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Review

Notch signaling in the division of germ layers in bilaterian embryos

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ABSTRACT

Bilaterian embryos are triploblastic organisms which develop three complete germ layers (ectoderm, mesoderm, and endoderm). While the ectoderm develops mainly from the animal hemisphere, there is diversity in the location from where the endoderm and the mesoderm arise in relation to the animal-vegetal axis, ranging from endoderm being specified between the ectoderm and mesoderm in echinoderms, and the mesoderm being specified between the ectoderm and the endoderm in vertebrates. A common feature is that part of the mesoderm segregates from an ancient bipotential endomesodermal domain. The process of segregation is noisy during the initial steps but it is gradually refined. In this review, we discuss the role of the Notch pathway in the establishment and refinement of boundaries between germ layers in bilaterians, with special focus on its interaction with the Wnt/β-catenin pathway.

1. Introduction

One of the first steps in the diversification of pluripotent cells in metazoans is the formation of the germ layers, from which all tissues and organs derive. Germ layers undergo segregation during gastrulation, a process that drives the internalization of cells by a diversity of morphogenetic movements throughout the animal kingdom (Keller et al., 2003; Stower and Bertocchini, 2017), leading to the development of two-layered (diploblastic) or three-layered (triploblastic) embryos (Martindale, 2005; Martindale and Hejnol, 2009). Triploblastic organisms comprise all bilaterian embryos. They develop a distinct mesodermal layer between the ectoderm and the endoderm (Martindale, 2005; Martindale and Hejnol, 2009). In vertebrates, the ectoderm gives rise to the central nervous system, the epidermis, and to all the neural crests derivatives, including the peripheric nervous system and facial bones. The notochord, axial skeleton, connective tissue, trunk muscles, kidneys, and the cardiovascular system descend from the mesoderm. The epithelium of the gastrointestinal and respiratory tracts, and several endocrine glands develop from the endoderm (Gilbert, 2014).

An important feature is the diversity in the location from where germ layers develop in relation to the egg's primary animal-vegetal axis. While in hemichordates and echinoderms, the mesoderm arises at the vegetal pole, in vertebrates, the mesoderm arises at the equatorial region (Martindale, 2005), and cephalochordates show other important differences (see below) (Holland and Holland, 2007).

Studies of germ layer formation in bilaterian animal models including nematodes, echinoderms, and vertebrates indicate that the mesoderm derives from two sources. One portion arises from a bipotential and ancient endomesodermal precursor domain, which initially has the potential to develop as either endoderm or mesoderm, and later divides into separate layers by the activation of their respective specification programs in exclusive subpopulation of cells. The second portion of mesoderm (which mostly gives rise to muscles in nematodes, to skeletogenic mesenchyme in euechinoid sea urchins, and to somitic mesoderm in vertebrates) does not share a common origin with the endodermal layer (Wray, 1999; Logan and McClay, 1999; Kimelman and Griffin, 2000; Rodaway and Patient, 2001; Oliveri et al., 2002; Peter and Davidson, 2010).

The induction and specification of germ layers have been thoroughly investigated (Arnold and Robertson, 2009; Zorn and Wells, 2009; Kiecker et al., 2016; Charney et al., 2017). Less is known about how the boundaries among them are established and refined during their segregation, which ultimately will determine the correct proportion and location of cells that at last will populate each layer. In this context, several key processes are not completely understood, for example, how signals are modulated in the transition zone between germ layers with such fine tuning that two adjacent cells (which could even be sisters) adopt different fates. This process of segregation is significantly noisy during the initial steps but it is gradually refined, as was elegantly demonstrated by single cell RT-PCR analysis in amphibian embryos (Wardle and Smith, 2004). These authors showed that individual cells at the marginal zone of the early gastrula may express markers of two or even the three germ layers, but become progressively and asynchronously committed to one layer during gastrulation.

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Moreover, lineage potency is not irreversibly restricted upon germ layer formation, as demonstrated by numerous heterotopic transplant experiments of cells fated to one germ layer into another germ layer, which adopted the fate of the surrounding tissue in gastrulating mouse embryos (Tam and Gad, 2004).

The establishment of the body plan depends on a delicately orchestrated series of molecular interactions which rely on the relative positions of cells, in such a way that their allocation into the different germ layers will have influence on the inductive signals that cells will encounter on their path and which will initiate developmental programs that lead to the differentiation of specific cell types. Anomalies in the formation and segregation of germ layers, with the resulting disturbance of cell migration and inductive interactions between tissues during organogenesis may eventually cause developmental defects with a final outcome which, depending on the severity, will vary from congenital malformations and pathologies to embryonic lethality (Herion et al., 2014).

The potential to differentiate embryonic stem cells (ESc) into any cell type is still a promise for regenerative medicine. A deep comprehension of the mechanisms underlying tissues formation during the development of an organism would help to recapitulate the mechanisms during in vitro differentiation. To fulfill rational protocols, differentiation into the desired cell type should occur at the right moment and in the right place. Since the formation of germ layers is the first step in the differentiation of all embryonic tissues, research in the gene regulatory networks (GRN) involved in the different phases in this process (induction, specification, segregation) is important to unravel how they can be employed in a directional way with therapeutic purposes. Since several of these concepts arose from in vivo studies in model organisms, it is important to understand similarities and differences between these programs among the different models and their evolutive implications.

In this review, we will focus on the role of the Notch pathway in the establishment and refinement of tissue boundaries during the segregation of germ layers in some representative bilaterians, paying special attention to its interaction with the Wnt/β-catenin pathway. The models discussed are: *Caenorhabditis elegans* (nematodes), sea urchins and sea stars (echinoderms), amphioxus (cephalochordates), zebrafish *Danio rerio* (teleosts), *Xenopus* (amphibians), chick embryos (birds); mouse embryos, and human and mouse ESc (mammals).

2. The Notch pathway

Canonical Notch signaling is activated through the interaction of receptors and ligands expressed by two neighboring cells. The receiving cell expresses the transmembrane receptor Notch, while the sending cell presents the transmembrane ligand belonging to the DSL (Delta, Serrate, Lag-2) family. According to their structural similarity to the two *Drosophila* members Delta and Serrate, ligands are classified into two categories: Delta like (Dll1, Dll3 and Dll4 in mammals) and Jagged (Jag1 and Jag2 in mammals), respectively (Lai, 2004). A key component in canonical Notch signaling is the sequence-specific DNA-binding protein CSL (from the names in vertebrates, fly, and worm: CBF1, Su (H), and Lag-1, respectively; also known as RBPJ). In the absence of Notch signaling, a complex containing CSL and co-repressors is present in the enhancers of Notch target genes, silencing them by recruiting histone deacetylases or other modifying enzymes. Upon ligand binding, the mature Notch receptor undergoes two successive proteolytic cleavages, the last one catalyzed by the γ-secretase intramembrane protease complex. This cleavage releases the intracellular Notch domain (NICD), which is the active Notch form in the canonical pathway and signals without further amplification. Once released, NICD enters the cell nucleus and forms a complex with the DNA binding protein CSL, which recruits the co-activator Mastermind-like (MAML). This trans-activating complex is thought to displace the CSL repressor complex that was bound to the enhancers of Notch target genes, and in this way, activates their transcription (Bray, 2016).

Classical Notch transcriptional targets belong to the bHLH genes of the HES/HEY families, which encode sequence-specific DNA binding proteins that function as transcriptional repressors of their target genes, mostly by recruiting transcriptional corepressors of the Groucho family through their conserved C-terminal tetrapeptide motif WRPW/Y. However, the number of direct Notch/CSL targets that do not belong to the HES/HEY families is constantly growing, and includes genes involved in diverse processes such as proliferation, apoptosis, cell fate choice, signaling pathways, metabolism and cytoskeletal regulators (Bray and Bernard, 2010; Meier-Stiegen et al., 2010). In this review, we will see some examples of these non HES/HEY targets operating in GRN controlling early cell fate choices.

The DSL/Notch canonical pathway is used iteratively during development in two main ways: 1) In lateral inhibition, Notch executes binary cell-fate choices in populations of equal developmental potential. In this mode, the sending cell signals to its neighbors to repress ligand expression and the fate of the sending cell, concomitantly instructing the receiving cells to adopt a different, alternative fate. This mode of action usually results in a “salt and pepper” pattern of cell specification, with more or less regular spacing between specific cells types within a field (Gazave et al., 2009; Sjöqvist and Andersson, 2017). 2) In lateral induction, the sending cell generally induces the expression of the ligand in its neighbors and instructs them to adopt the same fate (Sjöqvist and Andersson, 2017). In this mode, boundary formation sometimes involves signaling between two cells populations which results in the adoption of a third fate at their border, establishing a boundary within the field (Gazave et al., 2009).

There are numerous examples of lateral inhibition processes in vertebrates and invertebrates by which Notch restricts cell fates in a group of initially equipotent cells, preventing that all adopt the same fate. The classic paradigm is the neural-epidermal choice in *Drosophila*. In the proneural clusters, all cells express proneural proteins, which are basic helix-loop-helix (bHLH) transcriptional activators that confer these cells equal competence to become neural progenitors. However, only one cell in the proneural cluster will differentiate as neuron, as it signals through Delta to the neighboring cells. These, in turn, activate the Notch cascade, which represses proneural genes through the action of transcriptional repressors of the HES/HEY family. Consequently, cells surrounding the differentiating neuroblast are inhibited to adopt the neural fate and become, instead, epidermal precursors. Notch-mediated lateral inhibition also operates in binary cell-fate choices in several processes in metazoans, including the repression of neurogenesis and myogenesis in vertebrates, among other examples (reviewed in Bray, 1998; Lewis, 1998; Lai, 2004; Sjöqvist and Andersson, 2017).

A classic example of lateral induction is the establishment of the boundary between the dorsal and ventral compartments of the *Drosophila* wing. During this process, Delta and Serrate become preferentially expressed along the dorsal-ventral border of the wing primordium, with Serrate signaling from the dorsal to the ventral cells, and Delta signaling from the ventral to the dorsal cells. This results in Notch activation at the interface between the dorsal and the ventral fields, with Delta and Serrate reciprocally inducing their expression across the border by a positive feedback loop through Notch activation. In this way, Notch activity establishes an organizing center which coordinates growth and patterning of the wing and prevents intermixing between the dorsal and ventral cell populations (Irvine and Vogt, 1997; Bray, 1998; Irvine et al., 1999). Since a positive outcome results from Notch activation at the boundary (in this case, the induction of positive regulatory molecules with the acquisition of organizer properties), this kind of Notch signaling was termed as inductive (in opposition to lateral inhibition) (Bray, 1998; Lai, 2004). Lateral induction also takes place in the development of multilayered vascular smooth muscle in mammals, which involves direct up-regulation of Jag1 by Notch in a positive feedback wave that propagates smooth muscle differentiation throughout the mesenchyme surrounding the endothelial cells

(Manderfield et al., 2012). Interestingly, lateral induction and lateral inhibition can operate sequentially. A fine example is the development of the inner ear in vertebrates. In this system, Notch and Jag1 are initially involved in a positive feedback loop that amplifies the same cell fate, promoting the development of prosensory patches by lateral induction. Later, Notch-dependent lateral inhibition controls the generation of neurons and the mosaic pattern of hair and supporting cells of sensory organs (Neves et al., 2013). In addition, regardless of the regulation of DSL ligands expression by Notch (whether known or not), some authors regarded as lateral induction certain processes in which DSL/Notch induces contiguous domains of cells with the same fate, as opposed to the mosaic pattern generated by lateral inhibition. Examples of these processes are the induction of the neural crest domain at the boundary between the ectoderm and the neural plate in frogs and the establishment of somite boundaries in vertebrates (reviewed in Lewis, 1998; Cornell and Eisen, 2005). However, in the strict sense of how Notch regulates DSL ligands, the most recent models of somitogenesis propose that Notch down-regulates Delta expression cell-autonomously (which is what would be expected from a lateral inhibition mechanism), but synchronizes the expression of oscillating genes like Delta itself between neighboring cells by a complex, still incompletely understood system of feedback circuits through Delta/Notch intercellular communication that operates in the segmentation clock (Oates et al., 2012; Shimojo and Kageyama, 2016). Interestingly, oscillating but non-synchronous Delta/Notch dynamics operates in neural stem cells (Shimojo and Kageyama, 2016). Thus, simplistic models of lateral inhibition and lateral induction sometimes are not sufficient to thoroughly understand the complex mechanisms of cell fate decisions and boundary formation processes controlled by Notch.

From a phylogenetic survey of the core components of the Notch pathway and auxiliary modulating factors through 8 major eukaryotic clades, it was concluded that Notch signaling emerged in metazoans through the evolutionary acquisition of novel, metazoan-specific proteins, such as the Notch receptors and the DSL ligands, and the co-option of pre-metazoan, eukaryote proteins (Gazave et al., 2009). The Notch receptor is encoded by a single gene in most species, except in vertebrates, which contain from 2 to 4 Notch genes. This is due to the well-known two events of whole genome duplication (WGD) that occurred in this lineage, before the divergence of teleosts and tetrapods (Theodosiou et al., 2009; Gazave et al., 2009). The Notch2 group appeared in the first round of WGD, the Notch3 group is phylogenetically closer to Notch2, and the Notch4 group is only present in mammals, showing greater divergence from the other three Notch genes.

There is growing evidence that the core components of the Notch pathway described above are involved in several non-canonical functions. Depending on the cell context, Notch target genes can also be activated by NICD-dependent, CSL-independent mechanisms, or by NICD-independent mechanisms which can or cannot be mediated by CSL (Sanalkumar et al., 2010; Tanigaki and Honjo, 2010). In addition, ligand- and transcription/CSL-independent Notch activities have been reported both in vertebrates and invertebrates, mainly involving an antagonistic role on the Wnt/β-catenin pathway (Wnt/β-cat) (Hayward et al., 2005; Hayward et al., 2008; Sanders et al., 2009; Muñoz-Descalzo et al., 2010; Acosta et al., 2011; Kwon et al., 2011). In this pathway, membrane-bound Notch titrates transcriptionally active, hypophosphorylated β-cat through a physical, post-translational interaction that leads to its lysosomal degradation (reviewed in Andersen et al., 2012). In this way, Notch lowers the levels of transcriptionally active nuclear β-cat (nβ-cat), which, complexed to the TCF DNA binding protein, is the effector of canonical Wnt signaling. Another non-nuclear mechanism involving Notch, and which is independent of cleavage by γ-secretase and CSL, is related with the cytoplasmic tyrosine kinase Abl in axon growing in *Drosophila* (Heitzler, 2010). More non-canonical Notch functions were described, including interactions with non-DSL ligands and with other nuclear partners and signaling pathways (Wnt, BMP, F-kB, etc.) (D'Souza et al., 2010; Heitzler, 2010). Strikingly, it was recently proposed that non-canonical Notch signaling is the ancestral mechanism for regulating cell differentiation in metazoans, while the canonical pathway is more recent in evolutive terms, appearing in bilaterians (Layden and Martindale, 2014).

While canonical Notch signaling is the most studied during the development of germ layers, little is known about the possible role of non-canonical Notch pathways in this process. Experiments employing dominant negative CSL (CSL^{neg}) or γ-secretase inhibitors are useful to drive general conclusions about the role of canonical Notch signaling, without distinguishing between Notch paralogues. To arrive to more specific conclusions for each paralogue, antisense morpholino (MO) knock-down approaches or knock-out strategies are needed. Although specific for each paralogue, these tools impair both canonical and non-canonical functions. Thus, to dissect more precisely the role of Notch signaling in any biological context, a combination of approaches are necessary.

3. Notch and nβ-cat signaling in germ layers specification in nematodes

C. elegans contains two Notch genes (*glp-1* and *lin-12*) which arose through an independent duplication event (Theodosiou et al., 2009). Both are functionally redundant in some cell fate decisions, where *glp-1* can be substituted by *lin-12* (Greenwald et al., 1983; Austin and Kimble, 1989; Evans et al., 1994). However, *glp-1* was described to be involved during early development (Evans et al., 1994), while *lin-12* participates at later stages (Moskowitz and Rothman, 1996). Two Delta orthologues were described, *apx-1* and *lag-2* (Mello et al., 1994; Henderson et al., 1994), whereas *lag-1* corresponds to CSL (Lambie and Kimble, 1991; Christensen et al., 1996). The first division in the *C. elegans* embryo produces two cells, known as AB and P1. P1 is then asymmetrically divided along the anterior/posterior axis, giving rise to two daughter cells, EMS (endomesoderm precursor) and P2 (Fig. 1). On the other hand, AB is divided along the transverse axis and generates one anterior (ABa) and one posterior (ABp) daughter (Fig. 1). ABa descendants are mesoderm (pharynx) and ectoderm precursors, whereas ABp descendants are only fated to become ectoderm (Sulston et al., 1983). Both ABa and ABp express maternal Notch (*glp-1*) (green outline, Fig. 1), while P2 expresses maternal Delta (*apx-1*) (cyan outline, Fig. 1). The division of the EMS cell generates an anterior daughter called MS (a predominantly mesodermal precursor) and a posterior one called E (the only source of endodermal progenitors) (Evans et al., 1994) (Fig. 1).

