

## **Endoderm specification**

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### **Abstract**

In this chapter I focus on the emergence of endoderm, the origin of these cells and their organization in space. I also discuss the molecular events that lead to endoderm formation and how endoderm can be molecularly defined. Although largely based on knowledge generated using the mouse model system, data from other model organisms are used when they provide important information missing in mice. This article presents endoderm engineering from ES cells and provides molecular triggers and landmarks that may be used for optimized engineering based on normal development. Due to the similarity of markers between definitive and extraembryonic endoderm, the generation of these lineages is also discussed. Although endoderm stem cells, that is stem cells endowed with the ability to give rise to all endodermal derivatives but not ectoderm or mesoderm, have not been reported yet, there are stem cells in specific endodermal organs which will be discussed in the following chapters.

#### **1. Introduction**

The endoderm is classically defined as the inner germ layer of the embryo. The main derivative is the epithelial outlining of the digestive tract but it does also contribute to many other organs detailed in Figure 1 (Place Figure 1 here). However, the terminology in Amniotes is confusing as different types of cells that contribute to extraembryonic structures have also been called endoderm, such as the primitive (PrE), visceral (VE) and parietal endoderm (PE). Figure 2 depicts the location and lineage of the different types of endoderm. The fact that these extraembryonic structures share many molecular markers with definitive endoderm adds to the confusion. (Place Figure 2 here).

## 2. Cellular aspects of endoderm emergence

### 2a. Emergence of definitive endoderm at gastrulation

Definitive endoderm in Amniotes arises at the time of gastrulation, during which endoderm precursors initially located in the epiblast ingress in the anterior primitive streak (Figure 1). Definitive endoderm cells egress from the primitive streak and insert into the visceral endoderm, progressively displacing this layer anteriorly and laterally over the extraembryonic regions of the conceptus (Poelmann, 1981; PMID) (Lawson et al., 1986; PMID; Tam and Beddington, 1992; PMID 1516473; Tam et al., 2004; PMID 15355796; Tam et al., 1993; PMID 8305722). Definitive endoderm movement is accompanied by epithelial-mesenchymal transition and requires *Snail* or the related gene *Slug* that repress E-cadherin (Blanco et al., 2007; PMID 17965052; Nakaya et al., 2008; PMID 18552836; Thisse et al., 1995; PMID 7589816). The directionality of movement is controlled by Sdf1 acting as a chemoattractant on Cxcr4-expressing endoderm in zebrafish and *Xenopus* (Fukui et al., 2007; PMID 17239342; Mizoguchi et al., 2008; PMID 18579679). Mosaic genetic modifications in Zebrafish have shown that at least in this model, endoderm gastrulation is a combination of active movements of cells and passive movements whereby a cell is mobilized by its neighbours (Carmany-Rampey and Schier, 2001; PMID 11525740; Pezeron et al., 2008; PMID 18291651). Subpopulations of animal pole cells forced to express Sox17- an inducer of endoderm- either migrate towards the endodermal layer or die. These observations suggest that there is a feed-back check on the matching between the differentiation status of a cell and its environment (Clements and Woodland, 2000; PMID 11091074). The latter considerations, in the context of ES cell cultures may allow endodermal cells to aggregate in a heterogeneous culture or die depending on their neighbours.

### 2b. Timing of endoderm specification

In mouse and chick, heterotopic grafting experiments have shown that determination to form endoderm occurs after the cells have left the streak (Kimura et al., 2006; PMID 16337933; Kinder et al., 2001; PMID). Whether the EMT is crucial for endoderm differentiation is unclear at the moment but the ability of endoderm to differentiate prior to gastrulation in several species argues against this hypothesis (Laufer et al., 1980; PMID 7363324; Leung et al., 1999; PMID 10588867; Priess and Thomson, 1987; PMID 3802194; Schroeder and McGhee, 1998; PMID 9811572). Nevertheless in Amniotes, cells are exposed to signalling centers during their migration. In chick, endoderm progenitors in Koller's sickle undergoing their characteristic 'Polonaise movement' are thought to become specified as they receive signals from the

posterior marginal zone which activate the Nodal signaling pathway. These signals include Wnt ligands (Skromne and Stern, 2001; PMID). At a later stage, mesoderm and endoderm in passing may receive instructive patterning signals from the node (Brennan et al., 2002; PMID 12231623; Pagan-Westphal and Tabin, 1998; PMID). Such signaling centers are formed in ES cell cultures aggregated into embryoid bodies and most likely in dense monolayer cultures (Leahy et al., 1999; PMID 10368935). By sensing their position relative to signaling center, moving cells thus might coordinate their differentiation.

### 2c. Mesendoderm

With the notable exception of sea urchin, most species initially segregate ectoderm precursors from progenitors that give rise to endoderm and mesoderm. In *C. elegans*, sea urchin and zebrafish mesoderm and endoderm derive from bipotential progenitors (Rodaway and Patient, 2001; PMID). In Amniotes a similar mesendoderm population has been postulated based on coexpression of endoderm and mesoderm markers in the anterior streak (Rodaway et al., 1999; PMID), and the observation that certain signalling cascades induce both types of cells (Lemaire et al., 1998; PMID 9609820; Reiter et al., 1999; PMID; Rodaway and Patient, 2001; PMID). In space, endodermal/mesendodermal progenitors tend to be located in the anterior streak whereas mesodermal progenitors extend to the posterior streak. However, single cell lineage tracing has never formally demonstrated the existence of bipotential cells in Amniotes.

## **3. Molecular control of definitive endoderm formation**

A comprehensive analysis of the regulatory gene network responsible for endoderm differentiation has been carried out in sea urchin and largely corroborated in *Xenopus* (Davidson et al., 2002; PMID 11872831; Davidson et al., 2002; PMID 12027441; Loose and Patient, 2004; PMID 15223347). In non-Amniotes endoderm is initially induced by maternal proteins which will not be discussed here ( $\beta$ -catenin, VegT, Otx). They do not have a maternal activity in Amniotes although  $\beta$ -catenin and eomesodermin (VegT-like) have a zygotic activity in endoderm induction. In Amniotes endoderm is initially induced by secreted factors.

### 3a. The TGF $\beta$ s Nodal, Gdf1 and Gdf3 are endoderm inducers in Vertebrates

Insights into the inductive mechanisms underlying endoderm formation in vertebrates initially came from studies in *Xenopus* using a dominant-negative activin receptor which blocks secreted TGF $\beta$ -related

activities including Activin, Vg1, and *Xenopus* nodal-related proteins (Xnr). Vegetal pole endoderm explants of embryos injected with this construct express mesodermal and ectodermal marker genes at the expense of endoderm (Henry et al., 1996; PMID). This led to the idea that Vg1 and/or Xnr's are endogenous endoderm inducers. Further analysis of Nodal functions in *Xenopus* and zebrafish confirmed that mesodermal and endodermal cell fates are specified by different levels of Nodal signaling (Agius et al., 2000; PMID; Dougan et al., 2003; PMID 12642489; Schier et al., 1997; PMID; Thisse et al., 2000; PMID 10667793). Also in the mouse, Nodal induces both mesoderm and endoderm (Brennan et al., 2001; PMID; Conlon et al., 1994; PMID; Zhou et al., 1993; PMID), but endoderm populations appear to be selectively lost in embryos carrying a hypomorphic *Nodal* allele, mutations in Nodal proteolytic activation site or gradual reductions in the gene dosage of Smad 2 and 3 (Ben-Haim et al., 2006; PMID 16950123; Dunn et al., 2004; PMID 15084457; Liu et al., 2004; PMID 15183723; Lowe et al., 2001; PMID; Norris et al., 2002; PMID). Conversely, targeted inactivation of the Nodal antagonist Lefty2 leads to excess endoderm formation (Meno et al., 1999; PMID). Studies in zebrafish suggest that Nodal proteins establish a morphogen gradient to pattern the marginal zone along the animal-vegetal axis, with peak levels specifying blastomeres closest to the margin to form endoderm. In contrast, cells farther away from the Nodal source respond by expressing mesodermal genes, presumably because they are exposed to lower concentrations of Nodal, and/or a shorter duration of signaling (Chen and Schier, 2001; PMID). The regulation of Nodal expression is discussed in the next chapter (Figure 3). Homologues to the second activin receptor binding protein, Vg1, have been identified in zebrafish, chick and more recently in mouse where the more distant Gdf1 and 3 recapitulate Vg1 activity (Dohrmann et al., 1996; PMID; Helde and Grunwald, 1993; PMID 8405668; Seleiro et al., 1996; PMID 8939612; Shah et al., 1997; PMID) (Andersson et al., 2007; PMID 17936261; Bertocchini et al., 2004; PMID 15226255; Skromne and Stern, 2001; PMID). *Gdf1* and 3 are expressed in the node like Nodal and recent experiments show that Gdf1 potentiates Nodal activity by forming heterodimers that signal at a longer range (Tanaka et al., 2007; PMID 18079174) (Figure 3).

