of the regulatory mechanisms driving stomach and intestine differentiation are represented diagrammatically in Figure 4, where the cell regulatory state of each gut domain is causally explained (at least partially) through experimentally confirmed GRN nodes and interactions.

The involvement of Xlox in stomach differentiation and in the formation of the pyloric sphincter has been further demonstrated by showing that SpXlox is required for the activation of stomach terminal differentiation genes (at least *ManrC1A* and *ChP*), and for the transcription of the muscle specific Myosin Heavy Chain (*MHC*) gene in those cells of the gut that will give rise to the pyloric sphincter. Genome-wide differential transcriptomic analysis of SpXlox knock-down morphants has provided a large amount of information regarding the composition and structure of the genetic network downstream of SpXlox (Annunziata and Arnone, unpublished data). Down regulation of a number of genes encoding for metabolic enzymes, muscle associated proteins, and neuronal factors are the consequence of the knockdown of *Xlox* (using a sequence specific morpholino strategy). These data suggest that Xlox forms part of the upstream regulatory network controlling cell type specification for a number of different cell types.

Characterization of the transcriptional machinery involved in the specification and patterning of the sea urchin endoderm has provided us with a detailed mechanistic explanation of intestine differentiation. A summary of the regulatory events involved follows next: In late gastrula embryos, cells expressing *Xlox* and *Cdx* also express *Hox11/13b, Bra* and *FoxA* genes (Figs. 3 and 4), together defining a specific cellular regulatory state exclusive to the most posterior cells of the late gastrula archenteron. Only one regulatory gene capable of upregulating *Xlox* transcription has been identified, *Blimp-1a*. However, *Blimp-1a* transcription occurs in a broader domain and several hours before the activation of *Xlox* (Livi and Davidson, 2006), suggesting that other unknown regulatory factors are recruited for *Xlox* expression. *Cdx* transcription is under the control of the genes *Hox11/13b*, *Bra*, *FoxA* and *Xlox*, with *Xlox* probably functioning as a temporal switch to control the initiation of *Cdx* transcription. Moreover, in the larval intestine, *Cdx* becomes recruited for its own tran-

scription, "locking-in" the regulatory state of intestinal cells. This is further supported by the expression dynamics of the SpCdx protein, whose localization overlaps with the corresponding mRNA expression domain within the developing intestine at all stages analyzed (Annunziata and Arnone, unpublished data).

SpCdx is responsible for the activation of *Wnt10* ligand transcription (probably in concert with other posterior gut cell regulatory genes such as *Hox11/13b* and *Bra*) in the most posterior cells of the gut of the late gastrula: Wnt10 ligands diffuse towards the anterior side of the gut progressively clearing *Xlox* (and probably other) transcripts from the intestinal cells. All intestinal cells are depleted of *Xlox* transcripts in the 72 h larva. This clearing of active transcription is also reflected in SpXlox protein expression dynamics. While during gastrulation the protein and mRNA expression domains are overlapping, at larval stages the protein domain is clearly broader than that of the mRNA: whereas the protein is detected in about 30 cells that cover the pyloric sphincter and a portion of the aboral posterior stomach, Xlox transcripts are confined to around 10 cells of the pyloric sphincter.

In parallel with the activation of *Cdx, Endo-16* transcription (a terminal differentiation gene whose transcripts are accumulated in both midgut and hindgut cells until the late gastrula stage) is also progressively cleared from the intestinal cells and become confined to the stomach larval cells. The mechanism underlying this repression is unknown but the involvement of Wnt10 cannot be excluded.

The molecular understanding of the A–P patterning in the sea urchin embryo remains far from complete. We are still missing important information on many other levels of regulation, such as the involvement of small noncoding RNAs, the role of post-translational modifications and protein-protein interactions, plus the possible involvement of chromatin remodeling. Further studies combining multiple approaches will be needed to continue to unravel the complex mechanisms governing organogenesis of the sea urchin embryonic digestive system.