A first Delta/Notch interaction takes place in the 4-cell stage embryo between P2 and ABp (cyan arrow, Fig. 1), resulting in Notch activation in ABp, leading its descendants to adopt an ectodermal fate (Priess et al., 1987). Although ABp contacts MS, ABp descendants are not normally under regulation by MS signaling. However, when this first Notch interaction is blocked by either physically removing P2 or by Delta (*apx-1*) mutations, MS induces these ABp descendants, changing their fate from ectodermal to mesodermal (Mello et al., 1994; Moskowitz et al., 1994). In this situation, the expression of *tbx-37* and *tbx-38* (T-box transcription factors) in the ABp descendants increases, indicating that normally, the P2 signal inhibits the mesodermal fate. Therefore, by repressing *tbx-37* and *tbx-38* expression, the first Delta/Notch interaction prevents the ABp descendants from adopting the same cell fate as the ABa descendants (Good, 2004). Thus, the first activation of Notch promotes ectodermal fates (blue) and inhibits mesodermal fates (red) (Fig. 1).

A second Notch interaction occurs at the 12-cell stage (cyan arrow, Fig. 1). All ABa descendants express Notch (*glp-1*) (green outline in Fig. 1), but this second interaction takes place between MS and only two ABa descendants (ABalp and ABara) due to their specific orientation. MS expresses Delta (*lag-2*) (cyan outline, Fig. 1), which activates Notch in these ABa granddaughters, inducing them to adopt a mesodermal fate (red) instead of the ectodermal fate of their sisters (blue) (Fig. 1) (Good, 2004; Moskowitz et al., 1994).

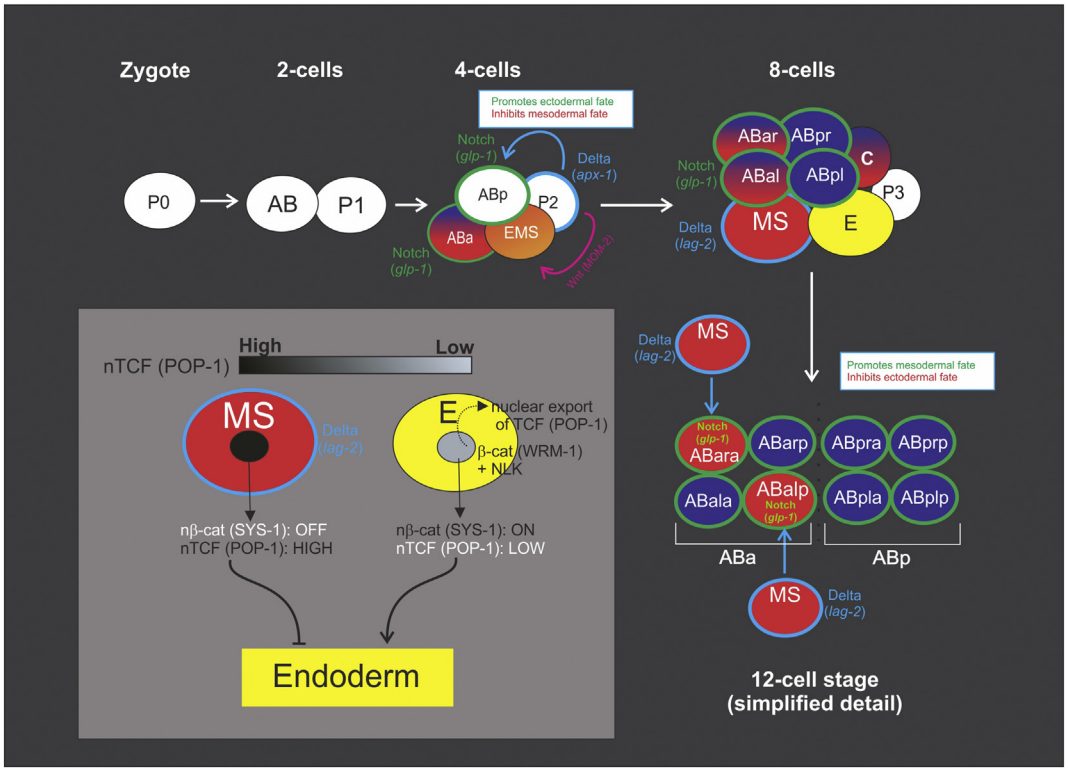

Fig. 1. Cell and molecular interactions in the subdivision of germ layers in *C. elegans*. Role of Notch signaling (main panel). Role of Wnt/nβ-cat signaling (inset). The green outline in cells indicates Notch expression. The cyan outline indicates Delta expression. See main text for explanation and references.

Simultaneously with the first Notch interaction at the 4-cell stage, several genes belonging to the Wnt/β-cat pathway are involved in an interaction between P2 and EMS (Fig. 1, magenta arrow and inset). This pathway controls endomesoderm segregation by regulating the asymmetric cell division of EMS and deciding the fates of its descendants. At the 4-cell stage, TCF (POP-1) is localized in the EMS nucleus, while this cell is being polarized by Wnt (Mom2) secreted by the neighboring P2 (magenta arrow, Fig. 1). When the subsequent cell division occurs (Fig. 1, inset), this polarized Wnt signal results in nuclear accumulation of a divergent β-cat (SYS-1) and concomitant nuclear export of TCF in the E daughter in which intervenes another atypical β-cat like protein (WRM-1) and a Nemo-like kinase (NLK). Active Wnt/β-cat signaling thus promotes endodermal specification (yellow, Fig. 1). Meanwhile, the MS daughter, which does not receive the Wnt signal, maintains high nuclear levels of TCF, which represses the endodermal GRN, thus promoting the mesodermal fate during the 8-cell stage (Thorpe et al., 1997; Rocheleau et al., 1997; Korswagen, 2002; Lo et al., 2004; Shetty et al., 2005; Maduro, 2009; Phillips and Kimble, 2009). Therefore, in the nematode, Wnt/β-cat segregates the endomesoderm, favoring endodermal fates.

4. Notch and nβ-cat signaling in the segregation of germ layers in echinoderms

Echinoderms comprise five extant classes, among which two will be discussed here: the class Echinoidea (the sea urchins, with two subclasses: Euechinodea and Cidaroidea), and the class Asterozoa (with two subclasses: Asteroidea or sea stars, and Ophiuroidea or brittle stars) (Cary and Hinman, 2017). The subclass Euechinodea encompasses four orders, including the order Camarodonta, which is the best studied (Minokawa, 2017).

4.1. Subclass Euechinodea, order Camarodonta

The segregation of germ layers has been studied with exquisite detail in some species of euechinoids from the order Camarodonta such as *Strongylocentrotus purpuratus* and *Lytechinus variegatus*. There is a complex interplay between Notch and Wnt/β-catenin pathways in this process, as we will see in more detail below. In these sea urchins, the ectoderm arises from the animal hemisphere and the veg1 tier; the endoderm, from the veg1 and the veg2 tiers. The mesoderm derives from three sources: 1) The skeletogenic mesoderm (SKM) descends from the large micromeres and gives rise to the larval skeleton. It is formed by the primary mesenchyme cells (PMCs), which are the first to internalize into the blastocoel. They do it from the vegetal pole, by delamination and ingression. 2) The non-skeletogenic mesoderm (NSKM) derives from the veg2 tier and gives rise to the pigment cells, immunocytes, muscle and part of the coelomic walls. It is formed by the secondary mesenchyme cells (SMCs), which internalize a few hours later than the SKM, by invagination of the vegetal plate. SMCs lead the archenteron formation, with the endodermal cells behind. Their segregation from endomesoderm depends on Delta/Notch signaling. 3) The larval coelomic pouches and the adult mesoderm derive from the small micromeres (Davidson et al., 1998; Davidson et al., 1998; Ransick and Davidson, 1998; Wray, 1999; Logan and McClay, 1999), and mesodermal specification of the small micromeres depends on Delta/Notch signaling. Thus, part of the ectoderm and part of the endoderm segregate from veg1 tier descendants. The veg2 tier is initially specified as endomesoderm, from which part of the mesoderm (the NSKM) and part of the endoderm later segregate (see below) (Fig. 2A).

All major components of the Notch signaling pathway (including the ligands Delta and Jagged, the effector CSL, *hes* genes, and modulators like γ-secretase, *fringe*, *numb*, and *deltex* were identified in the euechinoids genome (Walton et al., 2006). Notch protein is uniformly distributed on the cell surfaces at the 60-cells stage, but it is down-regulated in the vegetal plate of the blastula. From the mid-

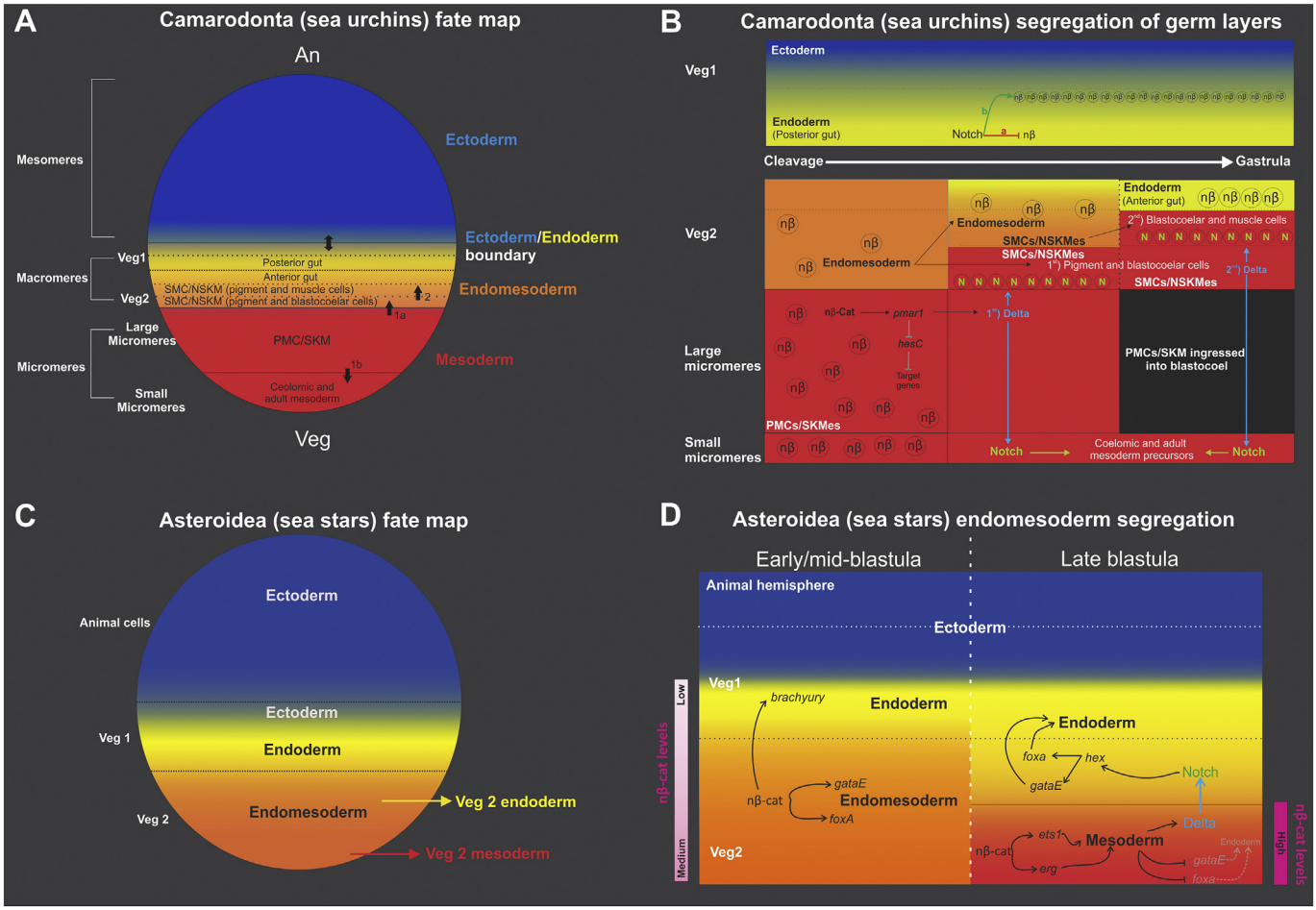

Fig. 2. Segregation of germ layers in Echinoderms. (A, B) Camarodonta sea urchins. (C, D) Asteroidea (sea stars). (A) In Camarodonta sea urchins, the 60-cells embryo is divided into the animal An1 and An2 cell tiers (which result from the division of mesomeres), whereas the vegetal hemisphere is divided (from the equator of the embryo to the vegetal pole) into veg1 and veg2 cells tiers (which derive from the macromeres), a large micromeres tier, and a cluster of small micromeres at the vegetal tip. The ectoderm (blue) arises from the animal hemisphere and the veg1 tier; the endoderm (yellow), from the veg1 tier (posterior gut) and the veg2 tier (anterior gut), and the mesoderm (red), from the veg2 tier and the micromeres. PMC/SKM: primary mesenchyme cells/skeletogenic mesoderm. SMC/NSKM: secondary mesenchyme cells/non-skeletogenic mesoderm. The small micromeres contribute to the larval coelomic pouches and to the adult mesoderm. (B) Role of Notch and nβ-cat in establishing the ectoderm/endoderm boundary (upper panel) and in mesoderm specification and endomesoderm segregation (lower panel) as development progresses from cleavage to gastrulation. nβ, nuclear β-cat; N, active Notch signaling. Notch positions the ectoderm/endoderm boundary by inhibiting nuclear localization of β-cat cell-autonomously in the vegetal cells, but enhancing it non-cell autonomously in more animal positions (upper panel). The lower panel shows that nβ-cat/TCF signaling exclusively activates the expression of the homeodomain transcription factor *pmar1* in the micromeres. *HesC* is a ubiquitous repressor, but Pmar1 represses it, thus de-repressing a set of genes that in this way become exclusively expressed in the micromeres (but are repressed elsewhere by HesC). This device is known as the "double negative gate", which is exclusive for Camarodonta sea urchins. Some of the de-repressed genes initiate the specification program of the SKM (Oliveri et al., 2008). In addition, Pmar1 activates two intercellular signals that emanate from the micromere lineage. 1) The earlier signal is still unknown and signals to the adjacent precursors of the veg2 cells between the fourth and sixth cleavages. 2) The later one, emitted by the large micromeres, corresponds to the first wave of Delta signaling (1st Delta), which activates Notch in the adjacent veg2 lineage between the seventh and ninth cleavage to induce a first subset of NSKM precursors (1st pigment and blastocoelar cells) and also, to the small micromeres to induce coelomic and adult mesoderm precursors. The second wave of Delta (2nd Delta) emanates from the first subset of specified NSKM to activate Notch in neighboring veg2 descendants and further segregate more SKM from the veg2 endomesoderm (2nd blastocoelar and muscle cells). As PMC have ingressed into the blastocoel (black box), the first subset of specified NSKM is brought in contact with the small micromeres population, in such way that the 2nd Delta also maintains the induction of coelomic and adult mesoderm precursors. (C) The sea star embryo lacks micromeres and neither develops SKM nor embryonic pigment cells. Ectoderm (blue) arises mostly from the animal cells, with some contribution from the veg1 tier. Veg1 endoderm (yellow). Endomesoderm (orange) segregates into veg2 endoderm (yellow) and veg2 mesoderm (red) as shown in (D), as a result of opposite actions of nβ-cat and Notch signaling. At late blastula, nβ-cat triggers a circuit that represses endoderm specification in the mesoderm domain (represented as a turned-off circuit by transparent grey), while Notch turns-on the circuit for endoderm specification in the endoderm subdomain. See main text for further explanation and references. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mesenchyme blastula stage (when ingression of PMCs is taking place) and during gastrulation, Notch protein accumulates in the apical membrane of presumptive and invaginated endodermal cells, but not on the apical membrane of presumptive and invaginated SMCs, which contain, instead, intracellular vesicles carrying Notch, suggesting that the pathway is active in these cells (see below). Low levels of Notch protein are present throughout the presumptive ectoderm (Sherwood and McClay, 1997; Sherwood and McClay, 1999).