### 3b. Nodal expression is induced by the canonical Wnt pathway and positive feedback signaling

Among the signals which activate *Nodal* expression is the Wnt pathway. Mouse embryos lacking Nodal, or  $\beta$ -catenin fail to form a primitive streak (Conlon et al., 1994; PMID; Huelsken et al., 2000; PMID), suggesting that the canonical Wnt pathway and Nodal act in synergy to specify definitive endoderm. Several *Wnt* genes are expressed before and during gastrulation (Kemp et al., 2005; PMID 15880404). Analysis of Wnt null alleles demonstrates that germ layer formation and expression of mesendoderm markers in the mouse depends on *Wnt3* (Liu et al., 1999; PMID). At the onset of gastrulation, *Wnt3* is

initially expressed in the posterior visceral endoderm and proximal epiblast region shortly before prospective mesendoderm cells begin to ingress into the primitive streak. *Wnt3* and *Nodal* mutually activate each other (Ben-Haim et al., 2006; PMID 16950123; Brennan et al., 2001; PMID; Liu et al., 1999; PMID). Thus, the canonical Wnt pathway may promote endoderm formation in mammals primarily by locally stimulating Nodal feedback signalling (Figure 3). (Place figure 3 here)

Residual Nodal signaling in *Wnt3* and  $\beta$ -*catenin* mutants indicates that Nodal expression is regulated by additional signals. Peak levels of *Nodal* expression depend on an autoregulatory loop mediated by the binding of FoxH1 on a FAST binding site in the *Nodal* regulatory region (Hoodless et al., 2001; PMID; Norris et al., 2002; PMID; Yamamoto et al., 2001; PMID). This autoregulation is potentiated by cripto, an EGF-CFC family GPI-anchored glycoprotein, that can directly associate with Nodal and its signaling receptor Alk4 (Reissmann et al., 2001; PMID; Yeo and Whitman, 2001; PMID). Although Cripto promotes mesendoderm formation primarily by stimulating Nodal signalling, recent data suggests that Cripto binds Wnt11 and stimulates activation of  $\beta$ -catenin (Tao et al., 2005; PMID 15797385).

Similar to other TGF $\beta$  family members, Nodal is derived from a precursor protein by redundant proteolytic activities of the subtilisin-like proprotein convertases Furin or Pace4 (Constam and Robertson, 1999; PMID). Embryo explant experiments and analysis of *Furin*<sup>-/-</sup>; *Pace4*<sup>-/-</sup> double mutants suggested that Nodal processing is essential to stimulate autoinduction early after implantation, but that uncleaved Nodal during gastrulation may activate at least a subset of mesodermal markers (Beck et al., 2002; PMID; Mesnard et al., 2006; PMID 16728477). Endoderm formation depends on two sequential positive feedback loops mediated by Cripto and Bmp4/Wnt3 that are activated by mature or uncleaved Nodal, respectively, to sustain Nodal signaling from implantation throughout gastrulation (Ben-Haim et al., 2006; PMID 16950123). According to a recent mathematical model as well as evidence in Zebrafish, the choice between mesodermal and mesendodermal fates depends on how long a particular cell and its ancestors have been exposed to Nodal and its effectors, rather than a concentration gradient (Ben-Haim et al., 2006; PMID 16950123; Hagos and Dougan, 2007; PMID 17391517). Recent experiments have demonstrated that the VegT homologue Eomesodermin, expressed in the trophoctoderm like Furin and Pace 4, synergizes with Nodal (Arnold et al., 2008; PMID 18171685). It may participate in one of the feed-back loops (Figure 3).

This important role of Nodal emerged recently as it has different functions in Echinoderms, Ascidian and possibly Amphioxus (Duboc et al., 2004; PMID 15030762; Hudson and Yasuo, 2006; PMID 16835438; Yu et al., 2002; PMID 12492142). The role of Nodal in endoderm and mesoderm induction in vertebrates may

have derived from its original function in dorso-ventral axis specification. The role of  $\beta$ -catenin and Wnt signalling in endoderm induction is prominent in *C.elegans*, Echinoderms and all vertebrates (Reviewed in (Grapin-Botton and Constam, 2007; PMID 17307341). Nodal autoregulation and induction by Wnts/ $\beta$ -catenin functions in echinoderms and must predate chordates. Clearance of FoxQ2, a Nodal repressor, by  $\beta$ -catenin maintains Nodal expression in Sea Urchin (Yaguchi et al., 2008; PMID 18194656). Although FoxQ2 identification in Vertebrates is awaiting, its expression in Chordates suggests that this function may be evolutionary conserved (Yu et al., 2003; PMID 12632180).

### 3c. GATA factors are expressed in mesendoderm and required for endoderm differentiation

Several transcription factors are acting downstream of secreted factors responsible for endoderm induction in a network tentatively represented in figure 4 although the direct regulatory mechanisms are still largely unknown. Several Gata family members are acting downstream of Nodal although the proteins leading to Gata activation are different in several model organisms (Grapin-Botton and Constam, 2007; PMID 17307341). (Place figure 4 here)

Forkhead transcription factors of the FoxA family and GATA factors are key players of the endodermal network of transcription factors in all triploblasts studied so far. Several family members are expressed in endoderm or mesoderm in most species. Inactivation of *Serpent*, one of the *Drosophila* Gatas precludes endoderm formation (Rehorn et al., 1996; PMID 9012522; Reuter, 1994; PMID 7913013). Among the 6 vertebrate *Gata* genes, *Gata4*, *5* and *6* play partially redundant roles in endoderm development. Zebrafish *faust* mutants lacking *Gata5*, contain about 60% of the wild type number of endodermal cells (Reiter et al., 1999; PMID). *Gata4* (Jacobsen et al., 2002; PMID 11784093; Soudais et al., 1995; PMID 8582296) and *6* (Koutsourakis et al., 1999; PMID; Morrisey et al., 1998; PMID), but not *Gata5* (Molkentin et al., 2000; PMID 10866681) knockout mice show impaired visceral and definitive endoderm development. In *Xenopus*, *Gata4*, *5* and *6* are expressed in endoderm and convert ectomesoderm into endoderm in a redundant manner (Afouda et al., 2005; PMID 15659482; Gao et al., 1998; PMID 9566909; Jiang and Evans, 1996; PMID; Weber et al., 2000; PMID 11003835). In vertebrates, GATA factors have been shown to activate genes involved in adult endodermal cell function (Intestinal fatty acid binding protein, hepatic nuclear factor 4/HNF4, gastric H<sup>+</sup>/K<sup>+</sup> ATPase) and in some cases such as albumin bind their promoter (Bossard and Zaret, 1998; PMID 9811575; Gao et al., 1998; PMID 9566909; Maeda et al., 1996; PMID 8867276; Morrisey et al., 1998; PMID).

### 3d. Forkhead factor expression and requirement for endoderm differentiation

Forkhead genes of the FoxA class are also expressed in endoderm all triploblasts (Harada et al., 1996; PMID 9025069) (Ang et al., 1993; PMID; Horner et al., 1998; PMID 9649499; Kaestner et al., 2000; PMID 10702024; Kalb et al., 1998; PMID 9584117; Monaghan et al., 1993; PMID; Odenthal and Nusslein-Volhard, 1998; PMID 9683740; Sasaki and Hogan, 1993; PMID; Schier et al., 1997; PMID; Strahle et al., 1993; PMID 7687227; Weigel et al., 1989; PMID 2566386). Their inactivation perturbs but does not abolish endoderm development. Interestingly, they are often expressed in a subpopulation of endodermal cells and their inactivation usually inhibits the development of parts of the gut. In mouse, neither FoxA1- nor FoxA3-inactivated mutants exhibit any early phenotype (Kaestner et al., 1998; PMID 9632808). By contrast, FoxA2, which is expressed at the onset of gastrulation, is required for fore- and midgut formation (Ang et al., 1993; PMID; Dufort et al., 1998; PMID; Sasaki and Hogan, 1993; PMID; Weinstein et al., 1994; PMID).

In zebrafish, *Gata5/fau* is expressed before FoxA2, suggesting that it is upstream of this forkhead transcription factor, as also described in sea urchin, *C. elegans* and *Drosophila* (Azzaria et al., 1996; PMID 8812130; Casanova, 1990; PMID 2133557; Horner et al., 1998; PMID 9649499; Kalb et al., 1998; PMID 9584117; Mango et al., 1994; PMID 7607089; Weigel et al., 1989; PMID 2566386). Direct regulation by Tcfs and T-boxes have been characterized in sea urchin and *Ciona* but remain to be investigated in other species (Davidson et al., 2002; PMID 12027441; Di Gregorio et al., 2001; PMID 11133152). Furthermore, the presence of Smad2 binding elements in the *Xenopus Foxa2* promoter raises the possibility that it is a direct nodal target (Howell and Hill, 1997; PMID 9405370). Autoregulatory loops-positive or negative- have been demonstrated in different species. (Davidson et al., 2002; PMID 12027441; Di Gregorio et al., 2001; PMID 11133152).

FoxA targets have been studied comprehensively in *C. elegans* (Gaudet and Mango, 2002; PMID 11823633). Beyond compiling a list of targets, Mango and co-authors have shown that the late targets have lower affinity binding sites and thus are only induced once the levels of FoxA reach a critical threshold. Several studies have proposed that GATAs and FoxA together form a preinitiation complex that is required but not sufficient for endoderm gene transcription (Bossard and Zaret, 1998; PMID 9811575; Cirillo et al., 2002; PMID 11864602).

### 3e. Sox and Mix, vertebrate players

Specific to vertebrates, other key components of the network downstream of Nodal comprise *Sox17*, *Mix*, and several related genes.