EVOLUTIONARY RELATIONSHIPS BETWEEN ECHINOID AND CHORDATE GUT PATTERNING SYSTEMS

The early digestive system in most deuterostome embryos arises through common developmental processes during gastrulation. Gastrulation results in the internalization of the endoderm, by invagination through the blastopores of sea urchin and frog embryos, or by ingression involving combined involution and migration movements through the primitive streak of avian and mammalian epiblast. In all cases, the first cells to enter the embryo contribute to the anterior
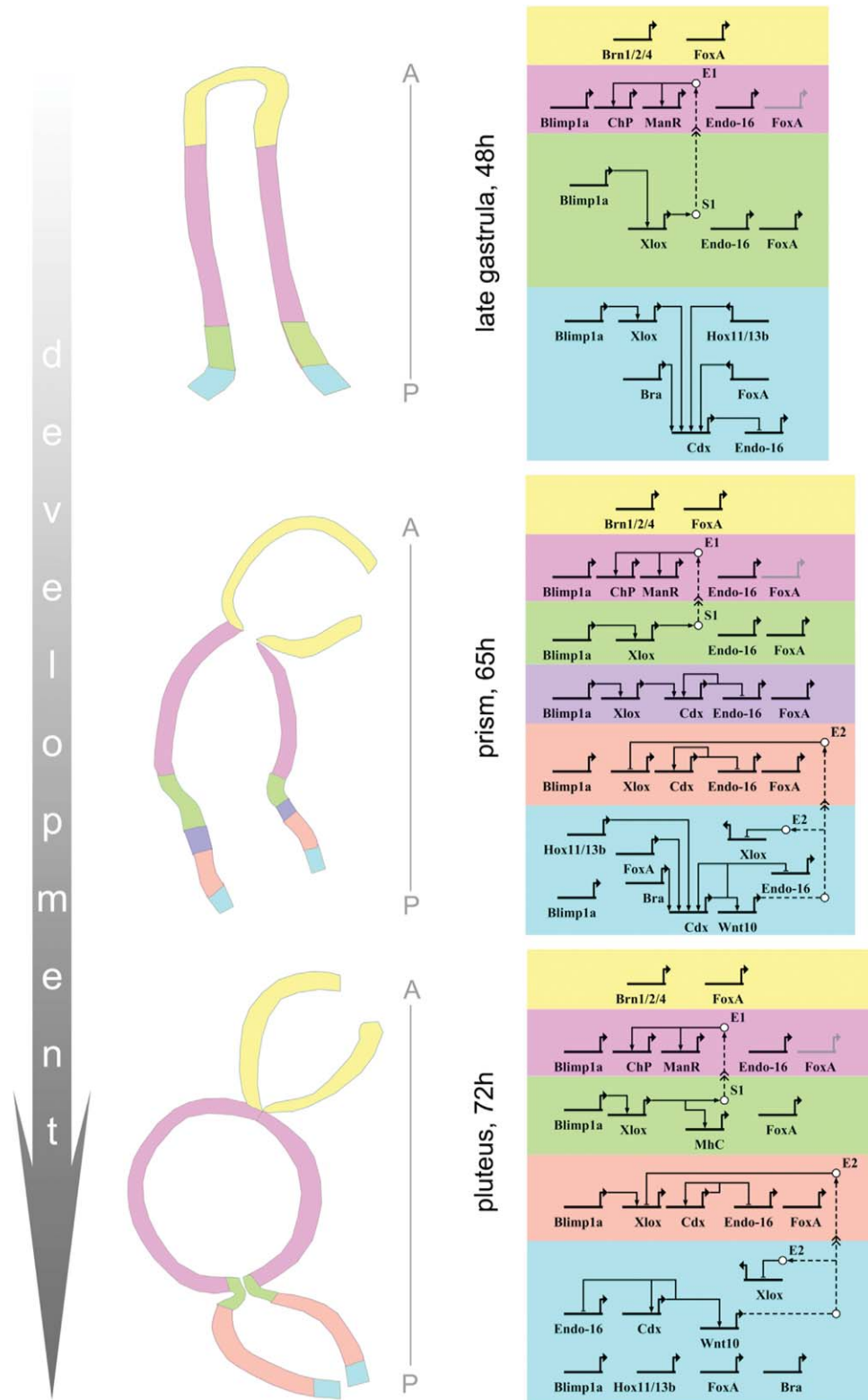
FIG. 4. Dynamics of gene interactions along the AP axis during sea urchin gut differentiation, a gene regulatory network perspective. Similar to figure 3, three stages of the sea urchin embryonic developing gut are depicted in lateral view, but different colors are used for compartments showing exclusive regulatory states. In the right part of the figure the regulatory states and the gene interactions are summarized, using the same color code present in the schematic diagram. The wiring within the gene network indicate known gene interactions, however to date none of these interactions have been demonstrated to be direct. Arrows represent positive regulation; bars represent repression. The white bullets, together with the black dashed line, indicate signaling events: S1 represents an unknown signaling molecule under the control of Xlox and directing stomach