The Notch pathway is not involved in SKM specification (Sherwood and McClay, 2001). This fate is autonomously specified by maternal nβ-cat, which accumulates in the four large micromeres from their time of birth (Fig. 2B). During cleavage, maternal nβ-cat continues accumulating in a graded fashion and in a cell-autonomous way throughout the vegetal hemisphere, with the highest levels in the small micromeres and the lowest ones in the veg2 cells (Davidson et al., 1998; Logan et al., 1999). This accumulation of nβ-cat is essential for mesoderm and

endoderm development, since antagonizing nβ-cat/TCF signaling gave rise to embryos consisting of a hollow ball of ectoderm, devoid of endoderm and all mesodermal types (see Davidson et al., 2002 and references therein). By perturbing different key regulatory genes with a number of strategies and compiling the data available in the literature, Davidson et al. began to construct a GRN of endomesoderm specification in these euechinoid embryos (Davidson et al., 2002; Davidson, 2002), which is permanently being updated in http://www.echinobase.org/endomes/.

From this effort, we now know that nβ-cat/TCF signaling initiates the specification program of the SKM (Oliveri et al., 2008) and indirectly activates in a cell-autonomous fashion two intercellular signals that emanate from the micromere lineage. 1) The earlier one is still unknown and signals to the adjacent precursors of the veg2 cells between the fourth and sixth cleavages. 2) The later one, emitted by the large micromeres, is Delta. It signals to the adjacent veg2 lineage between the seventh and ninth cleavage to induce a first subset of NSKM precursors (black arrow 1a in Fig. 2A) (see below) (Oliveri et al., 2002; Davidson, 2002; Davidson et al., 2002; Revilla-i-Domingo et al., 2004; Revilla-i-Domingo et al., 2007; Yaguchi et al., 2008; Oliveri et al., 2008; Sethi et al., 2009). This Delta signal also initiates mesoderm specification in the neighboring small micromeres at the vegetal pole (black arrow 1b in Fig. 2A), by canonically activating the direct Notch/CSL target *foxY*, which is critical for the development of their derivatives. Mesoderm specification of this lineage requires continuous Delta signaling, first by the large micromeres and later, by specified NSKM precursors (Fig. 2B) (Materna and Davidson, 2012; Materna et al., 2013).

Notably, nβ-cat begins to disappear stochastically and in a progressive way from the PMCs and the veg2 lineage after the midblastula stage, in such way that their descendants completely lack nβ-cat after being internalized. At late blastula, however, a subset of veg1 descendants begins to accumulate nβ-cat in presumptive endoderm cells bordering the presumptive ectoderm (Logan et al., 1999) (Fig. 2B). While the endodermal GRN of the veg2 descendants depends on the early accumulation of nβ-cat at cleavage stages, the specification of endoderm in veg1 descendants begins later, as a consequence of this second wave of nβ-cat accumulation (Davidson et al., 2002), which is related with the segregation of endoderm from ectoderm (see below).

4.1.1. Segregation between endoderm and mesoderm from veg2 endomesoderm

Sherwood and McClay first addressed whether Notch could control the boundary between endoderm and mesoderm, since the veg2 tier gives rise to both germ layers. By activating and blocking the pathway with a constitutively active form of the receptor (N^{act}, the intracellular Notch domain) and a dominant negative form (N^{neg}, the extracellular Notch domain), they found that N^{act} increased the number of SMCs at the expense of the endodermal precursors during gastrulation, whereas N^{neg} produced the opposite results. This suggested that initially, Notch is involved in establishing the mesoderm/endoderm boundary by promoting the SMCs fate at the expense of the endoderm (Sherwood and McClay, 2001). Thus, Notch autonomously specifies the mesodermal SMCs within the endomesodermal descendants of the veg2 tier. In addition, the authors mentioned unpublished observations that N^{act} was unable to induce mesodermal derivatives in isolated animal caps (Sherwood and McClay, 1999). This suggests that Notch is not involved in distinguishing between the ectodermal and mesodermal germ layers, which seems reasonable since these fates are not intermingled in the fate map of the 60 cells embryo, where the endoderm arises between the ectoderm and the mesoderm, in contrast to vertebrate models in which the mesoderm arises between the ectoderm and the endoderm (see the Introduction section).

Delta has a biphasic expression pattern during early embryogenesis. Elegant experiments combining overexpression (*delta* mRNA injection) or blocking strategies (Delta MO injection) in whole or chimeric embryos allowed to discern the role of Delta in each phase (Sweet et al., 2002). Both activate transcriptional Notch signaling and account for the down-regulation of the apical Notch protein and the appearance of Notch + intracellular vesicles in the vegetal plate (presumptive SMCs) described above.

1. At blastula stage, *delta* mRNA is transiently expressed in the mesodermal PMCs precursors, but they later turn-off this gene (Sweet et al., 2002). This early phase is indirectly driven by maternal nβ-cat in a cell-autonomous way, within the GRN that controls PMCs specification (Revilla-i-Domingo et al., 2004; Revilla-i-Domingo et al., 2007; Oliveri et al., 2008). This first Delta signal emitted by mesodermal PMCs precursors activates Notch in neighboring veg2 cells (black arrow 1a in Fig. 2A), leading to the specification of a first population of NSKM/SMCs cells, which gives rise to a subset of SMCs derivatives (pigment and blastocoelar cells) (Sweet et al., 2002). This early Delta signal also inhibits endoderm specification in a subset of veg2 derivatives (Croce and McClay, 2010) (Fig. 2B).
2. In the second phase, while the PMCs ingress into the blastocoel, *delta* transcripts appear in the mesodermal SMCs precursors, and persist in this population during early gastrula, when they begin to invaginate (Sweet et al., 2002). This second Delta signal is emitted by the first subset of specified SMCs, and activates Notch in neighboring veg2 descendants (Black arrow 2 in Fig. 2A). Notch, in turn, specifies a second subset of SMCs (which gives rise to blastocoelar and muscle cells) through the direct Notch/CSL target *foxY* (Sweet et al., 2002; Materna et al., 2013), which does not belong to the classic family of HES/HEY Notch targets. The regulatory input to the second phase of delta expression in veg2 descendants is currently unknown, but it is reasonable to believe that it depends on the first phase of Delta/Notch signaling, as the source of the second phase of Delta expression are the first NSKM cells specified by the first Delta signal. In overall, the different phases of NSKM segregation from the endomesoderm represent a beautiful example of lateral induction involving Delta/Notch signaling (Fig. 2B).

More recent work analyzed the change in the expression of a battery of genes involved in endoderm and mesoderm specification after knocking-down notch and delta functions with MOs (Peter and Davidson, 2010; Peter and Davidson, 2011). This allowed to precisely discern the place that Delta/Notch signaling occupies in the GRN that controls mesoderm and endoderm segregation from the endomesoderm. Delta/Notch signaling operates in the segregation of mesoderm from anterior endoderm in the veg2 descendants, while the specification of veg1 derived endoderm (which populates the posterior gut) is independent of Delta/Notch signaling. In the proposed model, nβ-cat/TCF directly activates cell-autonomously the endodermal GRN program in the veg2 tier, while Delta, emitted by the large micromeres, activates Notch in the neighboring veg2 cells, inducing the mesodermal GRN program in all of them at the 7th cleavage stage (Fig. 2B). Thus, at this time, the endomesodermal progenitors consist of a vegetal ring of veg2 cells, where both the endodermal and the mesodermal GRNs are co-expressed. As the veg2 tier divides, two concentric rings of descendants are formed in the vegetal hemisphere. Only the daughters in direct contact with the large micromere lineage (the inner ring) will become mesodermal founders, while those veg2-daughters that were left distal to the source of Delta (the external ring) will become endodermal founders (Fig. 2B). Thus, around 15–18 hpf, the external ring of cells expresses only endodermal markers, while the inner ring still co-expresses endodermal and mesodermal markers. Around 18–20 hpf, Notch down-regulates the endodermal GRN program in the inner ring, while the mesodermal GRN program persists in these cells. In this way, Notch segregates the mesodermal SMCs from the endomesodermal veg2 derived population (Fig. 2B), but this does not imply a binary decision, because the down-regulation of the endodermal GRN by Notch is independent of the up-regulation of the mesodermal GRN per se (Peter

and Davidson, 2011). The down-regulation of the endodermal program in the nascent mesodermal progenitors relies on the active repression of endodermal GRN genes by TCF, because deletion of the TCF binding site in the cis-regulatory module of the endodermal specifier gene *foxA* leads to ectopic expression of the reporter GFP throughout the embryo, indicating that *foxA* is repressed if β-cat is not available (de Leon and Davidson, 2010). Since injection of Notch MO prevents the clearance of *foxA* from the mesodermal territory, the down-regulation of the endodermal program in the mesodermal precursors might be due to a decrease in the availability of β-cat protein in the cells where Notch is active, rendering TCF as a repressor of the endodermal GRN, as it recruits the transcriptional co-repressor Groucho in the absence of nβ-cat (de Leon and Davidson, 2010; Peter and Davidson, 2011). While this occurs around 18–20 hpf, other study proposed that just before the onset of gastrulation (which begins at 30 hpf), Delta/Notch signaling clears TCF from the nuclei in the mesodermal lineage by up-regulating a Nemo-like kinase (NLK) (Röttinger et al., 2006; Sethi et al., 2012). Since NLK is known to phosphorylate TCF during endomesoderm segregation in the nematode *C. elegans*, promoting its export from the nucleus (Lo et al., 2004), it was proposed that after nuclear export of TCF, the mesoderm segregated from the veg2 lineage becomes refractory to Wnt signaling, while the veg2-derived endoderm preserves nuclear TCF and Wnt responsiveness in the sea urchin (Röttinger et al., 2006; Sethi et al., 2012). However, it was not addressed whether the gene encoding NLK is a direct Notch/CSL target. In addition, NLK is also known from other systems for inhibiting canonical Notch/CSL signaling by phosphorylating NICD and preventing the formation of the activating ternary complex with CSL and MAML (Ishitani et al., 2010; Wada et al., 2013).

Noteworthy, it was proposed that Notch initiates endomesoderm segregation from the veg2 lineage around the hatching blastula stage by repressing *hox11/13b*, a positive regulator of a subset of genes which are necessary to complete endoderm specification. While this *hox11/13b*-controlled circuit becomes inactive in the mesodermal lineage, it continues working in the endodermal lineage, which does not receive Delta signaling any more from the large micromeres descendants (Sethi et al., 2012). However, this study did not address whether *hox11/13b* is a direct Notch target depending on CSL, while it is known that it is positively regulated by nβcat/TCF. Thus, down-regulation of the nβ-cat/TCF pathway by Notch by an unknown mechanism might explain the repression of *hox11/13b* by Notch, as can be seen in the current model of the endomesoderm GRN, through the mediation of a hypothetical gene X: http://www.echinobase.org/endomes/.

The *gcm* gene is a marker of SMCs and is necessary for specification of pigment cells, a mesodermal SMCs derivative. This gene is another direct Notch/CSL target which does not belong to the HES/HEY family. CSL binding sites were found in its regulatory region, and they are necessary for its specific expression in SMCs (where Notch signal is active) and for repressing its ectopic expression where Notch signal is inactive. Blocking CSL-dependent signaling with a dominant negative CSL (CSL^{neg}) suppressed the expression of *gcm* at the mesenchyme blastula stage and resulted in loss of SMCs at gastrula and of pigment cells in pluteus larva. Gut formation at larval stage was not analyzed in this study (Ransick and Davidson, 2006). Quantitative transcriptome analysis showed that in CSL^{neg} embryos, only a few genes were directly or indirectly down-regulated at pre-gastrula stages, and most were involved in mesodermal SMCs specification, including the direct Notch targets *gcm* and *gataE*. At early gastrula, most known mesodermal regulatory genes expression was lost or strongly reduced. Interestingly, however, the endodermal GRN was minimally affected or not affected at all (Materna and Davidson, 2012). Thus, while canonical Notch, CSL-dependent signaling certainly operates in mesoderm specification during endomesoderm segregation of veg2 derivatives, we would like to suggest as a testable hypothesis that a CSL-independent, Notch-mediated mechanism might be operating in the repression of the endodermal fate. A possible explanation might be the down-regulation of nβ-cat (see Introduction) and/or promotion of nuclear export of TCF discussed above in specified veg2 derived mesoderm.

4.1.2. Establishment of the ectoderm/endoderm boundary

Sherwood and McClay also addressed the role of Notch in positioning the ectoderm/endoderm boundary, since the veg1 tier gives rise to both germ layers (Sherwood and McClay, 2001). They made use of the distribution of nβ-cat since it is present in presumptive endoderm cells bordering the presumptive ectoderm in the late mesenchyme blastula (Logan et al., 1999). N^{act} down-regulated nβ-cat cell-autonomously in vegetal cells (Fig. 2B, upper panel, red "a" line) but up-regulated nβ-cat non-cell autonomously in neighboring animal cells (Fig. 2B, upper panel, green "b" arrow) (Sherwood and McClay, 2001). We notice that this again indicates a state where nβ-cat is excluded from cells expressing Notch, and also, that these Notch + /nβ-cat − cells emit a signal (perhaps Wnt) that induces β-cat nuclear translocation in the neighboring cells. Moreover, opposite gradients of nβ-cat and membrane-bound Notch seem to be established during early development in sea urchins, when we compare their published localizations (Sherwood and McClay, 1997; Logan et al., 1999). It would be interesting to study if Notch is involved here in a non-canonical relationship with β-cat.

When examined at larval stage, N^{act}-injected embryos showed an expansion of endodermal at the expense of ectodermal derivatives. Lineage tracing experiments indicated that Notch shifts the ectodermal/endodermal boundary animally in a dual fashion: non-cell autonomously from vegetal cells, and cell autonomously in animal cells (double headed black arrow in Fig. 2A). This was confirmed by blocking Notch activity with N^{neg}, which gave the opposite results (Sherwood and McClay, 2001). The position of the ectoderm/endoderm boundary is dependent on Fringe activity, since the endodermal border of nβ-cat was lost in Fringe morphants (Peterson and McClay, 2005). The exact place than Notch occupies in the GRN that controls the limit between ectoderm and endoderm in the veg1 tier has not been currently solved (see http://www.echinobase.org/endomes/#Veg1Ectoderm).

4.1.3. Modulators of the Notch pathway

The interaction of the Notch pathway with other Notch modulators during endomesoderm segregation is also beginning to be clarified in sea urchins. In Camarodonta, Fringe protein is present in all cells through early cleavage and is sequentially lost, first from PMCs as they ingress, and later, from the SMCs and endoderm, as they invaginate during gastrulation. Afterwards, only ectodermal expression persists. Perturbing *fringe* function with a specific MO indicates that *fringe* is necessary for mesodermal specification of SMCs. Although it appears that *fringe* is not required for PMCs and endoderm specification, as judged by specific markers, Fringe morphants showed a stronger phenotype than N^{neg}- or Notch MO injected embryos, with a general failure in gastrulation and no archenteron invagination. However, co-injection of N^{neg} and Notch MO phenocopied the Fringe morphants, indicating that *fringe* is necessary for maternal and zygotic Notch signaling (Peterson and McClay, 2005).

Numb protein is located in apical membranes in presumptive SMCs from blastula stages. Both overexpression experiments and MO injections demonstrated that Numb acts synergistically together with Notch to specify the three types of mesodermal SMCs derivatives in Camarodonta (Range et al., 2008). Since during gastrulation, Numb protein is present in apical membranes in the endoderm overlapping with Notch in aboral endoderm, while Fringe protein disappeared from the invaginated archenteron, the authors leave open the question if another Notch ligand, such as Jagged, which is known from other models to require the Notch receptor unmodified by Fringe activity, might be participating in later aspects of NSKM and endoderm specification (Range et al., 2008).

4.2. Subclass Asteroidea (sea stars)

Unlike the sea urchins, the sea star embryo lacks micromeres and neither develop an embryonic skeleton nor embryonic pigment cells (Fig. 2C). Since among the five classes of echinoderms, only the sea urchins develop a skeletogenic micromere lineage, the sea stars represent the pleisiomorphic state from which the apomorphic state (represented by the sea urchins) derived (Hinman and Davidson, 2007). Both sea stars and sea urchins develop mesoderm from the vegetal plate, which is surrounded by cells fated to form endoderm (Fig. 2). However, the GRNs for endomesoderm segregation are wired differently, with a major change in the outcome of nβ-cat/TCF and Delta-Notch signaling, as was discovered in the sea stars *Asterina miniata* and *Patiria miniata* (Hinman and Davidson, 2007; McCauley et al., 2015).