## *Sox17*

*Sox17* was first implicated in endoderm development in *Xenopus* and has also been extensively studied in Zebrafish (Hudson et al., 1997; PMID 9363948). In mouse, *Sox17* is first expressed in visceral endoderm nearest to the ectoplacental cone at 6.0 dpc and progressively spreads to the entire extraembryonic VE. It is also expressed in definitive endoderm from 7.5-8.5 dpc (Kanai-Azuma et al., 2002; PMID). Mid- and hindgut expression persists until 8.5 dpc, whereas foregut expression decreases by 8 dpc. In *Sox17* knockout mice, definitive endoderm is depleted and visceral endoderm-like tissue replaces it in the most posterior and lateral regions. Anterior endoderm is generated, but posterior and lateral endoderm down from the midgut level are reduced and later fail to expand (Kanai-Azuma et al., 2002; PMID). In contrast to *Foxa2*-knockout cells that can form hindgut but not fore- and midgut, *Sox17* mutant cells can contribute to some extent to the foregut but not mid- and hindgut (Dufort et al., 1998; PMID). Elevated levels of apoptosis in the foregut later lead to foregut reduction suggesting that *Sox17* is also a maintenance factor for endoderm. Promoter studies in *Xenopus* have demonstrated that *Sox17* is directly regulated by TGF $\beta$ s (Vg1 or Nodal) and through a different promoter element cooperation between *Sox17* itself and VegT (Howard et al., 2007; PMID 17719026). Mixer also participates in *Sox17* regulation by stimulating an autoregulatory loop which also involves GATAs (Sinner et al., 2006; PMID 16651540). *Sox17* directly activates the endodermal genes *HNF1 $\beta$* , *FoxA1*, *FoxA2* and *Endodermin* in *Xenopus*, in part through synergistic interactions with  $\beta$ -catenin (Ahmed et al., 2004; PMID 15157240; Sinner et al., 2004; PMID 15163629). Other endodermal genes are exclusively under transcriptional control by Mixer or require synergy between Mixer and *Sox17* (Sinner et al., 2006; PMID 16651540). In mice, *Sox7* and *Sox17* may be redundant in extraembryonic visceral endoderm.

## *Mix family*

The Mix family encodes homeodomain proteins initially described in *Xenopus*. Mixer is predominantly expressed at the endoderm/mesoderm boundary, and is the only gene of the family that induces endoderm specifically (Henry and Melton, 1998; PMID; Kofron et al., 2004; PMID 15128672; Sinner et al., 2006; PMID 16651540). *Mix1* and *Bix1/Mix4* induce endoderm at high levels and can repress mesodermal genes like *Xbra* whereas at low level, they induce mesoderm (Henry and Melton, 1998; PMID; Latinkic and Smith, 1999; PMID 10079237; Latinkic et al., 1997; PMID 9389657; Lemaire et al., 1998; PMID 9609820; Tada et al., 1998; PMID 9735361).



Only one *Mix* gene has been found in Amniotes (Peale et al., 1998; PMID 9739137; Pearce and Evans, 1999; PMID 10495285; Robb et al., 2000; PMID 11084649; Stein et al., 1998; PMID 9739135). Mouse *Mixl1* is first detected in the visceral endoderm and later in nascent primitive streak, but not in the node or definitive endoderm. Mice lacking *Mixl1* have reduced definitive endoderm and mid- hindgut lies at the level of the foregut (Hart et al., 2002; PMID 12117810; Tam et al., 2007; PMID 17151016). However, visceral endoderm is displaced normally to the periphery. *Mixl1* mutant cells in chimeras contribute to all organs but the hindgut. Conversely, *Mixl1* overexpression in frog injection assays can induce excess endoderm formation (Hart et al., 2002; PMID 12117810). *Nodal* expression is expanded in *Mixl1* mutants suggesting that a feedback loop regulates NODAL. SMAD2/4 dimers bind the activin-responsive element of the *mix2* promoter (Howell et al., 1999; PMID). Mixer recruits SMAD2/4 to activin responsive elements of mesendodermal genes such as *Gsc* (Germain et al., 2000; PMID 10691736).

In addition to these genes, many genes have been described in endoderm and may be used as markers as compiled in Table1. (Place table 1 here)

#### **4. Engineering endoderm**

##### **4a. Generating endoderm from ES cells using Activin or Nodal**

ES cells spontaneously form endoderm, including definitive endoderm, but in a small proportion (Itskovitz-Eldor et al., 2000; PMID 10859025). Early work aimed at generating clinically relevant endodermal derivatives from ES cells did not characterize intermediate steps in the differentiation and rather focused on the end products such as hepatocytes or pancreatic beta cells. Such attempts were characterized by either controversy in their reproducibility at generating functional differentiated cells (Hori et al., 2002; PMID 12441403; Lumelsky et al., 2001; PMID 11326082; Rajagopal et al., 2003; PMID 12532008) or their low efficiency (Hamazaki and Terada, 2003; PMID 14696353; Jones et al., 2002; PMID 11740861) (Blyszczuk et al., 2003; PMID 12525695; Vincent et al., 2006; PMID 17253956; Yamada et al., 2002; PMID 11897871; Yamada et al., 2002; PMID 11796921). Based on the knowledge of endoderm development, strategies have more recently been devised to generate endoderm from mouse and human embryonic stem cells (ESCs). Developmental knowledge provided triggers and markers in this process. As in vivo, *Nodal* is necessary for endoderm induction from ES cells (Gadue et al., 2006; PMID 17077151; McLean et al., 2007; PMID 17204604). Due to the limited availability and price of biologically active *Nodal* protein (Tada et al., 2005; PMID 16141227), most efforts have made use of *Activin* as a surrogate for *Nodal*. Initial experiments by Kubo et al. (Kubo et al., 2004; PMID 14998924) and Yasunaga et al. (Yasunaga et al., 2005; PMID 16311587) on mouse ES cells (mESCs) and D'Amour et al. (D'Amour et al., 2005; PMID 16258519) on

human ES cells (hESCs) have shown that endoderm is efficiently generated with both species in the presence of Activin and low serum. This protocol functions with mESCs aggregated into embryoid bodies (Kubo et al., 2004; PMID 14998924) as well as mESCs or hESCs cultured as a monolayer (D'Amour et al., 2005; PMID 16258519; Yasunaga et al., 2005; PMID 16311587). The efficiency of the protocol appears to vary largely depending on the cell lines used but has been successfully used in many laboratories (D'Amour et al., 2006; PMID 17053790). The low serum most likely limits phosphatidylinositol 3 kinase (PI3K) activity, a condition needed for definitive endoderm formation from ES cells (McLean et al., 2007; PMID 17204604). Nodal has also in some instances been provided by the use of MEF-conditioned medium (McLean et al., 2007; PMID 17204604).

As expected from the developmental response to Nodal morphogen the authors could demonstrate that 25-100ng/ml activin lead to 50-60% endoderm whereas lower doses of activin (1-10 ng/ml) induced skeletal muscle markers (Gadue et al., 2006; PMID 17077151; Kubo et al., 2004; PMID 14998924). The duration of activin exposure was explored in less detail but Kubo et al. reported that hematopoietic progenitors emerged after 5 days of activin (3-100 ng/ml) treatment whereas 6 days of exposure were necessary for induction of the endodermal markers Sox17 and Hex. Shorter durations of high activin exposure (3 days) can nevertheless induce endoderm (Yasunaga et al., 2005; PMID 16311587), in particular when Wnts are added (D'Amour et al., 2006; PMID 17053790).

More recently the tetracycline-inducible system was used to drive expression of Nodal and was demonstrated to be more effective at inducing endoderm than adding exogenous Activin (Takenaga et al., 2007). This may be due to different intrinsic properties of Nodal and activin. Although they share the same receptors, their mechanism of action is somewhat different as for example their different requirement for Cripto. The differences observed may however be due to the method of delivery: endogenously produced Nodal may be differently post-translationally processed, may traffic differently or may be expressed more evenly.

#### 4b. Molecular characterization of endoderm induced from ES cells

The expression of Foxa2 and Sox17 show that the endoderm induced is at least in majority definitive endoderm. This idea is also confirmed by the transient expression of the primitive streak markers such as Brachyury, Goosecoid, LHx1, MixL1, PDGFR $\alpha$  and Wnt3a (D'Amour et al., 2005; PMID 16258519; Gadue et al., 2006; PMID 17077151; Kubo et al., 2004; PMID 14998924; Yasunaga et al., 2005; PMID 16311587). ES cells have been used to decipher the hierarchy of protein activity downstream of activin, confirming that

Eomesodermin is acting upstream of Mixl1 during endoderm differentiation (Izumi et al., 2007; PMID 17446562; Russ et al., 2000; PMID). Of particular interest, a recent study comparing two protocols of endoderm induction from ES cells reported a list of potential new endoderm markers using microarray RNA profiling of the genes enriched in both conditions (McLean et al., 2007; PMID 17204604). An earlier microarray study provided several additional validated markers including Cxcr4 that can be used to sort cells (Yasunaga et al., 2005; PMID 16311587). The eventual test for their endodermal nature will be transplantation assays and the proof that they can integrate to mouse or chick endoderm and further differentiate and contribute to endodermal organs.

#### 4c. Role of the Wnt pathway in endoderm induction from ES cells

Although the most ancient signaling pathway for endoderm induction has been marginally used for endoderm induction, Wnt pathway activity is necessary during endoderm induction from ES cells (D'Amour et al., 2006; PMID 17053790; Gadue et al., 2006; PMID 17077151; Lindsley et al., 2006; PMID 16943279). Rather surprisingly, Wnt3 (10ng/ml or 100 ng/ml) on its own does not have the ability to induce endoderm but at least in some instances can potentiate Activin activity (D'Amour et al., 2006; PMID 17053790; Gadue et al., 2006; PMID 17077151). During development, many other genes described in the previous paragraphs are necessary to form endoderm but there is limited knowledge as to whether they may be sufficient to induce endoderm from ES cells.