genes activation through the action of an unknown effector gene (E1); E2 represents the effector gene responsible for *Xlox* repression in the intestinal cells. For the expression dynamics see references in figure 3. All regulatory interactions are from Annunziata and Arnone (unpublished data).
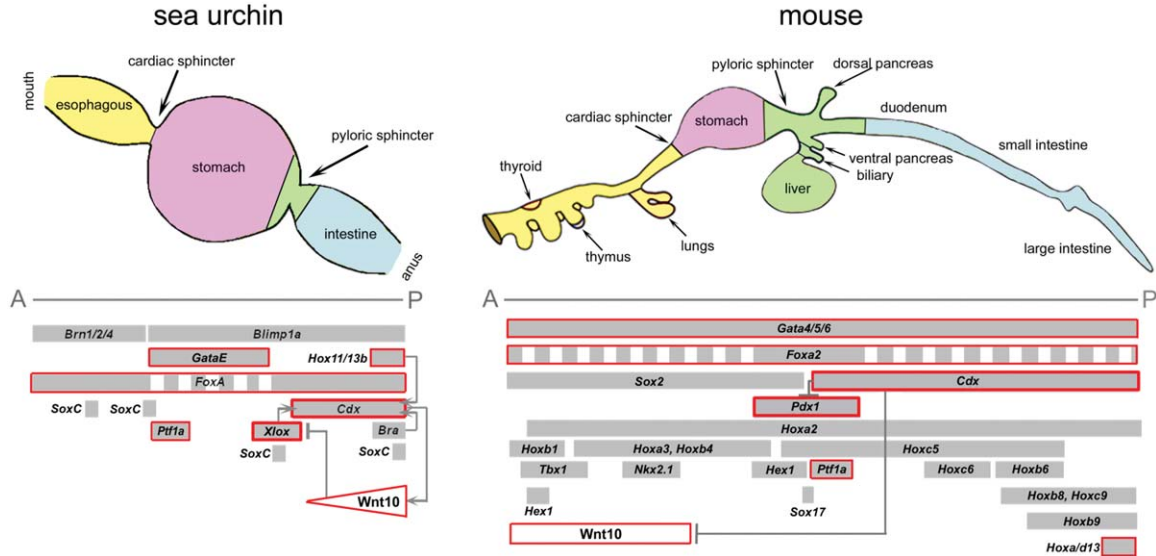
FIG. 5. Gut patterning along the AP axis in sea urchins as compared to vertebrates. The schematic diagrams have been assembled by putting together positional information of gene expression and known regulatory interactions for several transcription factors and signaling molecules drawn from the literature. For details regarding expression patterns, see Table 1 and Figure 3. The reported sea urchin regulatory interactions represented with lines and arrows are reported in (Cole *et al*., 2009) and (Annunziata and Arnone, unpublished data). The diagram shown for the mouse gut was adapted from (Zorn and Wells, 2009). The repressive regulatory interactions of *Cdx* on *Wnt10* and *Pdx1* are reported in Gao *et al*. (2009, 2010), respectively. *Gata4/5/6* expression is reported in (Ayanbule *et al*., 2011). *FoxA* is depicted in white and grey boxes to denote the low levels of expression in sea urchin midgut-stomach cells (Oliveri *et al*., 2006) and in mouse esophagus and intestine (Besnard *et al*., 2004). Conserved elements between sea urchin and mouse gut patterning components are highlighted in red.