Delta is expressed in the presumptive mesoderm in both subclasses, although the first phase of expression in the large micromere lineage (SKM lineage) described in sea urchins is not present at all in the sea star (which does not possess this lineage). Delta/Notch signaling is required for endomesoderm segregation in both, but while in sea urchins, this pathway is necessary for specifying mesoderm and limiting endoderm formation, knock-down experiments with Delta MO strikingly demonstrated that the opposite occurs in the sea star, with Delta/Notch promoting endodermal fates and restricting mesoderm specification. In addition, in the sea star, it simply does not exist the sea urchin sub-circuit that induces mesodermal pigment cells by direct contact between Delta expressing skeletogenic micromeres and the veg2 responding cells. The *gcm* gene, which is the direct Notch target in that sub-circuit, is not involved at all in this task in the sea star embryo (which does not possess pigment cells), since it is not expressed in the vegetal plate and is not subject to Delta control (Hinman and Davidson, 2007).

During early and midblastula, a gradient of maternal nβ-cat/TCF is also established in the vegetal hemisphere of the sea star embryo, with lower levels towards the equator and higher levels in the vegetal pole (Fig. 2D, left panel). This activity is sufficient to initiate the expression of the earliest zygotic transcription factors of endomesoderm specification in a dose-dependent response way. Low levels of activity induce *brachyury* (which is initially expressed in the whole vegetal hemisphere and is later restricted to the presumptive posterior endoderm). More vegetally, medium levels induce *gataE* and *foxA*, the earliest transcription factors of the endomesodermal GRN, which at this stage are broadly expressed in the vegetal hemisphere, but are later restricted to the endoderm. The highest levels of nβ-cat activity are reached at late blastula stage, in the presumptive mesoderm territory (Fig. 2D, right panel), whereas it is cleared from the presumptive endoderm (in striking contrast to what happens in sea urchins, which require maintaining high levels of nβ-cat/TCF activity for endoderm specification and clearing nβ-cat/TCF activity for mesoderm specification). These high levels of nβ-cat/TCF activity are necessary to induce the expression of est1 and erg, two pivotal transcription factors of the mesodermal GRN. This initiates a gene cascade which specifies mesodermal fates, induces delta expression, and represses *gataE* and *foxA* in the mesodermal lineage, thus limiting endodermal specification, which requires maintaining *gataE* and *foxA* expression in the presumptive endoderm. Since the endodermal lineage has lost maternal nβ-cat/TCF activity by the late blastula stage, maintaining *gataE* and *foxA* expression requires additional zygotic control, which is initiated by Delta signaling from the presumptive mesodermal cells. Thus, Notch is activated in presumptive endodermal cells, promoting the expression of the transcription factor hex, which establishes a positive feedback loop with *gataE* and *foxA*. In this way, Delta-Notch signaling maintains and potentiates the expression of the transcription factors that initiate the GRN for endoderm specification, stabilizing this fate and reinforcing the endoderm/mesoderm boundary previously outlined by the high levels of nβ-cat/TCF in the presumptive mesoderm (McCauley et al., 2015).

5. Notch signaling in the segregation of germ layers in chordates

5.1. Cephalochordates (amphioxus)

Cephalochordates (also called amphioxus or lancelets), urochordates, and vertebrates are the three subphyla composing the Chordate phylum. Phylogenomic analysis demonstrated that urochordates are the closest living relatives of vertebrates, whereas Cephalochordates are placed in the basal lineage within the phylum, being the sister group of urochordates and vertebrates (Delsuc et al., 2006). For the purpose of evo-devo studies, urochordates and amphioxus are strategically located in phylogenetic terms before the two complete rounds of genome duplication that took place in the vertebrate lineage (reviewed in Bertrand and Escriva, 2011). In comparison to amphioxus, which simply gastrulates by invagination, extensive tissue rearrangements occur during early development in urochordates and vertebrates, complicating the attempts to correlate axes orientation between early and later embryonic stages. In addition, while urochordates show specific derived features unrelated with the development of other chordates, it is thought that amphioxus retained many ancestral chordate characters, thus representing a key model to understand the transition from invertebrate chordates to vertebrates during evolution (Holland and Holland, 2007; Bertrand and Escriva, 2011).

A revised fate map of the amphioxus blastula shows a striking difference in the vegetal hemisphere in comparison to the fate map of the amphibian or fish blastulae. The presumptive ventral mesoderm territory is absent from the ventral vegetal hemisphere. Instead, the whole

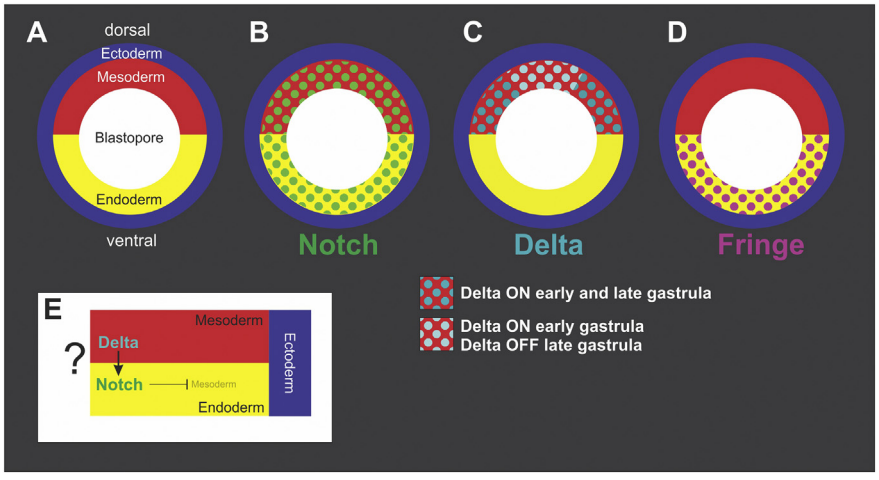

Fig. 3. Segregation of germ layers in Cephalochordates. (A) Fate map of the blastula in posterior view, depicting the position of the future blastopore. (B–D) Expression patterns of components of the Notch pathway during gastrulation (B, Notch; C, Delta; D, Fringe), overlaid on the segregating germ layers. From early to mid-gastrula, Delta is expressed in the prospective mesoderm, but at mid-gastrula is turned-off from the dorsal region from where the notochord arises. The absence or a lower expression of Dll1 in the mid-gastrula organizer in comparison to the non-organizer mesoderm appears to be a common feature of chordate development. (E) Proposed hypothesis for the role of Delta/Notch in controlling the position of the mesoderm/endoderm boundary. See main text for further explanation and references.

endoderm develops from this region in amphioxus (indeed, the ventral mesoderm arises from outgrowths of the somitic mesoderm during neurulation) (Fig. 3A). Another important difference in relation to vertebrates is that the amphioxus gastrula is bi-layered instead of three-layered, because both the endoderm and the mesoderm arise from the same single layer internalized by invagination during gastrulation (reviewed in Holland and Holland, 2007). The gut and the mesodermal derivatives are separated later.

Little is known about the role of Notch signaling during gastrulation in cephalochordates. *Notch*, *delta* and *fringe* were found as single genes, whereas two *jagged* genes are present in the genome of amphioxus. The expression patterns of some of them were analyzed by in situ hybridization (ISH) (reviewed in Bertrand et al., 2017). The spatial distribution of *notch*, *delta* and *fringe* transcripts strongly suggests the involvement of the Notch pathway in the early development of germ layers. *Notch* is first detected by ISH at the mid-gastrula stage in the whole endomesodermal ring, inside the open blastopore, thus encompassing the prospective endoderm, ventrally, and the prospective somitic and axial mesoderm, dorsally (Fig. 3B). Near the end of gastrulation, *notch* is down-regulated in the presumptive endoderm, but persists dorsally, in the presumptive notochord and somites, with higher levels in the posterior region and weak expression in the posterior neural plate (Holland et al., 2001). *Delta* transcripts are first detected at the early/mid-gastrula stage throughout the prospective mesoderm in the dorsal region, whereas ventrally, the prospective endoderm is devoid of delta transcripts (Fig. 3C) (Rasmussen et al., 2007), where *notch* transcripts are still present, suggesting that another Notch ligand or a non-canonical Notch pathway might be operating there (Bertrand et al., 2017). At mid/late gastrula stage, *delta* is down-regulated in the prospective axial mesoderm, leaving a gap between the bilateral bands of strong persisting expression in the presumptive paraxial mesoderm, which in late gastrula are resolved in stripes from where the first two pairs of rostral somites will later arise (Rasmussen et al., 2007). *Fringe* transcripts are first located at gastrula stages ventrally, in the presumptive endoderm, thus complementary to dorsal *delta* expression in the presumptive mesoderm (Fig. 3D), just bordering the presumptive rostral somites (Onai et al., 2015), and in early neurulae are distributed along the neural plate with a clear posterior limit (Mazet and Shimeld, 2003).

Interestingly, treatment with the γ-secretase inhibitor DAPT from late blastula stage suppressed *fringe* expression from the presumptive endoderm, but *delta* expression persisted in the dorsal region corresponding to the presumptive somites at mid/late gastrula stage, although reduced (indeed, we notice that this reduction might be due to the expansion of the *delta*-negative gap of the presumptive axial mesoderm in Fig. 2F in Onai et al. 2015). At larval stage, this treatment resulted in ectopic expression of a muscle differentiation marker expanding into the gut, whereas the boundary between both tissues was not clearly distinct anymore, as it would be expected at this stage. Thus, it was proposed that in amphioxus, the mesoderm/endoderm boundary is controlled by Notch signaling through the *delta/fringe* cassette (Onai et al., 2015). Although the authors do not discuss it further, it would seem that Notch does so by limiting mesoderm. Thus, it might be that the early expression of Notch in the presumptive endoderm of the early/mid gastrula limits mesoderm at the boundary, by being signaled by Delta from the adjacent presumptive mesoderm, while ventral Notch might be glycosylated through ventral Fringe to be responsive to Delta. Within the presumptive mesoderm, Notch might be exerting other functions, considering that *delta* is down-regulated in the presumptive axial mesoderm at late gastrula (Fig. 3E). In fact, DAPT treatments reduced the expression of markers of the rostral somites, and from this, the authors suggested that *notch* has a role in specifying the rostral somites rather that the entire mesoderm (Onai et al., 2015). Perhaps, making time-controlled experiments with DAPT in more limited periods would help to discern the different roles of *notch* in endoderm and mesoderm development during gastrulation in amphioxus.

5.2. *Danio rerio*

Zebrafish has four notch genes: *notch1a*, *notch1b*, *notch2*, and *notch3*. The two *notch1* genes appeared in teleosts in a recent duplication event, after their divergence from tetrapods (Theodosiou et al., 2009). Among them, *notch1a* transcripts are the only ones that can be detected by ISH as maternal mRNA, and are expressed ubiquitously in the animal region before the onset of epiboly. At early gastrula stage, they become diffuse, and zygotic transcripts accumulate throughout the germ ring in the involuting hypoblast (presumptive endomesoderm). As gastrulation proceeds, *notch1a* becomes highly expressed in the dorsal hypoblast of the embryonic shield (the fish dorsal organizer), which will give rise to the prechordal mesoderm and the notochord, and is later expressed throughout the presumptive neural ectoderm (Bierkamp and Campos-Ortega, 1993). During gastrulation, *notch1b* is transcribed in the notochord and is later detected also in the presomitic mesoderm, anterior axial neuroectoderm and posterior neural ectoderm, whereas *notch2* transcripts are first detected at late gastrula in the presumptive presomitic mesoderm, and are excluded from the notochord and from the ectoderm. *Notch3* is transcribed in epiblast cells behind the blastoderm margin that advances by epiboly, and fades-out as cells ingress during gastrulation (Westin and Lardelli, 1997).

DeltaA (*dla*) and *deltaD* (*dld*) are duplicated orthologues of human DLL1, whereas *deltaB* (*dlb*) and *deltaC* (*dlc*) are duplicated orthologues of human DLL3. Their expression is first detected by ISH during epiboly (Haddon et al., 1998). *Dla* is transcribed throughout the embryo at low levels at the onset of gastrulation, but from mid-gastrula stage and during the remainder of gastrulation, it is conspicuously expressed in a small group of deep cells in the fish organizer, where there is continuous involution. This represents a transient expression in organizer cells occurring at the time of their involution, as once they have entered the dorsal axial endomesoderm, they turn-off *dla* expression. *Dla* is also highly expressed by some epiblast cells in the dorsal midline during gastrulation (Appel et al., 1999). *Dld* transcripts are first visible at late blastula in the entire marginal region, persisting there through gastrulation, except for the organizer region, where transcription of *dld* ceases at the onset of this period (50% epiboly), leaving a gap corresponding to the future axial mesoderm. As gastrulation progresses, the *dld* domain in the marginal zone extends into the non-axial hypoblast (Dornseifer et al., 1997). A similar pattern was described for *dlc* (Haddon et al., 1998). At late gastrula (90% epiboly), *dla*, *dlb* and *dld* transcripts are detected by ISH in the epiblast (presumptive neural ectoderm), in (*dlb*) or near the axial midline (Haddon et al., 1998). Expression of dll4 was not reported during early and mid-gastrulation and is weak at late gastrula (Hsiao et al., 2007). Thus, mRNAs encoding for Notch receptors and DSL ligands are found in the regions where germ layers are segregating during gastrulation, both in the presumptive endomesoderm and in the contiguous epiblast, with some of them showing expression in the midline precursors and others excluded from the midline.

Over-activation of the pathway with N^{act} reduced the number of endodermal cells. Although pan-mesodermal markers were not analyzed, indirect evidence indicated that N^{act} favored somitic development, suggesting that Notch might play a role in the segregation of mesoderm from endoderm (Fig. 4B). However, neither *dlc* and *dld* knock-down nor DAPT treatments resulted in the opposite effects, and $Dll1^{neg}$ neither was able to promote endoderm specification (Kikuchi et al., 2004). This is the same Dll1 antimorphic construction used in the *Xenopus* study, which showed that Delta signaling was involved in refining the limit between ectoderm and mesoderm rather than between endoderm and mesoderm (Revinski et al., 2010) (see below), but this possibility was not explored in the zebrafish work. In addition, the morpholino approach was not used to knock-down the Notch receptor to further elucidate a possible role of Notch signaling in the division between germ layers in zebrafish, and the involvement of other DSL ligands was not addressed.

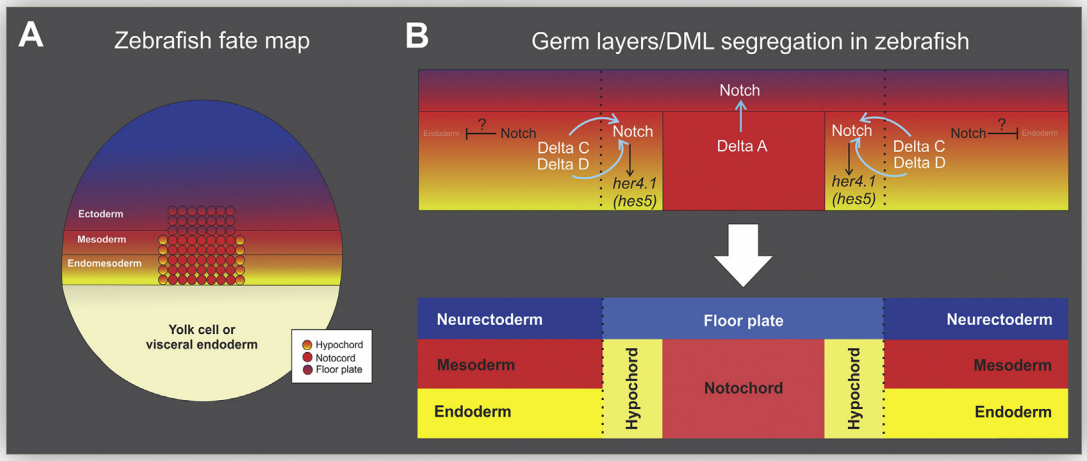

Fig. 4. Germ layers and DML segregation in zebrafish. (A) Fate map of the late blastula in dorsal view. (B) Proposed model for the role of Delta/Notch signaling in DML segregation. Delta C and Delta D from the non-axial mesoderm activates Notch in hypochord/notochord bipotential precursors, favoring hypochord and disfavoring notochord development through the action of a *hes5* orthologue. While Notch was more recently proposed to promote the proliferation of floor plate precursors, a role for Delta A/Notch signaling in a binary choice favoring floor plate development at the expense of the notochord cannot be ruled out. In addition, little is known about the role of Delta/Notch signaling in the segregation of germ layers in the non-axial territory, but gain-of-function experiments suggest that Notch inhibits endoderm development. See main text for discussion of the proposed model and references.