#### 4d. ES cells as a tool to answer developmental and medical questions

Experiments with ES cells are helpful to answer questions that may be difficult to address in mice or human. The best example is the demonstration using ES cells of mouse mesendoderm progenitors. Clonal analysis showed that sorted Gsc<sup>+</sup> single cells generated by activin treatment of ES cells could generate clones made of mesoderm only or mesoderm and endoderm (Tada et al., 2005; PMID 16141227). This experiment provides strong support in favor of mesendoderm progenitors and the lack of endoderm-only progenitors. ES cells may be very useful to study the antero-posterior commitment of endoderm cells. ES cells may also provide the number of cells needed to perform biochemistry or chromatin immunoprecipitations in endoderm-like cells. In the longer term, it would be interesting to have endodermal stem cells that can only give rise to endodermal lineages and be stably maintained as has been achieved for the ectoderm (Conti et al., 2005; PMID 16086633; Tada et al., 2005; PMID 16141227).

Lastly, human ES cells with mutated genes could also represent a wonderful tool to study endodermal organ disease.

## 5. Endoderm regionalization and morphogenesis

### 5a. Markers and fate maps reveal progressive patterning of endoderm into organs

In chick and mouse, the cells recruited early in the primitive streak will form more anterior endoderm derivatives. The position of endoderm progenitors along the primitive streak reflects their later antero-posterior (AP) and medio-lateral position (Franklin et al., 2008; PMID 18486455; Lawson et al., 1986; PMID; Tam et al., 2004; PMID 15355796). Subsequently, folding of the gut tube anteriorly brings the most anterior cells to the most ventral positions (Franklin et al., 2008; PMID 18486455). The endoderm at this stage appears to be roughly divided into anterior and posterior areas. *Cerberus 1 (m-Cer1/Cerr)1* (Belo et al., 1997; PMID; Biben et al., 1998; PMID; Shawlot et al., 1998; PMID 9600941), *Orthodenticle homologue 2 (Otx2)* (Ang et al., 1994; PMID 7607086), *Homeo box gene expressed in ES cells 1 (Hesx1)* (Thomas and Beddington, 1996; PMID) and *Hematopoietically expressed homeobox (Hex)* (Thomas et al., 1998; PMID) are restricted to anterior endoderm. Antero-posterior asymmetry of the endoderm at the same stages is also demonstrated by the specific ability of the anterior endoderm to induce heart differentiation in the mesoderm (Schultheiss et al., 1995; PMID). However, at this early stage association of the anterior endoderm half with posterior mesoderm can still induce posterior genes in endoderm and vice versa (Wells and Melton, 2000; PMID 10725233), suggesting that A-P patterning of the endoderm is not yet determined. At somitic stages, the identity of more regions exhibiting different gene expression profiles is progressively specified (Grapin-Botton, 2005; PMID 15906249; Grapin-Botton and Melton, 2000; PMID 10689353).

### 5b. Molecular mechanisms of endoderm patterning

The molecular mechanisms responsible for early patterning along the AP and medio-lateral/dorso-ventral axes in endoderm are beginning to emerge. They are schematized in figure 5. (Insert Figure 5 here) Thus far, they appear to be very similar to the mechanisms of AP patterning of the neurectoderm, involving wnts, Fgf4 and retinoic acid. Retinoic acid was recently shown to control AP patterning in endoderm at the time of gastrulation in *Xenopus*, *Zebrafish* and *Amphioxus* (Chen et al., 2004; PMID 15196957; Escriva et al., 2002; PMID 12050138; Schubert et al., 2005; PMID 15576409; Stafford et al., 2004; PMID 15322880; Stafford and Prince, 2002; PMID; Stafford et al., 2006; PMID 16452093). This work suggests that increasing levels of retinoic acid activity gradually induces posterior organs. In mouse retinoic acid is required to form the pancreas and pattern branchial arch endoderm (Huang et al., 2002; PMID 11891985; Huang et al., 1998; PMID; Martin et al., 2005; PMID 16026781; Matt et al., 2003; PMID 12668623; Molotkov et al.,

2005; PMID 15739227). However, it remains to demonstrate that graded activity orchestrates the relative position of organs along the entire AP axis in this layer. Retinoic acid is produced by the mesoderm, a tissue that sends patterning signals to endoderm (Kumar et al., 2003; PMID 12812792; Pan et al., 2007; PMID 17643968; Wells and Melton, 2000; PMID 10725233). Fgfs are also necessary for endoderm patterning from gastrulation to somitogenesis (Dessimoz et al., 2006; PMID 16326079; Serls et al., 2005; PMID 15576401; Wells and Melton, 2000; PMID 10725233). Exposing endoderm to FGF4, a node-derived factor, shifts posterior endoderm markers anteriorly and represses anterior markers at gastrulation. After gastrulation the patterning role of FGF4 becomes restricted to the mid- and hindgut, where increasing levels of signalling progressively induce more posterior fates in endodermal cells (Dessimoz et al., 2006; PMID 16326079; Wells and Melton, 2000; PMID 10725233). Although these pathways appear to pattern endoderm at least in part through direct signaling to this layer, more work is needed to elucidate how they cooperate (Dessimoz and Grapin-Botton, 2006; PMID 16322692; Huang et al., 2002; PMID 11891985; Pan et al., 2007; PMID 17643968; Stafford et al., 2006; PMID 16452093). Recent experiments in *Xenopus* show that endoderm is also patterned by the Wnt pathway: the foregut forms in the absence of Wnt activity whereas Wnt signalling is necessary to form the intestine (McLin et al., 2007; PMID 17507400). The lack of anterior Wnt activity is permitted by the expression of several Wnt inhibitors (Cerberus, dickkopf, Frzb) in anterior endoderm (Belo et al., 1997; PMID 9431803; Lewis et al., 2008; PMID 18403408; Mukhopadhyay et al., 2001; PMID 11702953; Pfeffer et al., 1997; PMID 9240561). The role of this pathway in endoderm development is likely to be conserved in rodents as mice deficient for Tcf1 and Tcf4 exhibit posterior endoderm defects (Gregorieff et al., 2004; PMID 15057272).

### 5c. Local signals lead to organ formation

There is evidence that in addition to the signals that lead to the regionalization of endoderm along the AP axis, other signals are necessary locally for the induction of organ primordia. These signals are discussed in more details in the chapters pertaining to specific endoderm organs. One such signal is Fgf2 secreted by the cardiac primordium which is necessary to liver and lung development (Deutsch et al., 2001; PMID 11222142; Gualdi et al., 1996; PMID; Jung et al., 1999; PMID 10373120; Serls et al., 2005; PMID 15576401). Similarly, BMP4 from the septum transversum is necessary for liver induction whereas the pancreas appears to form in the absence of BMPs (Rossi et al., 2001; PMID 11485993; Spagnoli and Brivanlou, 2008; PMID 18094028). In contrast to most of the digestive tract epithelium, the pancreas can only develop in an area free of Shh (Apelqvist et al., 1997; PMID 9368764). Lastly, Fgf10 is necessary for the development of several organs budding off the digestive tract such as the pancreas, caecum, lungs and

stomach glands (Bhushan et al., 2001; PMID 11748146; Burns et al., 2004; PMID 14697353; Nyeng et al., 2007; PMID 17196193; Sekine et al., 1999; PMID 9916808).

#### 5d. Patterning endodermalized ES cells

In spite of our limited knowledge of endoderm patterning, the exploitation of such information to generate organ-restricted progenitors is promising. Exposure to FGF and BMP after endoderm induction by activin enriched in liver progenitors (Gouon-Evans et al., 2006; PMID 17086172). The role of retinoic acid, Fgf10 and the absence of Shh in pancreas development were exploited to enrich endodermalized ES cells in pancreas progenitors (D'Amour et al., 2006; PMID 17053790; Kroon et al., 2008; PMID 18288110). Since Nodal and Wnts used to induce endoderm from ES cells also play a role in AP patterning, a careful investigation of the regional endoderm markers obtained after different amounts and duration of exposure to these morphogens would be interesting. Further, ES cells could be very valuable to investigate difficult questions such as the possible graded activities of Nodal, Wnt, RA and Fgf signaling pathways in endoderm patterning.

### **6. Differentiation of extraembryonic endoderm lineages**

In mouse, primitive endoderm (PrE) segregates from the inner cell mass (ICM) at the blastocyst stage as a squamous epithelium and covers the outside of the embryo (Weber et al., 1999; PMID) (Figure 2). Whereas some PrE cells remain attached to the basement membrane of the ICM and differentiate into cuboidal visceral endoderm (VE), others undergo an epithelial-mesenchymal transition to become parietal endoderm (PE). PE cells migrate along the basement membrane of trophectoderm (TE) cells which gives rise to Reichert's membrane of the parietal yolk sac. VE forms the epithelial lining of the yolk sac (Kadokawa et al., 1987; PMID 3607886). Until placentation, PE and VE lineages together are responsible for nutrient and waste exchange between maternal tissue and the foetus. VE can contribute in a minor way to embryonic gut in the fore- and hindgut (Tam and Beddington, 1992; PMID 1516473). What we know of the differentiation of these 3 lineages arises from a cross-talk between in vivo experiments and experiments with ES cells. ES cells are thus already an experimental model of embryo development.