endoderm, while cells that follow contribute to progressively more posterior domains. These common morphogenetic movements make it possible, and meaningful, to compare the molecular mechanisms described above for the sea urchin to what is known about chordate endodermal patterning.

As outlined before in Table 1 we provide a detailed description of the activities of several sea urchin orthologues of both transcription factors and terminal differentiation genes expressed in vertebrate digestive organs. The data are separated in three sections: organ specific genes, in green; stomach genes, in pink; intestine genes, in light blue. This comprehensive comparison highlights the remarkable similarities that echinoid and vertebrate digestive tracts share, extending from the upstream regulators that control the morphogenesis to the effectors that carry out all physiological functions associated to the gut. In parallel, and in order to better visualize the enormous amount of information contained in Table 1, we provide an alternative, schematic, representation of the overlapping expression domains of the principal transcription factors and terminal differentiation genes expressed along the A–P axis in the larval sea urchin gut, compared with expression domains of the homologous genes in the embryonic mouse gut (Fig. 5). It is clear that the expression pattern of the ParaHox genes *Xlox* and *Cdx* (*Pdx1* and *Cdx-2* in mouse) along the A–P axis of the gut are strikingly similar in both taxa, a first example of conserva-

tion of a key molecular mechanism involved in sea urchin and mouse gut development. Examples of additional elements showing conservation are, for instance, Ptf1a, FoxA, Brn4, and Wnt10 signaling, involved in both sea urchin and vertebrate gut patterning.

In the sea urchin, *SpXlox* is required for proper differentiation of the stomach: at least two stomach specific terminal differentiation genes (*ManrC1A* and *ChP*) disappear in Xlox morphants. *SpXlox* is also necessary for transcriptional activation of the muscle specific terminal differentiation gene, *SpMHC*, in endodermal cells that will from the pyloric sphincter. The absence of *SpMHC* transcription in the precursor cells of the pyloric sphincter in SpXlox knockdown larvae correlates well with the demonstrated absence of a pyloric structure (Cole *et al*., 2009). The mouse homolog of the Xlox gene, *Pdx1*, is well known for being involved in pancreas formation and differentiation, but also has a conserved function in pyloric sphincter morphogenesis (Offield *et al*., 1996). *Pdx1* is expressed in a gradient along the mouse intestinal tract and it plays a crucial role in both pancreas development and in the later maintenance of β-cell function (Monaghan *et al*., 1993; Stoffers *et al*., 1997). More specifically, *Pdx1* is expressed in the antrum, the pylorus, and the duodenum, with its expression delineating the domain of the tubular gut from which several accessory digestive organs (liver and gall bladder, as well as dorsal and ventral pancreas) will later emerge. *Pdx1*-null embryos

have a small, rudimentary pancreas that lacks insulin-secreting β-cells (Offield et al., 1996). The loss of *Pdx1* also disrupts the morphogenesis of the proximal duodenum causing a misshapen pyloric sphincter and deficiencies in gastric emptying, with an anterior duodenum resembling a posterior extension of the stomach (Jonsson et al., 1994; Offield et al., 1996). Since markers of the pyloric region (such as *Nkx2-5*) have not been examined in mice lacking *Pdx1*, it is still unclear whether or how this gene participates in pyloric development per se (Udager et al., 2010).

In sea urchins, restriction of Xlox expression within the developing hindgut is under the control of Cdx-mediated Wnt10 expression. *Cdx* appears to have a conserved repressive function in both vertebrates and echinoderms that is used for proper differentiation of the posterior gut epithelium. All vertebrates have three *Cdx* genes, *Cdx1, Cdx2*, and *Cdx4. Cdx1* and *Cdx4* knockout mice do not present any intestinal phenotype (Subramanian et al., 1995; van Nes et al., 2006) whilst intestinal growth is severely affected in *Cdx2* mutant mice (Gao et al., 2009). *Cdx2* null mice die at embryonic stage E3.5dpc (3.5 days postcoitum, commonly used terminology in medicine and biology to refer to the age of a mammalian embryo). Conditional loss of Cdx2 from stage E13.5dpc on led to a transformation of the small intestine into a pyloric-stomach-like structure and the ectopic expression of *Pdx1* (Grainger et al., 2010), a situation reminiscent to what has been described for sea urchins (Cole et al., 2009). Taken together, all these observations suggest conservation in vertebrates of the cross regulatory loop controlling gut patterning described in sea urchins.