5.3. *Xenopus*

The prospective germ layers are roughly depicted along the animal-vegetal axis in the 32-cell stage fate map of this amphibian (Dale and Slack, 1987). The animal blastomeres (A-tier) and the vegetal blastomeres (D-tier) mostly contribute to ectoderm and endoderm, respectively. Both equatorial tiers (B and C) mostly contribute to mesoderm, but B-tier descendants are also found in ectodermal derivatives in a significant proportion, while the C tier also contributes to endoderm (Fig. 5A). Therefore, the fates of the equatorial tears are significantly mixed in terms of germ layers. Recently, a *Xenopus* endomesoderm GRN from fertilization through early-gastrula was built (Charney et al., 2017), but a hypothetical role of Notch was not included in this assembly.

There are three *notch* paralogues identified in the *Xenopus* genome, corresponding to the vertebrate orthologues *notch1*, *notch2*, and *notch3*. *Notch1* transcripts are the most abundant at blastula stages and during gastrulation, followed by *notch3*. The levels of *notch2* transcripts begin to increase at neurula stages, making it more unlikely to intervene in the segregation of germ layers (analysis of RNAseq data from Session et al., 2016). The spatial expression of *notch3* is unknown. The first paralogue isolated from *Xenopus*, the most studied in general, and the only one studied in particular in the segregation of germ layers, is *notch1*. When gastrulation just begins, *notch1* transcripts are more abundant in the dorsal than in the ventral marginal zone (Coffman et al., 1990), comprising both the involuting (presumptive mesoderm and supra-blastoporal endoderm, which will form the archenteron roof) and the non-involuting region (presumptive neural ectoderm) (López et al., 2003). Soon, *notch1* transcripts are accumulated in a ring throughout the entire marginal zone (Miazga and McLaughlin, 2009; Favarolo and López, unpublished results). *Notch1* is not detected by ISH in the sub-blastoporal presumptive endoderm, which will form the floor of the archenteron. Thus, transcripts of the receptor are present throughout a region where limits between the three germ layers need to be defined, in order to be precisely allocated during gastrulation in their final destinations: ectoderm, mesoderm, and the supra-blastoporal endoderm.

Three paralogues of *dll* and two paralogues of *jag* were identified in the *Xenopus* genome, among the classical Notch ligands. Analysis of RNAseq data from (Session et al., 2016) indicates that *dll1* is maternally expressed at low levels. Zygotic expression rises abruptly at early gastrula (S10.5) for *dll1* and *dlc*, and both are highly expressed during gastrulation, whereas *dll4* is not expressed at these stages. ISH analysis showed that both ligands share a similar expression pattern around the blastopore in the presumptive mesoderm, except that at their onset of expression (S10.5), *dlc* is expressed in a complete ring (Peres et al., 2006), whereas *dll1* shows a gap of lower expression in the region corresponding to the Spemann-Mangold organizer, with isolated cells expressing the gene there in a "salt and pepper" pattern (López et al., 2005). Soon afterwards (S11), the *dlc* pattern also shows the same gap (Peres et al., 2006).

Time-controlled experiments revealed that activation of Notch signaling at the beginning of gastrulation resulted in an increase of many endodermal markers and to a decrease of paraxial, lateral plate and intermediate mesoderm markers, whereas blocking CSL-dependent Notch signaling at gastrulation produced the opposite results. This indicated that Notch participates in the division of endoderm and mesoderm, but the authors favored the hypothesis that Notch promotes a delay in the differentiation of mesoderm rather than a binary cell fate choice between endodermal and mesodermal fates (Contakos et al., 2005). Since the consequence of altering Notch signaling was analyzed at late neurula and more advanced stages, it was necessary to study this problem during gastrulation, when the segregation of germ layers indeed takes place. This was addressed in a more recent work, which proposed that *notch1* is involved in refining the segregation between the neural ectoderm, the mesoderm and the endoderm (Revinski et al., 2010). In the proposed model, Notch would act in two ways: (A) By refining the limit of involution between the endomesodermal cells in the involuting marginal zone (IMZ) and the neurectoderm in the non-involuting marginal zone (NIMZ). This is supported by the observation that constitutively active Notch1^{act} moved the limit of involution vegetalwards, whereas blocking Delta-Notch signaling with Notch1 MO or with an antimorphic Dll1 (Dll1^{neg}) moved it animalwards (type A decisions, Fig. 5B) by refining the segregation between mesoderm and supra-blastoporal endoderm within the endomesoderm in the IMZ. This is supported by the observation that Notch1^{act} favored endoderm

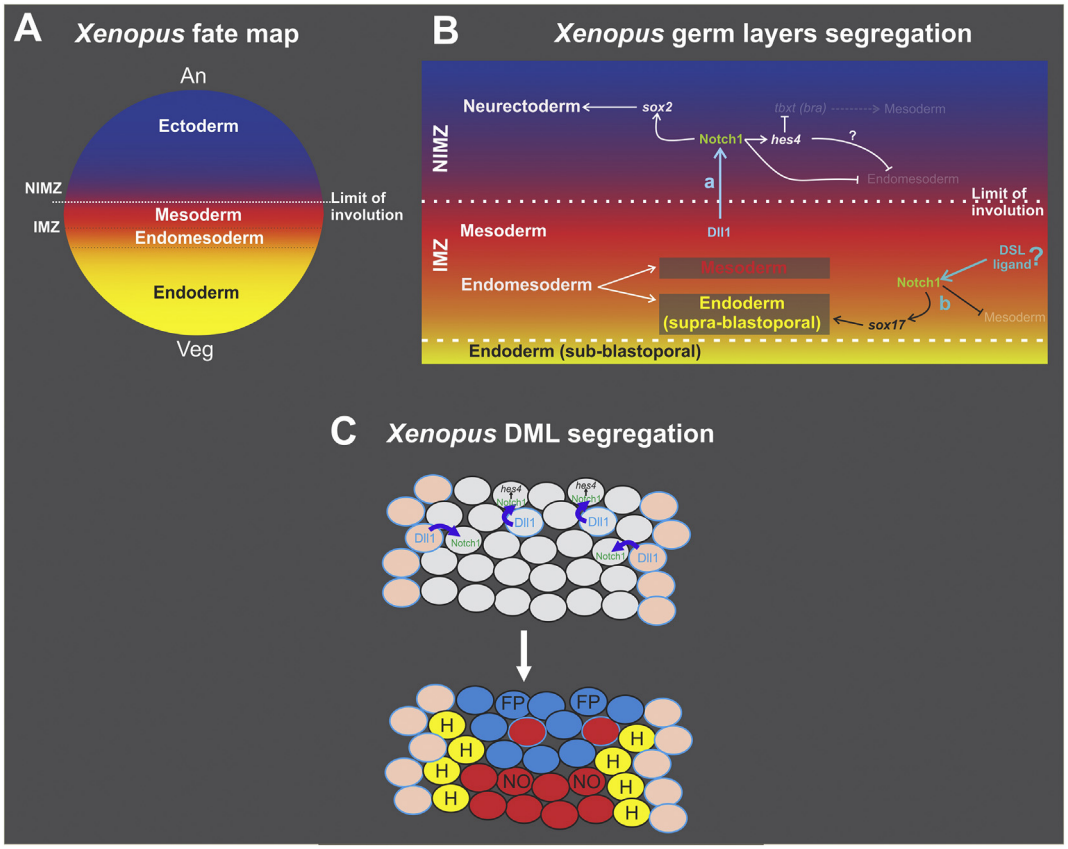

Fig. 5. Germ layers and DML segregation in the amphibian *Xenopus*. (A) Fate map of the *Xenopus* blastula, projecting the involuting marginal zone (IMZ), the non-IMZ (NIMZ) and the limit of involution. (B) Proposed model for Delta/Notch signaling in the segregation of germ layers. Dll1 (a, light blue arrow) controls the delimitation of the neurectoderm and the endomesdoderm by favoring neuroectodermal development at the boundary through the activation of Notch1/*hes4* in the NIMZ. This sub-circuit inhibits endomesoderm development in the NIMZ. In the IMZ, Notch is involved in endomesoderm segregation by favoring endoderm and inhibiting mesoderm specification (b). It is not known if a DSL ligand (?) induces this step. (C) Proposed model for Delta/Notch signaling in DML segregation. There is expression of Dll1 in isolated cells in the *Xenopus* dorsal marginal zone, in a salt-and-pepper pattern (white cells with cyan outline), whereas Dll1 is strongly and compactly expressed in the non-axial mesoderm (light pink cells with cyan outline). Similar to the proposed model for zebrafish, Dll1 from the non-organizer mesoderm activates Notch in hypochord/notochord bipotential precursors, favoring hypochord (H, yellow) and disfavoring notochord (NO, red) development. Within the axial precursors, some Dll1+ cells activate Notch1 in notochord/floor plate bipotential precursors. Notch1, in turn, activates *hes4*, which operates a binary cell-fate switch, preventing the involution of cells where it is expressed, favoring floor plate (FP, light blue) and disfavoring notochord (NO, red) development. See main text for further explanation and references. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

development at the expense of mesoderm, while the general blockade of Notch1 signaling with Notch1 MO gave the opposite result (type B decisions, Fig. 5B). Strikingly, Dll1^{neg} only shifted the limit of involution animalwards, favoring endomesodermal development at the expense of the neural ectoderm. This suggests that Dll1 is involved in type A decisions and raises questions about the ligand and mechanism underlying mesodermal vs. endodermal decisions (type B), which were only revealed by Notch1 MO (Revinski et al., 2010).

Interestingly, the Notch target *hes4* is expressed in the presumptive ectoderm from late blastula. *Hes4* refines its limit with the presumptive mesoderm progressively during gastrulation, when transcripts accumulate in the whole NIMZ, with highest levels in the dorsal NIMZ (prospective FP), establishing a complementary domain in relation to the pan-mesodermal marker *tbxt* (synonym: *brachyury*, *T*) (López et al., 2005; Aguirre et al., 2013). *Hes4* might be one Notch target involved in type A decisions, since its domain is expanded by Notch1^{act} in the NIMZ (López et al., 2005), *hes4* overexpression suppresses *tbxt* throughout the entire IMZ, and hes4 MO expands the *tbxt* domain, shifting the limit of involution animally (Aguirre et al., 2013) (Fig. 5B).

In addition, CSL^{neg} produced milder effects than Dll1^{neg} or Notch1 MO in the division of neural ectoderm and mesoderm (Revinski et al., 2010). Although this effect was analyzed at the neural plate stage instead of during gastrulation and does not allow to drive definitive conclusions, this suggests that non-canonical, CSL-independent pathways might also be operating in defining the ectoderm/mesoderm boundary. More work is needed to discern the intimacy of the role of the diverse DSL ligands and non-canonical Notch pathways in the segregation of germ layers.

5.4. Mammals

The first lineage segregation of the mammalian conceptus occurs before implantation, when the trophectoderm (TE) and the inner cell mass (ICM) form, both composing the blastocyst. These tissues have mutually exclusive fates, since the TE descendants only contribute to the trophoblast and extraembryonic ectoderm, whereas the ICM progeny segregates during implantation, giving rise to the primitive endoderm (hypoblast) and the epiblast. After implantation, the hypoblast originates the extraembryonic endoderm, whereas during gastrulation and body axis elongation, the epiblast gives rise to the extraembryonic mesoderm and the three primary embryonic germ layers (ectoderm, endoderm, and mesoderm), from which all the fetal tissues will develop (Beddington and Robertson, 1999; Wilson et al., 2009).

Cell-lineage analysis demonstrated that there is extensive cell

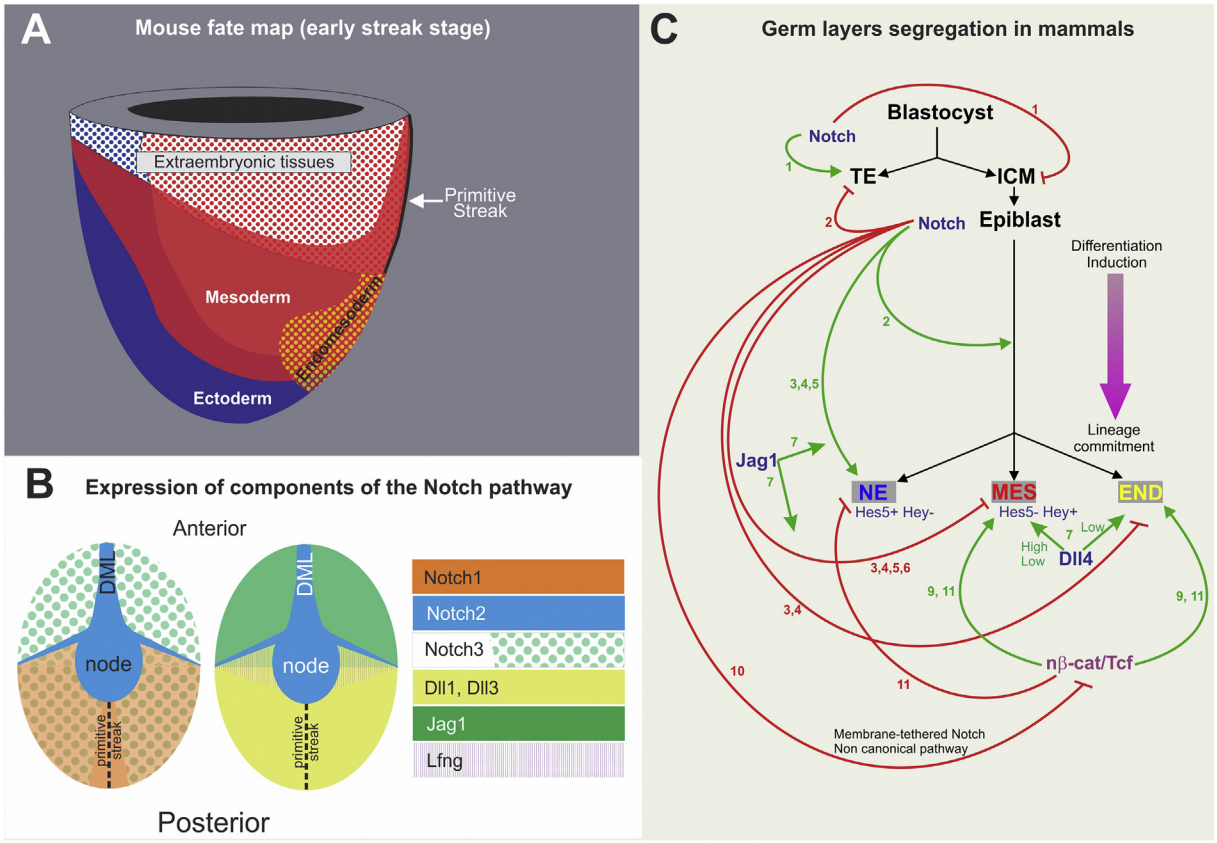

Fig. 6. Germ layers segregation in mammals. (A) Fate map of the mouse embryo at early streak stage (6.5 dpc). Individual cells have not fixed their specification at this stage, since their clonal descendants were found in more than one germ layer, as indicated by color overlap. Adapted from Tam and Behringer (1997). (B) Diagram of a mouse embryo in posterior view at late gastrula (neural plate, presomite stage) showing the expression of components of the Notch pathway. Adapted from Przemeck et al. (2003). (C) Diagram integrating Notch and β-cat signaling from the blastocyst stage to early lineage commitment into germ layers. Both in vivo studies in mouse embryos and in vitro studies in mESc and hESc were included. References: 1) (Rayon et al., 2014). Mouse, in vivo; 2) (Yu et al., 2008). hESc; 3) (Souilhol et al., 2015). Mouse, in vivo. $NICD^{Epi}$; 4) (Lowell et al., 2006). mESc, hESc; NICD, γ-secretase inhibitor, $CSL^{-/-}$; 5) (Nemir et al., 2006). mESc; NICD, CSL^{neg}; 6) (Schroeder et al., 2006). mESc; NICD; 7) (Ramasamy and Lenka, 2010). mESC, without LIF; 8) (Kageyama et al., 2010). mESc; 9) (Lindsley et al., 2006). mESc; 10) (Kwon et al., 2009). mESc; 11) (de Jaime-Soguero et al., 2018). mEpiSC, hESc.

intermingling in the mouse epiblast before the onset of gastrulation (pre-streak embryos) (Lawson et al., 1991; Tam and Behringer, 1997; Beddington and Robertson, 1999). There is substantial overlap of prospective germ layers in the early streak fate map (6.5 dpc) (Fig. 6A). Individual cells have not fixed their specification at this stage, since their clonal descendants were found in more than one germ layer (Lawson et al., 1991; Tam and Behringer, 1997; Lawson, 1999).