#### 6a. Primitive endoderm (PrE):

Before the segregation of epiblast and PrE, the inner cell mass (ICM) is a mosaic of cells expressing markers of one or the other lineage (Gerbe et al., 2008; PMID 18083160). In ES cell lines and in vivo, Pou5f1 (Oct3/4) is required to maintain pluripotency and to prevent differentiation into TE cells (Nichols et al., 1998; PMID; Niwa et al., 2000; PMID). On the other hand, using an inducible Pou5f1 transgene, less

than twofold increase in the expression level of Pou5f1 in ES cells is sufficient to induce markers of PrE and mesoderm differentiation (Niwa et al., 2000; PMID). Peak levels of Pou5f1 expression thus are likely to also specify PrE in the blastocyst. In normal ES cell cultures, differentiation is inhibited owing to the presence of LIF. However, if ES cells are aggregated to form embryoid bodies (EB), LIF can no longer prevent PrE differentiation and the segregation of these cells at surface of these aggregates (Murray and Edgar, 2001; PMID; Shen and Leder, 1992; PMID). Zebrafish MZ-Spg/Pou2/Oct4 mutants lack endodermal markers such as Cas/Sox32 and Sox17 but a similar activity in Amniotes may be hidden by early lethality (Lunde et al., 2004; PMID 14711414; Reim et al., 2004; PMID 14723850).

Communication between ICM cells is needed to segregate PrE from epiblast. Mice lacking either *Fgf4* or *Fgf receptor2* (*Fgfr2*) or its downstream effector Grb2 do not form PrE (Arman et al., 1998; PMID 9560232; Feldman et al., 1995; PMID 7809630; Wilder et al., 1997; PMID) (Chazaud et al., 2006; PMID 16678776; Cheng et al., 1998; PMID 9865697). In embryoid bodies, forced expression of a dominant-negative form of the Fgf receptor prevents formation of PrE, and overexpression of *Gata6* and *Gata4* rescues this phenotype (Li et al., 2004; PMID 15456727; Li et al., 2001; PMID 11352941). Gata factors are thus likely to control PrE formation downstream of Fgf signaling, although single knockouts for these genes do not elicit such early phenotypes, most likely due to redundancy (Koutsourakis et al., 1999; PMID; Morrisey et al., 1998; PMID). It is however unclear how a subset of ICM cells activate the FGF pathway. Several pieces of evidence suggest that Gatas control the sorting of PrE and epiblast cells (reviewed in (Yamanaka et al., 2006; PMID 16773657). Genes enriched in PrE have been identified by gene expression arrays (Gerbe et al., 2008; PMID 18083160). Extraembryonic stem cells (XEN) have been isolated from PrE and contribute to their lineage of origin in chimeras (Kunath et al., 2005; PMID 15753215). Overexpression of GATA factors in ES cells leads to cells molecularly very similar to XEN cells and both contribute preferentially to parietal rather than visceral endoderm in chimeras (Shimosato et al., 2007; PMID 17605826). Subsequent withdrawal of GATAs however leads to their differentiation into cells endowed with visceral endoderm characters (Shimosato et al., 2007; PMID 17605826). Although XEN cells require MEFs for their growth, GATA-induced ES cells do not, suggesting that MEF-derived factor(s) serve to maintain Gata expression.

#### 6b. Parietal endoderm (PE):

How PrE cells choose between PE and VE fates is poorly understood. Clonal analysis of cells from genetically marked E3.5 and E6.5-7.5 donor embryos revealed that PrE and VE both give rise to PE when transplanted into host blastocysts (Gardner, 1982; PMID). This suggests that PrE descendants adopt PE

fate because of environmental cues, rather than owing to a lack of competence to express the characteristics of VE.

Terminally differentiated PE cells have been derived *ex vivo* from ES cells upon forced expression of GATA4 or GATA6 (Fujikura et al., 2002; PMID). The signals governing the differentiation of extraembryonic endoderm have been studied best in F9 cells. Upon treatment with retinoic acid, this embryonal carcinoma cell line differentiates to become PrE and VE (Strickland and Mahdavi, 1978; PMID; Strickland et al., 1980; PMID). The transcription factor Sox7 is needed downstream of RA for the induction of GATA4 and GATA6, and subsequent parietal endoderm differentiation (Futaki et al., 2004; PMID 15542856). Although Sox17 is not required for PE and VE formation from PrE *in vivo* recent work with ES cells shows a requirement *in vitro* (Shimoda et al., 2007; PMID 17940068). The effect of RA on F9 cells appears to be mediated by activated Ras (Verheijen et al., 1999; PMID). Parathyroid hormone-related peptide (PTHrP) produced by trophoblast cells and the deciduum immediately adjacent to the implantation site or induces an epithelial to mesenchymal transition (EMT) in PrE cells and their conversion into a PE-like cells (Behrendtsen et al., 1995; PMID; Chan et al., 1990; PMID; Karperien et al., 1996; PMID; Smyth et al., 1999; PMID; Strickland et al., 1980; PMID; Veltmaat et al., 2000; PMID). cAMP, the intracellular mediator of PTHrP triggers the same effect.

#### 6c. visceral endoderm (VE):

Differentiation and survival of VE depends on a number of transcription factors, including the orphan nuclear receptor HNF4 (Chen et al., 1994; PMID; Duncan et al., 1997; PMID). HNF4 is expressed in PrE as early as day E4.5, but after E5.25 becomes restricted to the visceral yolk sac endoderm (Duncan et al., 1994; PMID; Mesnard et al., 2006; PMID 16728477). Signals upstream of HNF4 include BMP2/4 (Coucouvani and Martin, 1999; PMID), and the activin receptor Alk2 (Sirard et al., 1998; PMID) which is essential in extraembryonic lineages (Gu et al., 1999; PMID). Also the homeodomain protein HNF1 $\beta$  and GATA6 are required to induce HNF4 expression in the VE (Barbacci et al., 1999; PMID; Coffinier et al., 1999; PMID; Morrissey et al., 1998; PMID), suggesting that both of these transcription factors may act within or in parallel to the BMP pathway. HNF1 $\beta$  (Tcf2) also stimulates expression of HNF1 $\alpha$  (Tcf1) and Foxa2 (Barbacci et al., 1999; PMID). GATA6 is responsible for activating expression of GATA4 (Morrissey et al., 1998; PMID), which in turn acts in the VE lineage to enable ventral closure of the primitive gut tube (Molkentin et al., 1997; PMID; Narita et al., 1997; PMID). Together, these observations indicate that



differentiation of the VE lineage is brought about by the concerted action of a cascade of transcription factors which later also regulate gene expression in the definitive endoderm and its various derivatives.

The rules as to how specific combinations of these and other transcription factors might pattern the VE remain poorly understood. Initially, VE cells form a columnar epithelium which is subsequently patterned along the proximal distal axis of the conceptus by inductive interactions with adjacent ectodermal cells (Brennan et al., 2001; PMID; Dziadek, 1978; PMID; Gardner, 1982; PMID). Single-cell labelling at E5.5 shows that cells can contribute to embryonic and extraembryonic visceral endoderm (Perea-Gomez et al., 2007; PMID 17705827). The AVE is formed from both part of a PrE population of Cer1-expressing cells and cells that acquire Cer1 expression later (Torres-Padilla et al., 2007; PMID 17662710). Thus, expression of HNF1 $\beta$  and TTF (Transthyretin) is confined to the extraembryonic region, whereas VE cells overlying the egg cylinder express  $\alpha$ -fetoprotein (Dziadek and Adamson, 1978; PMID) and Ihh (Becker et al., 1997; PMID; Belaoussoff et al., 1998; PMID) and adopt a squamous morphology. Recent experiments have shown that Nodal is expressed in the PrE. Nodal signaling is essential to downregulate a subset of PrE markers and thus induce embryonic visceral endoderm (Mesnard et al., 2006; PMID 16728477). In the embryonic region, patterning of the VE further becomes evident with the expression of Wnt3 in the proximal-posterior region. By contrast, cells differentiating at the distal tip express elevated levels of Otx2, and in response move to the prospective anterior pole to become anterior visceral endoderm (AVE) (Kimura et al., 2000; PMID). Foxa2 binds Otx2 promoter and is essential for its expression in AVE (Kimura-Yoshida et al., 2007; PMID 17389379). The specific gene expression pattern of the distal tip is restricted by signalling from the extraembryonic ectoderm as well as nodal signalling (Mesnard et al., 2006; PMID 16728477; Rodriguez et al., 2005; PMID 15857911). The AVE also expresses a number of other specific markers, including Lefty-1 (Ebf) and Cerberus-like. These secreted proteins function redundantly as negative feedback inhibitors in the Nodal pathway and thereby confine primitive streak formation to the posterior epiblast (Perea-Gomez et al., 2002; PMID).

## **7. Concluding remarks**

Although our understanding of endoderm development is less extensive than that of ectoderm and mesoderm, it has been successfully exploited to generate endodermal cells from ES cells with high efficiency. This knowledge has also been helpful with regards to quality control by providing a set of markers that collectively define endoderm identity. This protocol is expensive and labour intensive. It would therefore be of great interest to develop endodermal stem cells endowed with the ability to self-renew and differentiate into all endodermal organs. These cells may be developed from ES cells or from

emerging embryonic endodermal cells. A major gap remains to be filled to understand how different organ primordia are induced from endoderm and use this strategy on endoderm cells in vitro. These recent advances in endoderm generation in vitro will most likely allow the development of new strategies to address questions that are difficult to address in vivo such as those requiring large numbers of cells or live monitoring of cells.