Cdx activation is bi-modal in chordates (Chawengsaksophak et al., 2004; Osborne et al., 2009; Reece-Hoyes et al., 2002): during gastrulation *Cdx* is required for axial elongation (in mice: (Chawengsaksophak et al., 2004)); later *Cdx* is involved in intestinal patterning. In the sea urchin embryo, *Cdx* function in the early phases of gastrulation has been lost and only its function in posterior gut patterning has been retained (note that a *Cdx* "biphasic" transcription profile has been described in other echinoderms, the sea stars, and in hemichordates (Annunziata et al., 2013; Ikuta et al., 2013). The loss of Cdx2 in mouse endoderm at an earlier stage (E9.5dpc) than that previously described (stage E13.5dpc), also results in a posterior to anterior gut transformation, leading to ectopic expression of anterior gut markers like *Sox2, Pax9* and *Wnt10* in the intestine (Gao et al., 2009) (in this case *Pdx* expression is not affected). In these mice the posterior Hox code was only transiently affected: 5 out of the 13 Hox genes examined at stage E12.5 showed reduced expression levels, which eventually returned back to normal levels by stage E14.5. In vitro studies on pancreatic cell differentiation have revealed that Wnt factors are necessary

for the down-regulation of the *Pdx* gene (Shi et al., 2013). However, in mice Wnt10a signaling is not activated by Cdx2; in fact Cdx2 seems to be actively inhibiting Wnt10a expression—a remarkable difference between the two systems. Given the presence of three functional *Cdx* genes and multiple Wnt factors in mice we cannot rule out the possibility that another Wnt member is repressing *Pdx* in the posterior intestine. Nonetheless, it is clear from conditional knockout studies that Cdx2 is initially required for establishing and maintaining posterior identity within the gut and, at later stages, it is necessary for maintaining A–P identity.

However, we have to stress that Cdx2 is not the only player of the gut differentiation process in vertebrates; a crucial role for several signaling pathways in A–P patterning of the endodermal tube has been demonstrated. The available data support a model in which posteriorizing factors such as Wnt, FGF4, and Cdx2 progressively demarcate more posterior regions of the gut. While they are initially broadly active in the midgut and hindgut and are suppressed in the presumptive foregut, later they become down-regulated specifically in the midgut (by early somite stages; stages e8/9dpc). In the hindgut, persistent FGF and Wnt signaling are required to maintain Cdx2 expression and define the anterior boundary of the intestine (Gregorieff et al., 2004). At the same time their inhibition is required for proper differentiation of anterior structures such as the stomach, liver and pancreas (Kim et al., 2005; McLin et al., 2007). For instance, (Kim et al., 2005) have shown that *Barx1*, a homeobox gene whose expression in restricted to stomach mesenchyme during gut formation, is responsible for the repression of Wnt signaling in the stomach through the action of frizzled related proteins (sFRPs), an activity that allows the proper differentiation of the gastric epithelium. These data put together suggest that intestinal differentiation represents a default state for gut endoderm, and that, active signals, in the form of Wnt inhibition, are needed to specify the stomach epithelium. In this context, these same authors published recently a paper in which it is demonstrated that micro-RNAs (miR-7a and miR-203) are required for proper digestive tract organogenesis controlling the expression of the stomach homeobox gene *Barx*1 (Kim et al., 2011).

The degree of conservation that is being revealed between sea urchin and mouse ParaHox gene activities in the gut patterning process is impressive. The repression of alternative regulatory states, as described here, is a recurring mechanism used during embryonic development for the patterning of structures (examples include: the repression of mesodermal fate for endoderm specification in sea urchins; ectoderm repression for pharyngeal cell specification in worms; proneuronal fate repression for sensory organ specification in flies; reviewed in (Oliveri and Davidson, 2007). The similar

ways in which ParaHox genes are used in vertebrates and echinoderms provides a precious opportunity to study a very complex morphogenetic process such as animal gut development in a simple and experimentally amenable model system, the sea urchin embryonic gut.

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