As the primitive streak elongates from posterior to anterior, diverse mesodermal lineages ingress through it from the epiblast. Their allocation on the cranial-caudal axis of the embryo depends on the order in which they are recruited (earlier, cranial; later, caudal), while the allocation in the dorsal-ventral axis of the embryo depends on the site of ingression in the primitive streak (anterior, dorsal; posterior, ventral) (Kinder et al., 1999; Arnold and Robertson, 2009; Tam and Gad, 2004). Thus, while the posterior end of the primitive streak contains lateral and extraembryonic mesoderm progenitors until 7.5 dpc., the posterior third of the 8.5 dpc primitive streak (late gastrula/early somites stage) contains mostly paraxial and some lateral/ventral mesodermal precursors in the mesodermal layer of the streak, whereas the ectodermal layer contains ectodermal, neuroectodermal and posterior mesoderm progenitors (Wilson and Beddington, 1996). Yet, at the late streak stage, there is still cell fate intermingling, mainly in the posterior region adjacent to the PS. Thus, posterior hindbrain and spinal cord fates still overlap with paraxial and lateral mesodermal fates (Tam and Behringer, 1997), indicating that these mesodermal populations have not completely segregated from posterior neuroectodermal fates by the end of gastrulation. Indeed, by the late-streak/no bud stage, complete layers of mesoderm and definitive endoderm (fore- and mid/hindgut) have been laid below the ectoderm, but this mesodermal layer only contains the more anterior precursors (heart mesoderm, cranial and upper trunk paraxial and lateral mesoderm) (Tam and Gad, 2004). Hence, lower trunk and tail mesoderm segregation from neural ectoderm continues after gastrulation, during axis elongation. However, gastrulation-like ingressive movements through epithelial-mesenchymal transition mechanisms still persist in the early tailbud both in mouse (E9.0) and in chick embryos (HH stage 17). They are gradually attenuated and finally cease in the late tailbud (E10.5, HH stage 24) (Ohta et al., 2007). Thus, it is reasonable to think that gastrulation as a process continues in the early tail bud although gastrulation in the conventional staging term has finished (Stern et al., 2006).

Most of the definitive endoderm (DE) is recruited from precursors in the posterior epiblast among the cells of the gastrula organizer. Recruitment begins at the early- to mid-streak stages and continues throughout gastrulation in a sequential way, according to the final A-P position of the different gut segments (Tam and Beddington, 1987; Tam and Gad, 2004). These DE precursors exit from the anterior primitive streak and migrate anteriorly and laterally together with mesodermal cells. By E7.0, nascent DE cells emerge from within the wings of mesoderm and progressively intercalate and intermingle with the existing visceral endoderm, which persists in 10% of the foregut, 15% of the midgut and 35% of the hindgut at E8.75 (Kwon et al., 2008; Arnold and Robertson, 2009; Viotti et al., 2014). In addition, there is evidence that

bipotential endomesodermal precursors exist in the mouse epiblast as it has been described for other metazoan embryos (Lewis and Tam, 2006; Tam et al., 1997; Tam and Gad, 2004; Viotti et al., 2014). However, only a subset of the mesodermal cells seems to arise from the endomesoderm (Lewis and Tam, 2006; Tzouanacou et al., 2009). Clonal analysis showed that endoderm progenitors begin to segregate from other lineages soon after the onset of gastrulation and that both surface ectoderm and DE finish their segregation before gastrulation ends (Tzouanacou et al., 2009). However, the old primitive streak still retains some ability to generate endoderm, although of posterior character (Tam and Beddington, 1987).

The expression of transcripts encoding members of the Notch pathway during preimplantation development in the mouse was globally analyzed by RT-PCR. *Notch1* and *2*, *Jag1* and *2*, *Dll3*, *Rbpj* (CSL) and *Deltex2* are expressed from unfertilized eggs until late blastocyst stage. *Notch3*, *Dll1* and *Deltex1* transcripts are detected in 2-cell embryos and in hatched blastocysts, but are absent or have a weak expression at the morula stage (Cormier et al., 2004). The distribution of transcripts encoding members of the Notch pathway was studied in post-implantation development by ISH analysis. In mid-streak embryos (7.0–7.5 dpc), *Notch1* was detected in the posterior ectoderm, adjacent to the primitive streak, and also in the nascent mesoderm, with the highest levels in the primitive streak, and lower levels in more lateral and anterior mesoderm (Del Amo et al., 1992), marking all the mesoderm that has migrated through the streak (Reaume et al., 1992). Except for the region around the streak, expression was absent from the ectoderm and neural ectoderm at the mid-streak stage (Del Amo et al., 1992; Reaume et al., 1992). As gastrulation proceeds, and mesoderm continues migrating toward the anterior region, *Notch1* becomes confined to the posterior, pre-somitic mesoderm, with a boundary just anterior to the node, which did not show *Notch1* expression (Del Amo et al., 1992; Reaume et al., 1992; Przemeck et al., 2003). Another study reported complementary patterns for the three Notch receptors during gastrulation (Williams et al., 1995). At 7.0 dpc, *Notch1* and *Notch2* transcripts were detected in the nascent notochord, whereas *Notch2* mRNA was also found in the node. In addition, *Notch2* and *Notch3* transcripts were detected in cells ingressing through the primitive streak. *Notch3* mRNA was also found throughout the embryonic ectoderm, but was absent from the node, the notochord and the developing dorsal midline. As gastrulation progresses, cells derived from the node continue expressing *Notch2* in the notochord and also in the presumptive floor plate at high levels, whereas cells migrating through the primitive streak (paraxial and lateral mesoderm precursors) activate *Notch1*, instead, coincident with the other studies, whereas *Notch3* is absent from the streak. These authors also show that Notch1 transcripts persist in the dorsal midline, showing some overlap with *Notch2* there (Williams et al., 1995). In addition, *Notch2* also shows bilateral stripes of expression anterior to the node and to the presomitic mesoderm domain of Notch1 (Przemeck et al., 2003) (Fig. 6B).

Two studies analyzed the early distribution of the canonical active mouse Notch1 by immunofluorescence. This was achieved with an antibody that only recognizes N1ICD after cleavage by γ-secretase, since the epitope is not exposed before cleavage. Del Monte et al. made their analysis in post implantation development from mid-streak stages onward, showing that active N1ICD is present in the nuclei of nascent embryonic mesodermal cells at 7.0 dpc, but is absent from the embryonic ectoderm. Later on, it is found in derivatives of the three germ layers (Del Monte et al., 2007).

The expression of *Dll1* and *Dll3* was analyzed from pre-streak stages onward by ISH (Bettenhausen et al., 1995; Dunwoodie et al., 1997). *Dll1* transcripts were first detected at the mid-streak stage in the primitive streak and ingressing mesoderm. As gastrulation progresses, expression continues in the primitive streak and becomes restricted to posterior (presomitic) mesoderm excluding the node and with a sharp boundary just anterior to the node, thus reminiscent to the patterns described in *Xenopus*, zebrafish, and cephalochordates around the blastopore for the corresponding orthologues. *Dll1* transcripts were neither detected by ISH in the embryonic ectoderm and neuroectoderm at early and mid-streak stages, nor in the axial mesoderm at later stages (Bettenhausen et al., 1995) (Fig. 6B)

Dll3 transcripts were detected earlier than *Dll1*, in the whole epiblast of pre-streak embryos. Once gastrulation begins, *Dll3* transcripts persist in low levels throughout the epiblast until mid-streak stages, and they become restricted to the cells adjacent to the primitive streak at late-streak stage. Strong expression of *Dll3* is found in the primitive streak itself and in the whole nascent mesoderm at mid-streak stage (Dunwoodie et al., 1997). This expression continues in the primitive streak, extending to its entire length, but is restricted to the posterior nascent mesoderm (presumptive paraxial mesoderm) at late-streak stages, whereas transcripts are neither detected in the node nor in the axial mesoderm (Dunwoodie et al., 1997; Przemeck et al., 2003) (Fig. 6B).

The expression pattern of *Jag1* was analyzed in late-streak embryos. Highest levels of transcripts were found in an anterior domain around the primitive streak and the node, complementary to the *Dll1* domain and corresponding presumably to embryonic endomesoderm (Mitsiadis et al., 1997; Przemeck et al., 2003), and lower levels were present in the caudal epiblast around the primitive streak (Przemeck et al., 2003). Interestingly, at the late streak stage, *Lfng (Lunatic fringe)* transcripts are restricted to a bilateral domain flanking the node (Przemeck et al., 2003) (Fig. 6B).

Thus, the dynamic expression patterns of the components of the Notch pathway show that they are present in a field of great cell-fate plasticity, where decisions of ingressing as mesoderm and endoderm or remaining in the ectoderm are continuously taking place.

Recently, highly sensitive single-cell analysis of RNAseq transcriptomics and quantitative RT-PCR data from cells isolated from post-implantation mouse embryos attempted to bring light to the molecular basis of cell-fate plasticity and germ layer lineage segregation at the cell level (Wen et al., 2017). This revealed that in the early epiblast (E5.5 and early E6.5), there is no lineage segregation in the major germ layers yet, but some cells begin to diverge and are molecularly categorized as pre-mesendoderm (pre-MEN), since they express high levels of endomesodermal markers and low levels of Sox3. Late E6.5 embryos also contained characteristic epiblast cells as well as pre-MEN, but in addition, there were cells co-expressing endomesoderm markers that also co-expressed primitive streak markers, and were thus characterized as endomesodermal progenitors (MEN cells). These late E6.5 embryos also contained cells that, according to their molecular signature, could be distinguished as belonging to either the definitive endoderm (DE) lineage or to the mesodermal lineage (extraembryonic and embryonic mesoderm). The authors proposed that the single cell transcriptome analysis of late E6.5 embryos reveals differentially expressed genes corresponding to signaling pathways and transcription factor networks that might underlie the segregation of mesoderm and DE in the mouse embryo (Wen et al., 2017). They suggested that Wnt signaling might be operating in both autocrine and paracrine ways in both lineages, since they identified transcripts of Wnt receptors, Wnt antagonists and other negative regulators of the pathway enriched in the DE group of cells in comparison to the mesodermal group, while Wnt agonists and at least one down-stream target of the pathway were enriched in the mesodermal group (Wen et al., 2017). Although they do not discuss the Notch pathway, from their RNAseq dataset (Table S6 in Wen et al., 2017) we notice that some components of the pathway were differentially regulated around the time when the segregation of the endomesoderm from the epiblast and the segregation of the endoderm and mesoderm from the endomesoderm is thought to occur. *Dll1*, *Dll3*, *Jag2*, *Hes6*, and *Dtx1* were down-regulated from early to late epiblast. A bit later, *Dll1* and *Dll3* were up-regulated, and *Dtx1* was down-regulated in the mesodermal lineage, whereas *Jag1* was up-regulated in the DE lineage in relation to the late epiblast cells, suggesting that Dll and Jag ligands might play different roles during endomesodermal segregation.

Given the apparent opposite roles of Notch and Wnt/nβ-catenin signaling in germ layer division across different phyla, it would be interesting to study their interplay in concert during this process in chordates.

Knock-out of several genes encoding members of the Notch pathway result in embryonic lethal phenotypes in mouse, with death occurring between E8.5 (early somite stages) and E12 (mid gestation, when caudal somitogenesis is still undergoing). They include *Notch1* (Conlon et al., 1995; Swiatek et al., 1994; de la Pompa et al., 1997; Zhang et al., 2002; Krebs et al., 2000; Krebs et al., 2003), *Notch2* (Hamada et al., 1999; Krebs et al., 2003), Dll1 (Hrabě de Angelis et al., 1997; Przemeck et al., 2003; Dunwoodie et al., 2002; Zhang et al., 2002), *Jag1* (Xue et al., 1999), *RBPJ* (CSL, zygotic and both maternal + zygotic) (Oka et al., 1995; de la Pompa et al., 1997; Barrantes et al., 1999; Souilhol et al., 2006), *Presenilin 1 + 2* (*Psen1* + *Psen2*, which encode the active component of γ-secretase, necessary for NICD signaling) (Donoviel et al., 1999; Herreman et al., 1999), and the O-fucosyltransferase encoding gene *Pofut1* (zygotic and both maternal + zygotic) (Shi and Stanley, 2003; Shi et al., 2005). Single knock-outs of *Psen1* (Wong et al., 1997) and *Jag2* (Jiang et al., 1998) are perinatally lethal, whereas knock-outs of *Dll3* (Kusumi et al., 1998; Dunwoodie et al., 2002; Zhang et al., 2002) and *Lfng* (Evrard et al., 1998; Zhang and Gridley, 1998; Zhang et al., 2002) survive postnatally (Tables S1 and S2). Although all germ layers are evidently present in these Notch pathway mutants, their derivatives do not develop properly. All of these mutants, together with *Dll1* and *Dll3* knock-outs, displayed defective somitogenesis and often showed an evident reduction in trunk/tail somite number, while *Jag1* and *Jag2* single knock-outs did not display somitogenic phenotypes, indicating that Jag ligands are not involved in somitogenesis. *Jag1* knock-out mouse embryos, on the other hand, show a severe vasculogenesis phenotype, but not the liver and heart abnormalities expected from the human mutations in this gene that are cause of the Alagille syndrome (Spinner et al., 2001), which indicate that *Jag1* indeed has a role in the development of mesoderm and endoderm derivatives in mammals. Deficient vasculogenesis was also observed in *Notch1*, *Notch1 + 4*, *Psen1* + 2 and *Pofut1* knock-outs, while *Dll1* and *Psen1* mutants showed severe hemorrhage, whereas defective cardiogenesis was found in *Notch1*, *RBPJ*, *Psen1 + 2*, and *Pofut1* knock-outs. Knock-outs with a general blockade of the Notch pathway (*RBPJ*, *Psen1 + 2*, *Pofut1*), and also, *Notch1*, *Notch2* and *Dll1* knock-outs show defects in neural development. However, a detailed study of germ layer recruitment, delimitation and allocation is lacking in mammals. Since maternal and zygotic *RBPJ* and *Pofut1* null embryos were indistinguishable from wild types or mutant heterozygotes at the onset of gastrulation, it was concluded that canonical Notch signaling is dispensable for preimplantation development, blastogenesis and the formation of germ layers prior to gastrulation in mouse (Shi et al., 2005; Souilhol et al., 2006). However, this conclusion was based on morphological criteria, without analysis of early markers of germ layer commitment. In addition, these approaches did not rule out the possibility that non-canonical Notch signaling could play an early role. Addressing this issue would require conditional gene deletion in oocytes of the four Notch receptors and/or the five ligands, which was not yet achieved. Nevertheless, in vitro studies in embryonic stem cells (ESc) indicate that Notch indeed plays a role in early germ layer commitment (see below). Moreover, in vivo studies in mouse showed more recently that Notch and the Hippo pathway are involved in the earliest cell-fate choice in mammals, cooperating in the specification of the trophectoderm lineage. Notch is active, while the Hippo pathway is inactive in the outer cells of the blastocyst. The trophectoderm enhancer of Cdx (which determines trophectoderm specification) contains functional binding sites for RBPJ and TEAD4 (the transcription factor involved in the Hippo pathway when Hippo signaling is down-regulated). This indicates that *Cdx* is another direct target of the Notch pathway which does not belong to the HES/HEY family. Notably, homozygous double knock-out embryos for both transcription factors were not recovered at E3.5, indicating that they died at an earlier stage, precluding the analysis of cell allocation in the blastocyst. However, double heterozygote embryos (*Rbpj*$^{+/-}$; *Tead4*$^{+/-}$) contained significantly fewer cells expressing a gene reporter under the *Cdx* trophectoderm enhancer than did control embryos, and this effect was more marked in *Rbpj*$^{-/-}$; *Tead4*$^{+/-}$ embryos. Although the number of Cdx+ cells was not affected, endogenous Cdx expression was decreased in these compound mutants, indicating that other pathways converge in Cdx regulation. Forced activation of NICD1 up-regulated *Cdx* and changed the allocation of ICM cells to the trophectoderm, indicating that Notch is involved in the earliest cell-fate choice in mammalian development, favoring allocation to the trophectoderm lineage vs. the ICM lineage (Rayon et al., 2014).