## 8. Figure legends

**Table 1 : Selected endoderm-enriched genes** Yellow: membrane proteins; green: membrane proteins used for cell sorting (HSCs) Hematopoietic stem cells (OV) Otic vesicle (YS) Yolk Sac (NT) Neural tube (Emap) [Edinburgh](#) mouse Atlas Project, (MGI) mouse genome Informatics

Gene name	ES	E7.5	E8.5 Fore	E8.5 Mid	E8.5 Hind	E 9.5 Fore	E9.5 Mid	E9.5 hind	E10.5 and +	Extraemb. endoderm	Other tissues	References
<b>General endoderm</b>												
Sox17	-	+	+/-	+	+	?	?	?	?	VE	HSCs, endothelia	(Kanai-Azuma et al., 2002; PMID) <a href="#">MGI</a>
Foxa2	-	+	+	+	+	+	+	+	+	PE,PrE,AVE	notochord, floor plate	(Kimura-Yoshida et al., 2007; PMID 17389379) (Ang et al., 1993; PMID) <a href="#">emap</a>
Claudin6	?		+	-	+	+	+	+	+	VE	epiblast, OV, mesonephos	(Anderson et al., 2008; PMID 18213590; Sousa-Nunes et al., 2003; PMID 14613977) <a href="#">MGI</a>
<b>CXCR4</b>	-	+	+	+	+/-	+	+	-	+/-	PE, YS	mesoderm, many after 8.5	(McGrath et al., 1999; PMID 10479460) (Yasunaga et al., 2005; PMID 16311587) <a href="#">MGI</a>
Gata4,6	?		+	+/-	+/-	+	+	+	+	VE,PrE	heart	Hou <a href="#">emap</a> (4) <a href="#">MGI</a> (4)
Tcf2/HNF1b	-	-	?	+	+	post	+	+	+	VE	notochord	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
<b>Tmprss2</b>	-	+	?	+	+	?	+	+	+	VE	Prostate	(Hou et al., 2007; PMID 17683524; Moore-Scott et al., 2007; PMID 17576135; Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
<b>Tacstd1 (EpCAM)</b>	+	+	+	+	+	+	+	+	+	VE	Ectoderm, lower after 8.5	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
<b>Embigin</b>	-	+	+	+	-	?	+/-	+/-	-	VE,YS	midbrain, kidney	(Huang et al., 1990; PMID 1965893; Sousa-Nunes et al., 2003; PMID 14613977) (Fan et al., 1998; PMID 9639355) <a href="#">MGI</a>
Hhex	-	+	+	-	-	+	-	-	+	PrE,PE,AVE	endothelia	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
Cer1	?	+	+	-	-	-	-	-	-	AVE	mesoderm	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
Dkk1	?	+	+	-	-	+	-	-	?	AVE	head mesoderm	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
<b>AnxA4</b>	+	?	-	+	+	-	+	+	?	VE	Notochord	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
Bnip1	+	?	+/-	+/-	+/-	-	+	+	?	VE	OV	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
<b>Cacna1b</b>	-	+	+			+			?	VE	heart	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
Clic6	-	?	?	+	+	+/-	+	+	+	VE	head	(Sherwood et al., 2007; PMID

											mesenchyme	17328885) (Hou et al., 2007; PMID 17683524) , <a href="#">emap</a> , <a href="#">MGI</a>
Dsg2	+	+	+			+			?	VE	mesoderm	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
Krt7	-	+	+			+			?	VE, YS	?	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
<b>Npnt</b>		?	?	?	?	?	+	+	?	VE++	branchial arch 1	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
Rab15	-	?	?	?	?	?	+/-	+/-	?	VE++	OV	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
Rbm35a	+	+/-	+/-	+/-	?	+	+/-	+/-	?	?	OV	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
Ripk4	-	+/-	+/-	?	+	+	+	+	?	VE	OV	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
Sh3gl2	+	+/-	+			+			?	VE	?	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
St14	+	+/-	+/-			-	+	+	?	VE	branchial arch 1	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
Spink3	-	+/-	+/-	+/-	+/-	?	?	?	?	YS	ectoderm	(Sherwood et al., 2007; PMID 17328885) (Hou et al., 2007; PMID 17683524) <a href="#">MGI</a>
Alpha Fetoprotein	-	-	+	+	+	liver	-	-	+	VE,YS	-	(Dziadek and Adamson, 1978; PMID) <a href="#">MGI</a>
<b>Enriched definitive endoderm</b>												
Foxa3		-	+/-	+	+	+/-	+	+	+	-	germ cells	(Ang et al., 1993; PMID; Monaghan et al., 1993; PMID) <a href="#">MGI</a>
Foxa1	-	+	+/-	+	+	+/-	+	+	+	-	forebrain	(Monaghan et al., 1993; PMID), <a href="#">emap</a> , <a href="#">MGI</a>
PYY	?	-	+	-	-	+	-	-	+	-	brain	(Hou et al., 2007; PMID 17683524) <a href="#">MGI</a>
5730521E12Rik /Nephrocan	-	+	-	+	+	-	+	-	+	-	kidney	(Hou et al., 2007; PMID 17683524; Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
NM_029639	?	+	-	-	+	+	+	+	+	-	Oral ectoderm	(Moore-Scott et al., 2007; PMID 17576135)
Klf5	?	+	-	+	+	-	+	+	+	-	Otic vesicle, somites, brain	(Moore-Scott et al., 2007; PMID 17576135) <a href="#">MGI</a>
Epha2	?	+	+	-	+	-	-	+	+	-	limb	(Moore-Scott et al., 2007; PMID 17576135) <a href="#">MGI</a>
Cdx 2	?	+	-	-	+	-	-	+	+	-	Trophectoderm NT, notochord	(Hou et al., 2007; PMID 17683524) <a href="#">emap</a> , <a href="#">MGI</a>
<b>Enriched in extraembryonic endoderm</b>												
<b>Ceacam1</b>	+	-	-	-	-	-	-	-	-	VE++ (8.25)	?	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
<b>Dpp4</b>	-	-	-	++	-	-	++	-	?	VE++ (8.25)	Somites+/-	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
Icam	+	-	-	-	-	-	-	-	-	VE++ (8.25)	?	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
<b>CD38</b>	+	-	-	-	-	-	-	-	-	VE++ (8.25)	?	(Sherwood et al., 2007; PMID 17328885)
Timd2	+	-	-	-	-	-	-	-	-	VE++ (8.25)	?	(Sherwood et al., 2007; PMID 17328885) <a href="#">MGI</a>
<b>DBA lectin</b>	+/-	-	-	-	-	-	-	-	-	VE (8.25)	?	(Sherwood et al., 2007; PMID 17328885)
<b>SSEA-4</b>	-	-	-	-	-	-	-	-	-	VE (8.25)	?	(Sherwood et al., 2007; PMID 17328885)
<b>Claudin9</b> , habp2, spp2, ttr	?	?	+/-	+/-	+/-	?	?	?	?	YS	?	(Hou et al., 2007; PMID 17683524)
<b>Amn</b>	-	-	-	-	-	?	?	?	?	PrE,VE	?	(Sherwood et al., 2007; PMID 17328885) (Kalantry et al., 2001; PMID 11279523) <a href="#">MGI</a>
HNF4alpha	-	-	-	-	-	-	-	-	liver	VE,YS		(Sherwood et al., 2007; PMID 17328885; Vincent and

												Robertson, 2004; PMID 15282747) <a href="#">MGI</a>
Cited1	?	-	-	-	-	-	-	-	-	VE	Heart, lateral mesoderm	(Rodriguez et al., 2004; PMID 14673158; Sherwood et al., 2007; PMID 17328885) <a href="#">emap</a> , <a href="#">MGI</a>
Sox7	?	-	-	-	-	-	-	-	-	VE	endothelia	(Kanai-Azuma et al., 2002; PMID) <a href="#">MGI</a>
Tdh	?	-	?	?	?	?	?	?	?	YS	?	(Hou et al., 2007; PMID 17683524) <a href="#">MGI</a>
Lgals1	?	-	?	?	?	?	?	?	?	YS	?	(Hou et al., 2007; PMID 17683524) <a href="#">MGI</a>
Cubn	?	-	?	?	?	?	?	?	?	YS	?	(Hou et al., 2007; PMID 17683524) <a href="#">MGI</a>
Pla2g12b	?	-	?	?	?	?	?	?	?	YS	?	(Hou et al., 2007; PMID 17683524) <a href="#">MGI</a>
Apoc2	?	-	?	?	?	?	?	?	?	YS	?	(Hou et al., 2007; PMID 17683524) <a href="#">MGI</a>
<b>Slc39a8</b>	?	-	+	+	+	+/-	+/-	+/-	?	VE	endothelia	(Moore-Scott et al., 2007; PMID 17576135; Sousa-Nunes et al., 2003; PMID 14613977) <a href="#">MGI</a>
Angiotenin	?	+	?	?	?	?	?	?	?	VE	?	(Moore-Scott et al., 2007; PMID 17576135; Shimono and Behringer, 2003; PMID 12676095) <a href="#">MGI</a>
Dp111/Reep6	?	-	?	?	?	-	-	+	+	VE	brain	(Moore-Scott et al., 2007; PMID 17576135) <a href="#">MGI</a>

**Figure 1: Endoderm derivatives**

**Figure 2: Organization of endodermal progenitors and extraembryonic endoderm at onset of gastrulation in chick and mouse embryos.** Extraembryonic endoderm lineages and the fate of epiblast cells are color-coded. The territories of prospective endoderm, mesoderm and neurectoderm are separated by sharp boundaries only for the sake of simplification. In the mouse, posterior VE is speculated to be the equivalent of the chick endoblast due to its analogous position. Likewise in the proximal epiblast, prechordal plate progenitor cells are predicted to reside in between definitive endoderm precursors and posterior VE in analogy to chick, even though the limited resolution of current fate maps cannot distinguish two separate populations in the mouse epiblast.

**Figure 3: Feedback loops of secreted factors induce endoderm.** Two positive feedback loops (in green and red) involving crosstalk between epiblast cells at the site of future primitive streak and trophectoderm allow to generate the high and long lasting levels of Nodal that induce endoderm. GDF1/3 act as Nodal co-factors in endoderm induction. A negative feedback loop (in blue) involving Lefty2 shuts down signalling activity to limit the amount of endoderm produced. Stars represent receptors.

**Figure 4: Transcription factor network in endoderm induction.** Grey arrows show inductions. Black is used when direct regulation was demonstrated. Red lines show molecular interactions. Some of the regulations have not been demonstrated in mice and may come from Zebrafish or Xenopus. Mix, Sox17 and Gatas each regulate a subset of differentiation genes (Sinner et al., 2006; PMID 16651540).

**Figure 5: Origin of endoderm cells eventually populating different gut areas in chick and mouse.** Yellow marks prospective dorsal foregut endoderm, green marks prospective ventral foregut endoderm, red marks prospective dorsal mid- hindgut endoderm and orange marks prospective ventral mid-hindgut endoderm. The panels on the left show cells in the streak just before migration. All other panels show their emergence in the endodermal layer. Ventral foregut endoderm progenitors shown in green have not been mapped in mouse and in the chick streak. Picture of early streak embryo courtesy of D. Mesnard.

**Figure 5: Regional markers of endoderm.** Endoderm can be schematized as a 2-D plane with AP and DV axes. On this plane organ domains emerge at specific locations. Markers with relatively stable expression patterns between 8.5/9.5 and 10.5 dpc are presented with colored boxes. Endoderm is regionalized through the activity of secreted factors, which origin is indicated. Among those at least Fgfs and retinoic acid have a graded activity along the AP axis. Secreted factors patterning the DV axis are awaiting.

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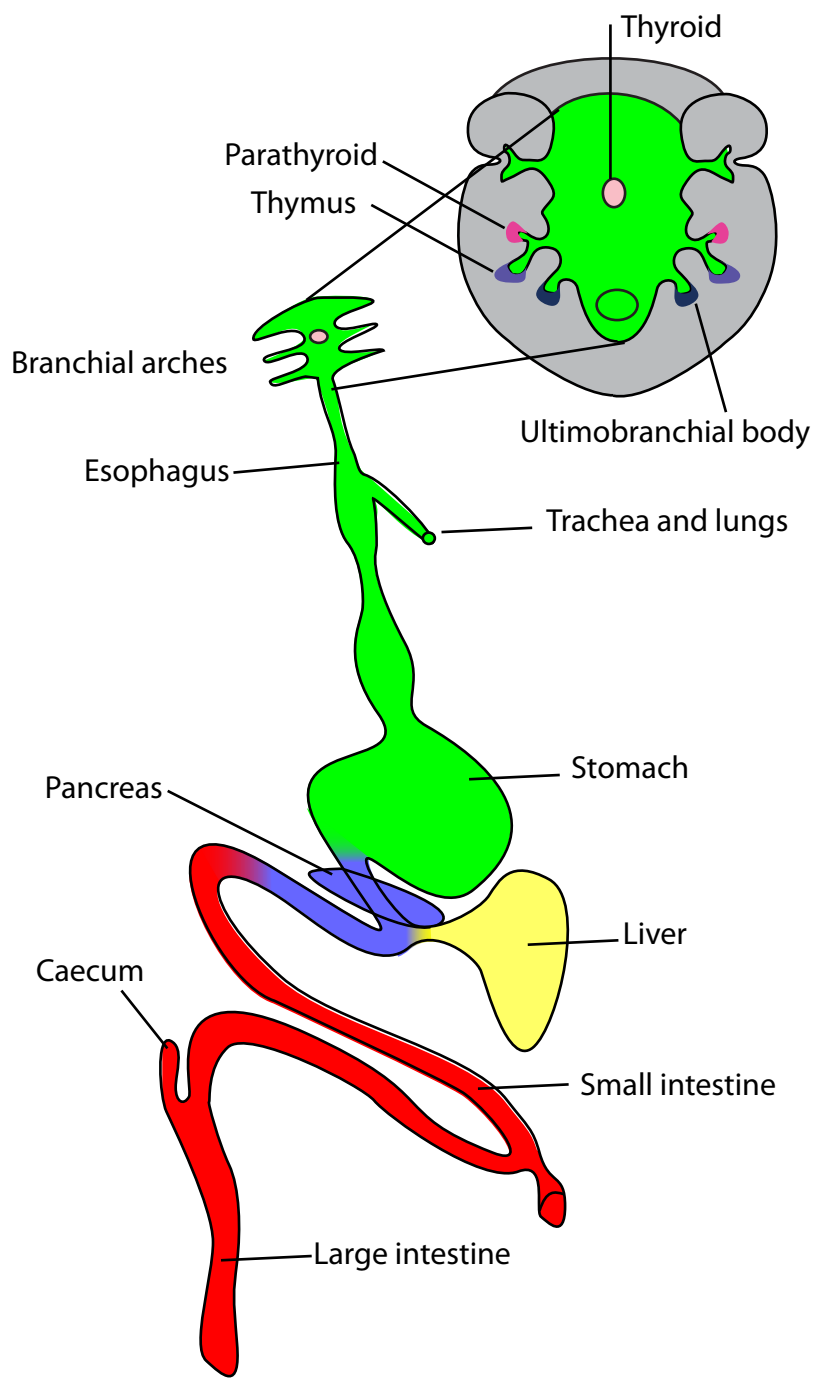
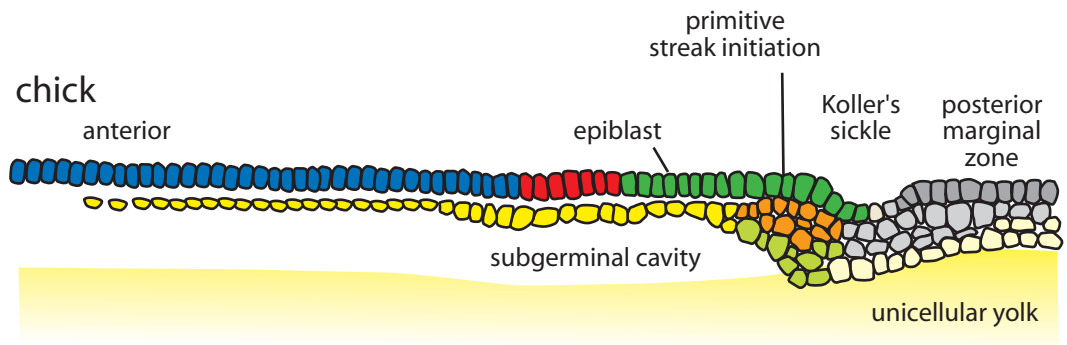
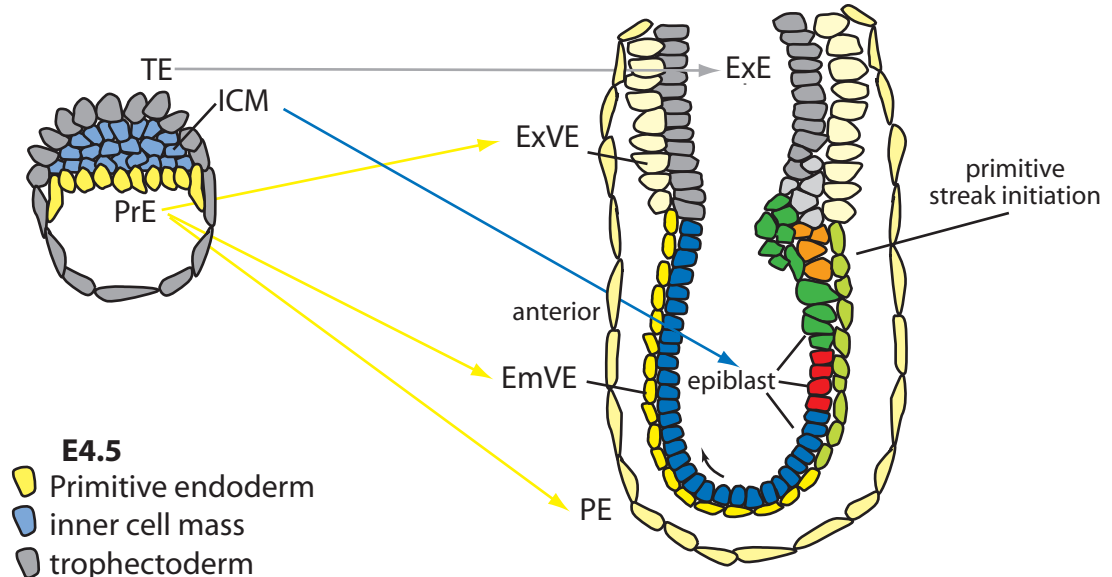





Figure 1



Mouse







**E4.5**

-  Primitive endoderm
-  inner cell mass
-  trophoctoderm

**E6**

**Epiblast**

-  future definitive endoderm
-  future prechordal plate
-  future mesoderm
-  future ectoderm

**Extraembryonic**







-  hypoblast (c), anterior visceral endoderm (m)
-  endoblast (c), posterior visceral endoderm (m)
-  extraembryonic visceral endoderm (m)
-  yolk (c), parietal endoderm (m)
-  extraembryonic mesoderm (c?, m)
-  extraembryonic ectoderm (ExE)