In particular, it was not analyzed whether there was an unbalanced allocation of cells among the different germ layers during gastrulation or axial elongation in knock-out mice of the Notch pathway, with the only exception of DML derivatives in *Dll1*$^{-/-}$ embryos (see below). However, there are some hints that might point to this (Tables S1 and S2). We notice, for example, that the expression of neurofilaments appears relatively more restricted whereas endothelial *Kdr*+ cells (a mesodermal lineage; synonym: *Flk*) and the expression of the mesodermal markers *Twist* and *Meox1* (synonym: *Mox1*) looks relatively more extended in *Notch1* knock-outs than in wild type embryos (Figs. 4 and 6 in Swiatek et al., 1994; Fig. 6 in Conlon et al., 1995). Interestingly, in the case of the *Dll1* knock-out, the authors noticed a hyperplasic CNS (Przemeck et al., 2003), whereas in a previous work, these mutants showed down-regulation and diffuse expression of sclerotome and dermomyotome markers (Hrabě de Angelis et al., 1997). In addition, the expression of other members of the Notch pathway was significantly perturbed in *Dll1* knock-out embryos: *Notch1* (which at the stage analyzed marks the primitive streak and nascent presomitic mesoderm) and *Lfng* domains were expanded and invaded the node, while *Notch2* was down-regulated in the node and also showed an unrestricted expression in the surrounding tissues (Przemeck et al., 2003). It is also intriguing that some markers that are normally expressed in the presomitic or somitic mesoderm are completely down-regulated from these tissues but ectopically activated in the neural tube in mouse embryos with complete blockade of the Notch pathway (*RBPJ*, *Psen1 + 2*, *Pofut1* knock-outs) and also in *Notch1* knock-outs. This might suggest that although cells are able to allocate to the neural-ectoderm layer in the absence of Notch signaling, they are not able to operate properly the gene network corresponding to this layer but continue expressing incorrectly some genes of the PSM/paraxial mesoderm specification network. Interestingly, massive cell death that causes embryonic lethality first appears in the neural ectoderm in *Notch1* and *Notch2* knock-outs, and it is known that apoptosis eliminates misplaced or mis-specified cells (Fuchs and Steller, 2011; Aguirre et al., 2013). Therefore, although loss of function of the Notch pathway was not apparently sufficient to completely divert cell allocation between neurectoderm and paraxial mesoderm, some specification cues might be misplaced between germ layers.

Notably, it was recently shown that forced activation of *Notch1* in the epiblast and its derivatives by directed expression of NICD1 exclusively in the epiblast (NICD1^{epi}) resulted in severe gastrulation defects, impairment of axial mesoderm and anterior definitive endoderm formation, with loss of other mesodermal lineages (cardiac, hemangioblast and endothelial precursors), with a concomitant enhancement of neurectoderm development (Souilhol et al., 2015), suggesting a possible role of Notch in cell fate choices among germ layers in mouse. This is supported by evidence from in vitro experiments with mammalian embryonic stem cells (ESc).

Mouse embryonic stem cells (mESc) are isolated from the inner cell mass of the pre-implantation blastocyst and can be maintained indefinitely in vitro. They exhibit a hallmark of naive pluripotency, consisting in their ability to contribute to all embryonic cell lineages, including germ cells, in chimeric embryos. They are a useful tool to

study peri-implantation development, since they mimic the morphological differentiation from epiblast to egg-cylinder stage in culture (Niwa, 2010). Transcripts of *Notch1* and its ligands *Jag1*, *Jag2* and *Dll3* were readily detected in positively selected undifferentiated mouse ESc (mESc). Interestingly, cell sorting showed that around half of the undifferentiated mESc expressed *Jag1* (Lowell et al., 2006).

By performing gain- and loss-of-function studies in human ESc (hESc) through genetic approaches and pharmacological treatments with a γ-secretase inhibitor, Yu et al. (2008) proposed that Notch is necessary during the transition from self-renewal to cell lineage commitment. They proposed that it first promotes the commitment of hESc to form the progeny of the three germ layers, while inhibiting the alternative trophoblast cell fate at this stage, resembling the phenomenon of lateral inhibition (Yu et al., 2008). In addition to this role, Notch is required later in another cell fate choice between germ layers, as discussed below.

Overexpression of NICD in mESc did not change the stem cell phenotype. However, when they were withdrawn of self-renewal and serum factors, NICD increased their rate and frequency of neural specification, concomitantly inhibiting pluripotency and also, mesodermal and endodermal specification. Interestingly, while control mESc cultures showed a random distribution of neural committed cells in a "salt and pepper" pattern, neural commitment was more uniform in NICD mESc cultures. In contrast, blocking Notch signaling with a γ-secretase inhibitor initially delayed lineage commitment, but after 5 days of treatment, mESc were progressively diverted to a nonneural fate. Consistent with this, *RBPJ*-null mESc retained pluripotency or initiated nonneural differentiation. However, although both interfering approaches inhibited the neural fate, they did not completely abolish it (Lowell et al., 2006). Moreover, when human ESc (hESc) were co-cultured with cells expressing Dll1 in the absence of self-renewal factors, hESc adopted a neural fate, and this effect was blocked by treatment with a γ-secretase inhibitor. Thus, Delta-Notch signaling also promotes neural development in hESc (Lowell et al., 2006). These authors proposed that in ESc, Notch promotes lateral induction, a kind of community effect that guarantees that cells adopt the same fate in certain contexts. In this way, Notch would amplify and consolidate neural specification by promoting the transition of ESc to mature epiblast and then, by synchronizing the timing when mature epiblast cells enter in the neural pathway, concomitantly inhibiting nonneural fates (Lowell et al., 2006). This is supported by other studies in mESc showing that: 1) NICD inhibits the expression of the mesodermal markers *Tbxt* (synonyms: *Brachyury*, *T*), *FGF8* (Nemir et al., 2006) and *Kdrl* (*Flk1*) (Schroeder et al., 2006) while concomitantly, cells adopt a neural fate (Nemir et al., 2006); 2) Notch inhibition by CSL^{neg} resulted in an up-regulation of *Tbtx* and *Fgf8*, while the neural fate was inhibited (Nemir et al., 2006). Time-controlled experiments indicate that activated Notch acts at the initial stages of ESc differentiation to block mesodermal differentiation (Schroeder et al., 2006).

The Notch ligand preference during mESc commitment into different germ layers was studied in another work by exposure to immobilized Dll4 or Jag1 (Ramasamy and Lenka, 2010). Usually, mESCs are maintained in a self-renewal state with LIF in serum-containing medium. While BMP (present in serum) inhibits neural commitment, LIF inhibits non-neural commitment. These authors found that even under maintenance conditions (with LIF), both ligands induced mESc differentiation, as judged by cell morphology and the down-regulation of the pluripotency marker Oct4. In these conditions, both ligands further inhibited early endodermal markers. In addition, Dll4 inhibited early ectodermal markers, and Jag1, early mesodermal markers. However, Jag1 showed a contrasting dose-dependent response on neural commitment, favoring specification of neural progenitors at the lower dose, but inhibiting neural commitment at the higher dose. In the absence of LIF, Dll4 promoted mesodermal commitment at both assayed doses and endodermal commitment at the lower dose only. In contrast, Jag1 favored neural and inhibited mesodermal commitment. Interestingly, neural commitment induced by Jag1 was correlated with enhanced Hes5 and inhibited Hey expression, while mesodermal commitment induced by Dll4 was correlated with the opposite changes in the expression of these Notch target genes (Ramasamy and Lenka, 2010).

Hes1 belongs to a very well-known family of Notch target genes and behaves as so in most contexts. However, it has been proved that in mESc, *Hes1* expression is under the control of the leukemia inhibitory factor (LIF) and BMP but not of Notch signaling. Moreover, *Hes1* expression oscillates in mESc with a period of about 3–5 h, and cell sorting demonstrated that when expression is high, they tend to adopt a mesodermal progenitor fate, whereas when the expression is low, they are prone to adopt a neural fate. Furthermore, *Hes1*-null mESc were diverted to the neural fate earlier and in a more uniform way than wild type cells, consistent with the idea that this gene is not under the control of the Notch pathway in this context, since inactivation of Notch induces mesodermal specification in mESc (Kobayashi et al., 2009). Moreover, ESc with sustained *Hes1* expression did not adopt the neural fate even in neural induction conditions, but chose the early mesodermal fate instead and down-regulated *Dll1* and *Jag1* expression. ChIP-chip analysis (chromatin immunoprecipitation followed by microarray analysis) confirmed that Hes1 protein directly binds *Dll1* and *Jag1* promoter regions (Kobayashi and Kageyama, 2010). Therefore, *Hes1* oscillation contributes to the heterogeneity of cell fate choices in mESc. When differentiation signals appear, high, sustained *Hes1* expression promotes mesodermal progenitor fates and inhibits neural fates by directly repressing Notch ligands, whereas at low *Hes1* levels, mESc tend to adopt the neural fate (Kageyama et al., 2010).

Canonical Wnt/nβ-cat signaling is absolutely required in vivo in the epiblast for primitive streak and mesoderm formation, since they are absent from knock-out mice embryos of β-cat or *Wnt3* (Huelsken et al., 2000; Liu et al., 1999). Inhibition of this pathway during differentiation of mESc first inhibited the expression of primitive streak markers and then blocked mesoderm and endoderm specification, as demonstrated by transcriptome analysis by microarrays, indicating that canonical Wnt is necessary for the formation of both germ layers. However, a stabilized β-cat form did not induce expression of primitive streak markers in vitro, indicating that in differentiating mESc, Wnt/nβ-cat modulates the responses to other effector pathways (Lindsley et al., 2006). Notably, under these differentiation conditions, a membrane-bound Notch, which is not cleavable by γ-secretase and antagonizes active β-cat by titration, significantly decreased the number of mesodermal precursors, as judged by the expression of Tbxt (Kwon et al., 2011). These results indicate that Notch inhibits mesoderm specification in mESc by a non-canonical, non-transcriptional mechanism, involving degradation of β-cat. In addition, in vitro studies have shown that activation of Wnt/β-cat drives mouse epiblast stem cells (mEpiSCs) and human embryonic stem cells (hESCs) towards endomesoderm specification, while active repression of this pathway promotes neuroectoderm specification in mEpiSCs (de Jaime-Soguero et al., 2018).

In overall, an interesting picture emerges from all these studies about the iterative role of the Notch pathway during early cell-fate commitment and germ layers segregation in mammalian development and its antagonistic relationship with the Wnt/β-cat pathway. We integrate all this evidence in the model proposed in Fig. 6B.

While segregation of endoderm, anterior neural plate and craniocervical paraxial mesoderm from the pluripotent epiblast occurs during gastrulation, bipotential neuromesodermal progenitors (NMPs) with self-renewal ability persist from early somite stages throughout posterior axial elongation. They are the source of the cells that populate the posterior neural plate and paraxial mesoderm caudal to the sixth somite level from around E8.0–E8.5 onwards (Tzouanacou et al., 2009; Takemoto, 2014). These NMPs are present in the caudal end of the embryo, in the node-streak border (NSB) and caudal lateral epiblast (CLE) adjacent to the primitive streak and later, in the chordoneural hinge (CNH) of the tail bud, which descends from the NSB and appears

at E10.0. These NMPs provide the source for elongation, since they progressively generate more posterior cells that populate the spinal cord and somites in the trunk and tail region (Cambray and Wilson, 2002; Cambray and Wilson, 2007; Wilson et al., 2009; Takemoto, 2014). Grafting experiments demonstrated that the neural or the mesodermal fate choice or the retention in the progenitor region of these NMPs depend on local cues and are not cell-intrinsic (Wymeersch et al., 2016). In vivo experiments combining lineage tracing and a conditional β-cat knock-out allowed to discern that Wnt/β-cat signaling is necessary for these NMPs populations to adopt the mesodermal fate (Wymeersch et al., 2016). Nascent mesodermal cells produce retinoic acid (RA), which is required to initiate NMPs generation and to induce neural differentiation, whereas single cell transcriptome analysis of CLE showed that the presomitic mesodermal lineage up-regulates *Dll1* in relation to NMPs (Gouti et al., 2017). Although not pointed out when the expression patterns were initially described, we notice that transcripts for the three Notch receptors persist around the primitive streak in the CLE, and *Notch2* transcripts also persist in the node, including the NSB (see Del Amo et al., 1992; Williams et al., 1995; Barrantes et al., 1999). Both *Dll1* and *Dll3* expression continues during caudal elongation in the primitive streak, surrounding the node, coincident with the CLE territory, and later continues in the tail bud (Bettenhausen et al., 1995; Dunwoodie et al., 1997), whereas *Jag1* and *Lfng* expression in the CLE continued during elongation (Zhang and Gridley, 1998; Johnston et al., 1997).

While embryonic lethal phenotypes of knock-outs of the Notch pathway precluded an exhaustive analysis of the development of germ layers derivatives, *Psen1*, *Dll3* and *Lfng* knock-outs show severe posterior vertebrae truncations (Wong et al., 1997; Kusumi et al., 1998; Dunwoodie et al., 2002; Zhang et al., 2002; Evrard et al., 1998; Zhang and Gridley, 1998; Zhang et al., 2002) (Tables S1 and S2). Embryonic lethal phenotypes resulting from a general blockade of the Notch pathway, like those of *RBPJ*, *Psen1 + 2* and *Pofut1* knock-outs, show posterior truncations with developmental arrest (Oka et al., 1995; de la Pompa et al., 1997; Barrantes et al., 1999; Souilhol et al., 2006; Donoviel et al., 1999; Herreman et al., 1999; Shi and Stanley, 2003; Shi et al., 2005) (Tables S1 and S2). Thus, Notch signaling is absolutely required for posterior axial elongation, suggesting a role in the maintenance of the NMP stem cells (Wilson et al., 2009).

Although the functional significance of the *Notch/Dll/Jag/Lfng* landscape around the node during gastrulation and axial elongation has neither been functionally explored nor discussed in terms of germ layer segregation, the *Lfng* domain at late streak gastrula corresponds to the region where the anterior *Jag1* and the posterior *Dll1* and *Dll3* meet around the node, suggesting that *Lfng* could be modulating *Dll/Jag1* signaling at this border, perhaps in relation with segregation of neuromesodermal precursors or with endomesoderm segregation. Thus, it would be interesting to thoroughly study the role of the Notch pathway in germ layer segregation during gastrulation and caudal elongation and its relationship with the β-cat pathway.

5.5. The dorsal midline in vertebrates

In vertebrate embryos, the dorsal midline (DML) is a key signaling center for the development of the surrounding tissues. Signals emitted by the DML are required for the specification of ventral neural fates and the sclerotome, proliferation and survival of neural precursors, axonal pathfinding, and patterning of the axial vasculature. Several studies in the past were focused on the segregation of the vertebrate organizer's descendants into the components of the DML that are progressively allocated in the three germ layers during gastrulation: the medial floor plate (MFP) in the midline of the neural plate, the notochord (axial mesoderm), and the dorsal midline of the endoderm (in amniotes) or the hypochord (in anamniotes) (discussed in López and Carrasco, 2006). In particular, the role of Notch signaling in the segregation of the DML components was addressed in fish, amphibian, avian, and mammalian embryos.

5.5.1. Zebrafish

Floor plate, notochord and hypochord precursors are intermingled in the zebrafish organizer (Shih and Fraser, 1995; Shih and Fraser, 1996; Melby et al., 1996; Latimer et al., 2002; Latimer and Appel, 2006). This intermingling appears to be regionalized. While cells occupying the dorsal margin of the shield give rise almost exclusively to notochord descendants, shield cells located at a distance or 4-8 cell diameters above the dorsal margin give rise to notochord and floor plate descendants, and cells located at the lateral edges of the shield give rise to notochord and hypochord descendants in the trunk (Fig. 4A). Specified hypochord cells then migrate toward the midline during gastrulation, extending in a row ventral to the notochord (Latimer et al., 2002; Latimer and Appel, 2006).