Figure 2

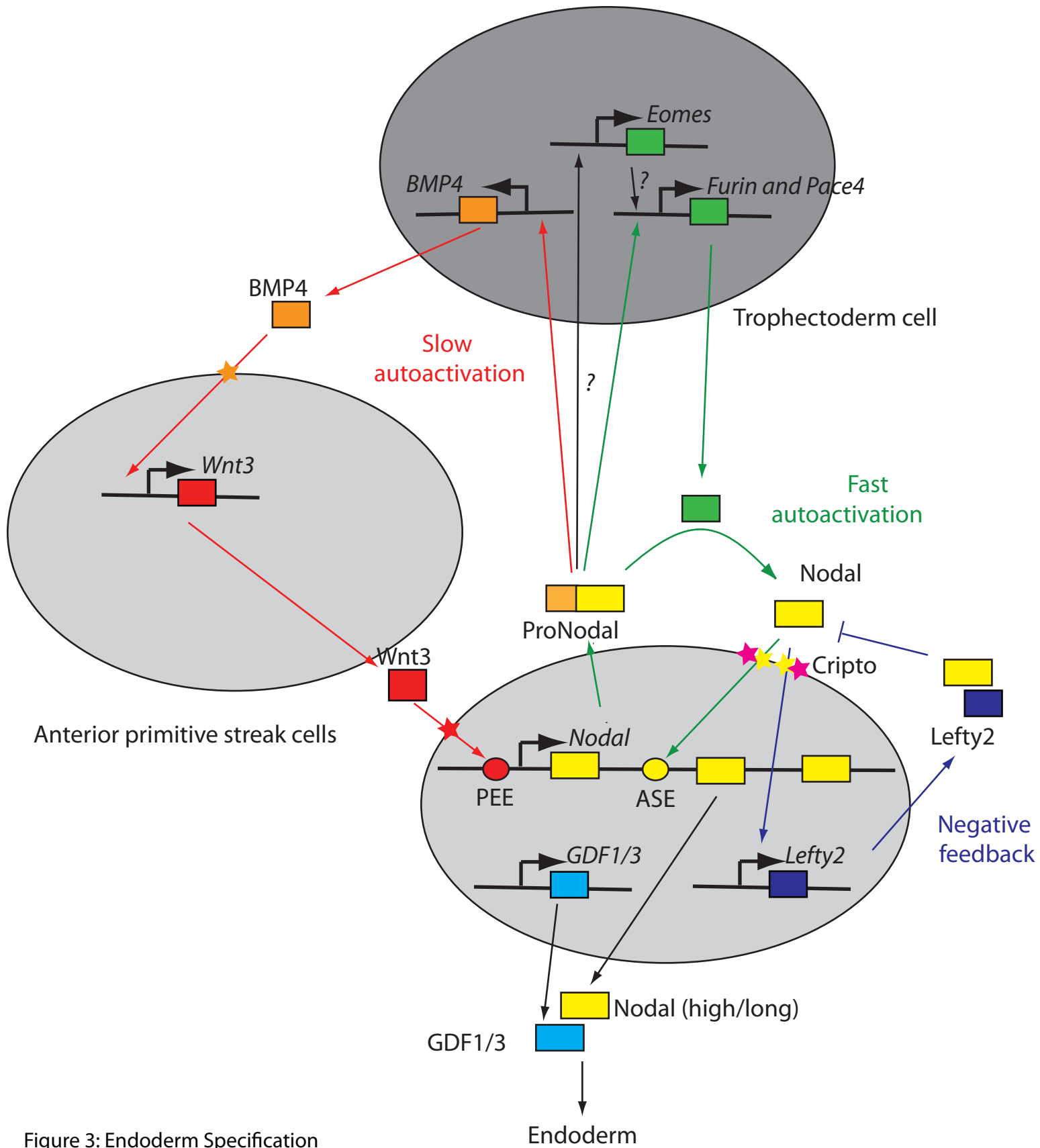


Figure 3: Endoderm Specification

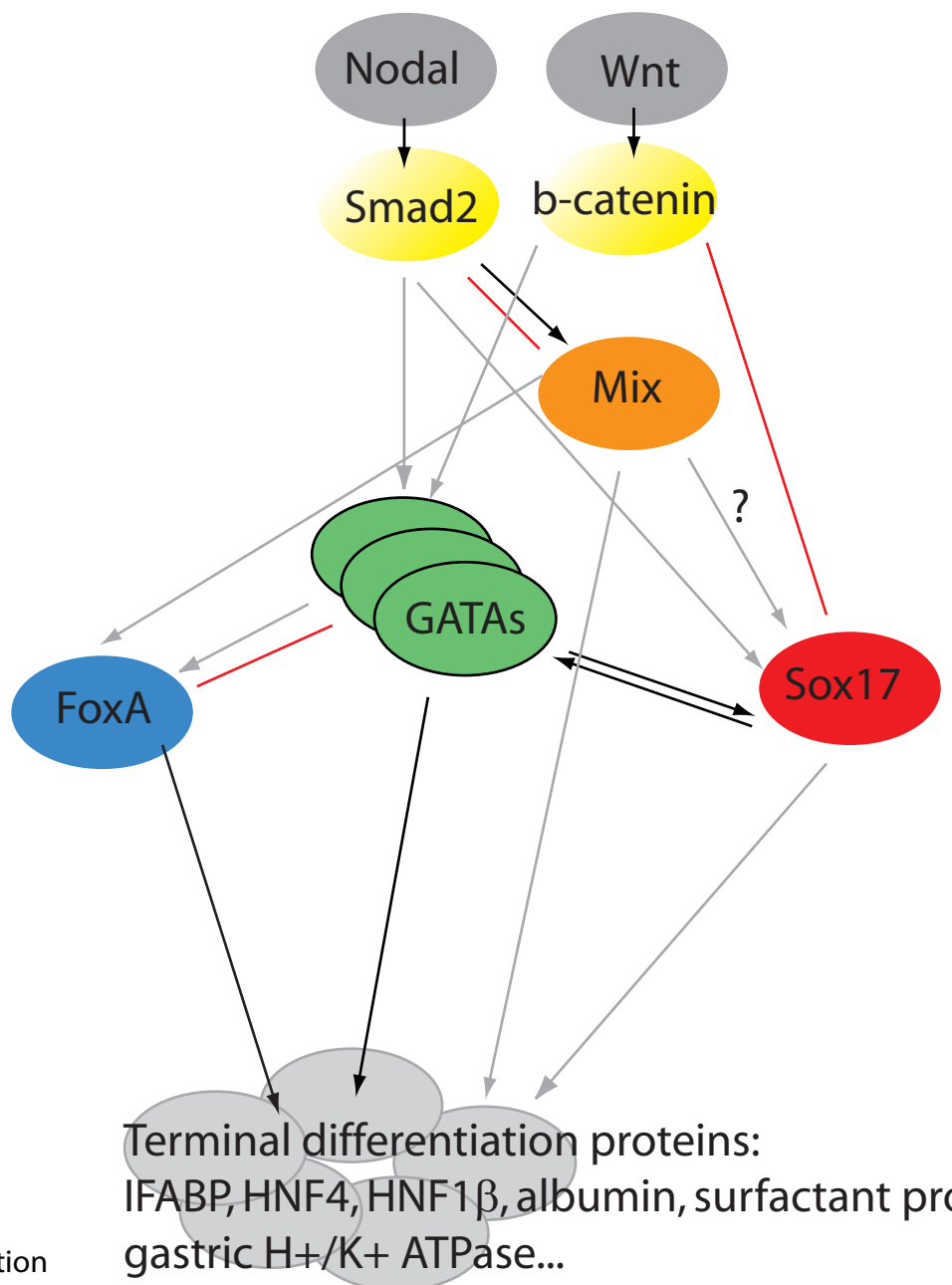


Figure 4: Endoderm Specification

Wnt(3a, 8c?) from primitive streak

Retinoic acid From mesoderm

Fgf(4?) From primitive streak

Anterior/rostral

Posterior/caudal

<p>Dorsal</p> <p>Hlxb9</p> <p>?</p> <p>Klf5</p> <p>Ventral</p>	Branchial pouch 1/2	Branchial pouch 3	Branchial pouch 4	Esophagus	Stomach	Pancreas	Posterior small intestine	Colon
	Branchial pouch 1/2	Parathyroid Gcm2	Branchial pouch 4	Esophagus	Stomach	Duodenum	Posterior small intestine	Colon
	Branchial pouch 1/2	Thymus Foxn1	Ultimobranchial body	Trachea/ Lungs Nkx2.1	Stomach	Pancreas	Posterior small intestine	Caecum
	Thyroid Nkx2.1 Hex	Branchial pouch 3	Branchial pouch 4	Esophagus	Stomach	Liver Dp111 Hex	Posterior small intestine	Colon

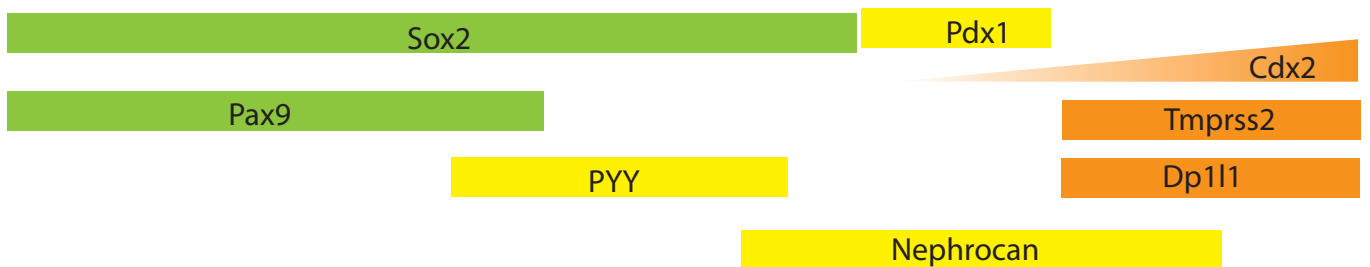


Figure 5: Endoderm Specification