Mutant embryos carrying a missense mutation that substitutes a critical cysteine in an EGF repeat of the extracellular domain of *dla* (*dla*^{dx2}) have reduced numbers of floor plate and hypochord cells and an excess of notochord cells in the trunk. Mutants for the ubiquitin ligase mind bomb (which is necessary for Delta endocytosis and Notch signaling) show a similar phenotype, whereas overexpression of *dla* results in the opposite phenotype (Appel et al., 1999). This suggested that the midline progenitors require Delta signaling prior to germ layer segregation in order to establish the correct proportion of cells that will populate the floor plate, the notochord and the hypochord, favoring floor plate and hypochord development and disfavoring notochord formation (Appel et al., 1999) (Fig. 4B). However, Notch1a MO only disrupted hypochord development, whereas notochord and floor plate appeared normal at pharyngula stage (Appel et al., 2003). Recent in vitro experiments in the mouse-derived cell line NIH3T3 demonstrated how Notch1 discriminates between ligands by being differentially modified by three members of the Fringe glycosyltransferases in the extracellular domain. Glycosylation by Lunatic fringe (Lfng), for example, enhances activation of Notch1 by Delta and inhibits Notch1 activation by Jagged (Kakuda and Haltiwanger, 2017). Interestingly, *lnfg* is expressed in the fish organizer, and knock-down experiments showed that it is necessary for hypochord specification, since the expression of a *hes5* ortholog (called in zebrafish *her4.1*) was suppressed at late gastrula stage (Appel et al., 2003). Although control MO-injected embryos were not shown to compare the expression patterns of early markers of specification of other midline populations with those of lnfg MO-injected embryos, the authors concluded that lnfg does not control medial floor plate, notochord or adaxial muscle specification, since the expression patterns of *twhh*, *tbxt* and *myoD* appeared normal at late gastrula stage (Appel et al., 2003). On the other hand, embryos lacking either *dld* or *dlc* function showed reduced hypochords in an incompletely penetrant and variable way. However, embryos lacking both *dlc* and *dld* functions showed very few hypochord cells at pharyngula stage and down-regulated *her4*.1 in hypochord precursors at late gastrula stage, while development of the floor plate was unaffected at pharyngula stage. While knock-down of *dla* seldom reduced the hypochord, nearly all *dld* mutants injected with dla MO showed reduced hypochords, indicating that *dla* also contributes to hypochord development, as was previously shown with *dla* mutants. In addition, constitutive activation of Notch signaling with *Xenopus* NICD1 resulted in ectopic expression of *her4.1* and suppression of *tbxt* (synonyms: *brachyury*, *ntl*) in a cell-autonomous way at late gastrula stage (Latimer et al., 2002). Consistent with these findings, time-controlled activation of zebrafish NICD1a at the shield stage, but not at tailbud stage, resulted in an expansion of the hypochord and a reduction of the notochord without apparently affecting the floor plate when the midline structures were analyzed at the pharyngula stage. However, time-controlled blocking of CSL function at the shield stage (but not at late gastrula) decreased the number of floor plate cells and almost deleted the trunk hypochord (analyzed at pharyngula stage). Moreover, inhibition of the γ-secretase-dependent cleavage of Notch with DAPT

treatment at the shield stage resulted in an important reduction in the number of floor plate cells and in nearly complete suppression of the hypochord when embryos were analyzed at pharyngula stages. These effects were gradually lost when DAPT was applied progressively later during gastrulation. In addition, cell lineage experiments employing photoactivation of a caged fluorescein tracer in small clusters of cells in the dorsal margin of the shield (which normally contains only notochord precursors) revealed that they changed their fate to hypochord (but not no floor plate) when NICD1a was activated at the shield stage. BrdU and phospho-histone-H3 labelling in embryos treated with DAPT at 15-somites stages indicates that Notch is necessary to maintain the number of floor plate cells by promoting their proliferation, rather than by promoting specification during gastrulation (Latimer and Appel, 2006). Thus, these authors proposed that Dlc and Dld signaling from the presumptive paraxial mesoderm activates Notch/*hes5* (*her4.1*) in the lateral edges of the organizer, inducing hypochord specification while suppressing the notochordal fate, and that that Notch is necessary to maintain the number of floor plate cells by promoting their proliferation, rather than by promoting specification during gastrulation, which would be in charge of Nodal signaling, instead (Latimer et al., 2002; Latimer and Appel, 2006). However, a role for Delta/Notch signaling in a cell fate choice between floor plate and notochord fates in zebrafish cannot be completely ruled out from these findings. The hypothesis proposed by these authors does not explain why the floor plate precursors population is most sensitive at the beginning of gastrulation in DAPT treatments, suggesting that a cell-fate choice involving Delta/Notch signaling might be taking place between notochord and floor plate, as previously suggested by *dla* and *mind bomb* mutants. Moreover, the effect on floor plate cells proliferation was assessed in embryos treated with DAPT at post-gastrulation stages. To address whether Notch signaling is required for binary cell fate choices between notochord and floor plate in fish embryos, it would be necessary to perform lineage tracing experiments by photoactivation in the region of the shield where floor plate and notochord fates are indeed mixed, comparing the allocation of descendants in the notochord and in the floor plate in control vs. DAPT-treated or CSL^{neg} transgenic embryos. In addition, multiple knock-down of notch receptors and analysis of an early marker of floor plate specification (like *twhh* in Appel et al., 2003 or *hes4* in *Xenopus*, see below) during gastrulation, when cell fate choices between germ layers are taking place, rather than analyzing the effects at later stages, would help to clarify this issue. In overall, the current evidence strongly supports the idea that Dlc and Dld from the presumptive paraxial mesoderm promotes hypochord at the expense of notochord development at the lateral edges of the organizer, while *dla*, which is transiently expressed in involuting cells in the organizer, appears to be also involved in notochord vs. floor plate cell fate binary decisions (Fig. 4B).

5.5.2. Xenopus

Several experimental perturbations of the Notch pathway, including N^{act} and time-controlled experiments with $Notch1^{act}$, Notch1 MO, CSL^{neg}, $Dll1^{neg}$, presenilin overexpression and knock-down, hes4 overexpression and knock-down indicate that during gastrulation, Notch promotes floor plate development at the expense of the notochord (López et al., 2003; López et al., 2005). In addition, by injecting $Notch1^{act}$ and CSL^{neg}, other authors later showed that, besides promoting FP and inhibiting notochord development, Notch also promotes hypochord formation (Peyrot et al., 2011). Considering this result, we can now update the model earlier proposed in (López et al., 2003) and (López et al., 2005) about the role of Notch signaling in controlling the allocation of SMO descendants in the three derived structures that populate the DML. The early SMO contains multipotential cells that may choose between MFP, notochord, or hypochord fates. *Dll1* expression starts at early gastrula in scattered cells on the SMO and in the non-SMO presumptive mesoderm and interacts with the Notch receptor in the surrounding cells, leading to the activation of hes4 and perhaps other related Notch targets which in turn repress the GRN that controls notochord development. Hes4 concomitantly impedes the movement of involution, and hes4 + cells gradually incorporate into a growing arc of hes4 + cells in the DNIMZ. This arc ultimately converges and extends along the anterior-posterior axis, forming the notoplate (prospective FP). By this mechanism involving *notch* and *hes4*, *dll1* executes a cell-fate switch that favors FP development at the expense of the notochord. On the other hand, Notch also favors hypochord development at the expense of the notochord, but the down-stream mechanism is unknown, since *hes4* is not expressed in hypochord precursors (Fig. 5C).

5.5.3. Birds

In avian embryos, Notch signaling controls the balance of progenitor cells that the Hensen's node contributes to the notochord and the floor plate, favoring floor plate development at the expense of the notochord (Gray and Dale, 2010), similar to what happens in *Xenopus* (López et al., 2003; López et al., 2005). This process depends on CSL and γ-secretase, as both CSL^{neg} electroporation and DAPT treatments altered the proportion of the Hensen's node descendants that populated each structure, increasing its contribution to the notochord (Gray and Dale, 2010). It is not known which is the Notch target gene that executes this decision, but at least, *hes1* (formerly known as *hairy2* in chicken) is expressed at the right time and the right place to be a good candidate. *Hes1* transcripts are present in the Hensen' node and its descendants, with much higher levels in the floor plate than in the notochord, and are down-regulated by DAPT treatments (Gray and Dale, 2010). However, functional evidence is still lacking to be sure that *hes1* mediates Notch signaling in controlling the allocation of the Hensen's node descendants along the DML in birds. Neither is known the ligand(s) involved. Interestingly, *dll1* transcripts are highly abundant along the primitive streak, including cells just posterior or around the Hensen's node, whereas the node itself seems to lack or expresses very low levels of *dll1* transcripts (Caprioli et al., 2002). This resembles to what happens in Cephalochordates, *Xenopus*, zebrafish and mouse, with strong *dll1* expression around the blastopore/primitive streak except in the organizer/node, where *dll1* is found in a few isolated cells in a salt-and-pepper pattern in *Xenopus* or was not detected by ISH in the other models (see above). This indicates that *dll1* expression must be differentially regulated in the places where axial or non-axial mesodermal cells are internalizing.

The strong accumulation of *notch1*, *notch2*, *delta1*, and *hes1* transcripts in the primitive streak of chick embryos during gastrulation (Caprioli et al., 2002; Gray and Dale, 2010) indicates that, apart from the role in DML cells allocation, Notch signaling plays a more general task during germ layers formation and/or segregation in avian embryos. However, we were unable to find relevant experimental data in the literature functionally addressing this hypothetical role in birds.

5.5.4. Mammals

The prechordal plate, the notochord, the floor plate and the dorsal midline of the endoderm descend progressively in the cranial-caudal direction from the mouse gastrula organizer, which appears anterior to the early streak (early gastrula organizer) and then locates in the anterior tip of the late streak (late gastrula organizer/node) (anterior primitive streak/node). While floor plate cells arise from the dorsal layer of the node, the endodermal descendants arise from its ventral layer (Beddington, 1994; Sulik et al., 1994; Wilson and Beddington, 1996; Kinder et al., 1999; Tam and Gad, 2004; Arnold and Robertson, 2009).

Forced activation of Notch1 in the epiblast ($NICD1^{epi}$) impairs the formation of the organizer and causes a loss of its DML derivatives in mouse, including prechordal plate, notochord, anterior definitive endoderm and floor plate (Souilhol et al., 2015). Consistent with this, *Notch1* transcripts are excluded from the node (see above) and in *Notch1* knockouts, the notochord is specified, as shown by *Tbxt* expression (Conlon et al., 1995). This indicates, that *Notch1* expression in

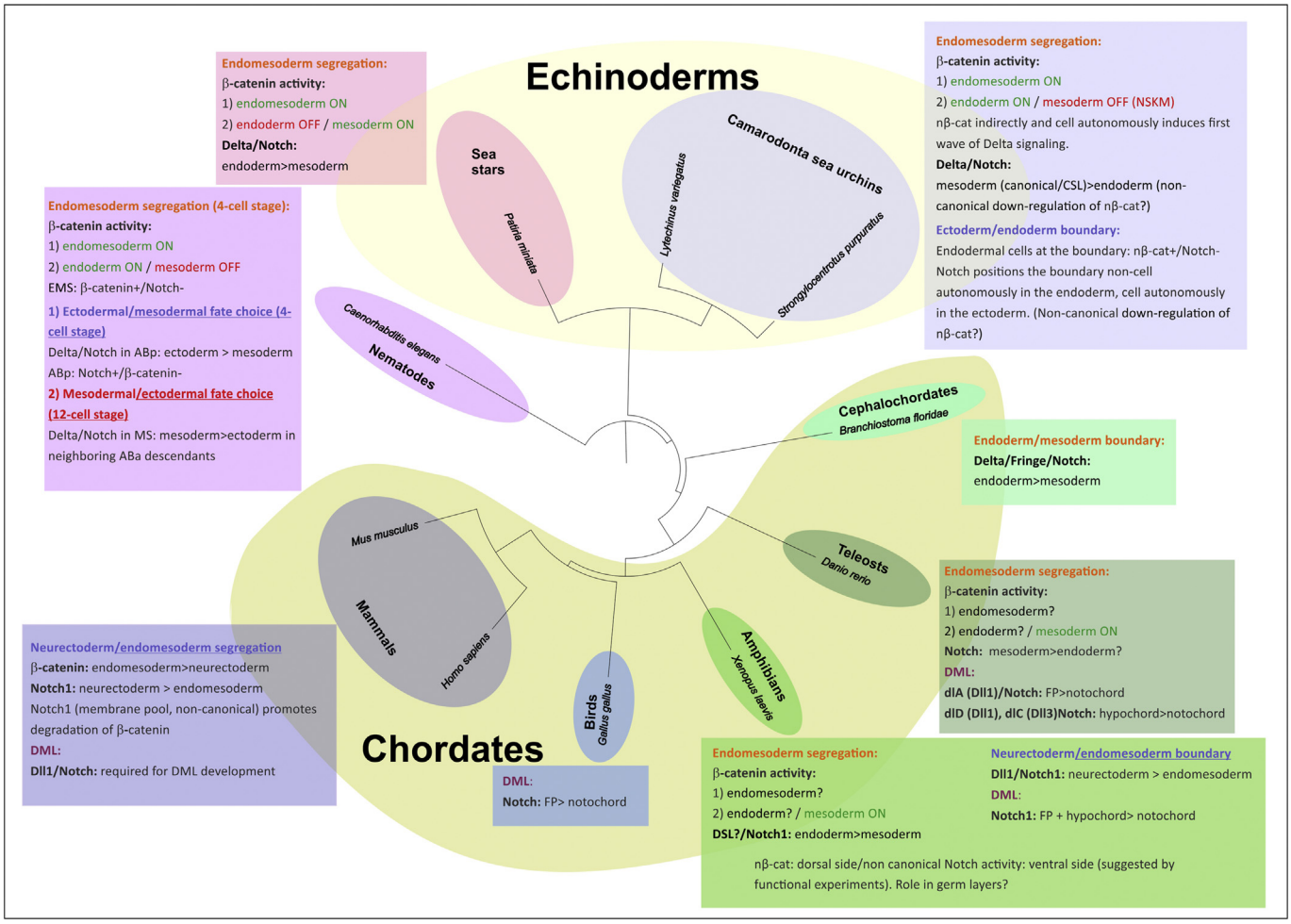

Fig. 7. Summary of Notch and β-cat signaling in germ layers segregation in bilaterians. A phylogenetic tree including the representative bilaterian models discussed in this review was generated with PhyloT: http://phylot.biobyte.de/

the primitive streak is rather related to development of non-organizer derived tissues in mouse and must be actively suppressed in the node for a normal development of its derivatives.

Loss-of-function of *Dll1* results in an excess of floor plate cells, while the notochord is reduced (Przemeck et al., 2003). In contrast, double homozygous mutants for *Psen1* and *2* lack floor plate but the notochord is present in some sections or is impossible to discern from the disorganized tissue in others. The ventral neural tube is disorganized and strongly express ectopic *Dll1* in a continuous A-P domain (which, at the stage analyzed, is normally expressed in a continuous fashion in the presomitic mesoderm), whereas the somitic mesoderm is also completely disorganized (Donoviel et al., 1999). This suggests that Notch activity might be required for FP specification, as shown in anamniotes. The opposite activity of Dll1 signaling in mouse vs. anamniotes embryos are intriguing. Nevertheless, the present evidence suggests that the Dll/Notch pathway is required in the development of the DML structures in mammals.

6. Conclusions

The main points of this work are summarized in Fig. 7. We have seen that the Delta/Notch pathway has a central role in mesoderm or endoderm induction and specification in invertebrates, with entire germ layers nearly absent when the pathway is blocked. In vertebrates, however, where induction of endomesoderm is mainly in charge of Nodal (Kiecker et al., 2016), DSL/Notch (perhaps with non-canonical routes involved) rather seems to be employed in refining the limits between germ layers.

Overall, whether lateral inhibition or lateral induction, in the strict sense of negative or positive feed-back regulation of the DSL ligands by Notch, respectively, operate in limiting germ layers in metazoans remains poorly understood (Table S3). However, we have seen a clear example of lateral induction in Camarodonta sea urchins, where a wave of Delta activation is responsible of the propagation of mesodermal specification. It would be necessary to study the precise patterns of ligands and receptors in space and time at the cell level and how Notch signaling regulates the expression or activity of DSL ligands to understand if this pathway controls the delimitation of germ layers by lateral inhibition or lateral induction. Moreover, it might be that this simplistic classification is insufficient to describe the role of the Notch pathway in the segregation of germ layers (see Introduction). In addition, understanding if Notch plays an instructive or a permissive role in favoring one from two alternative cell fates during germ layers segregation would help to clarify this point. More extensive work at the level of the GRN in the different models is required to solve this issue.

The localization of nβ-cat is causally related with the gastrulation site in metazoan development. There have been reorientations in nβ-cat location in relation to the animal-vegetal axis during evolution. While in pre-bilaterians, nβ-cat accumulates in the animal hemisphere (from where the endoderm arises, unlike in bilaterians), in non-chordate deuterostomes like echinoderms, it is found in the vegetal hemisphere. On the other hand, in chordates like *Xenopus* and zebrafish, maternal nβ-cat is first concentrated in the dorsal region, encompassing both animal and vegetal domains (reviewed in Martindale, 2005), being

functionally involved in the induction of the dorsal center (Weaver and Kimelman, 2004; Hikasa and Sokol, 2013). While the central role of nβ-cat in specifying endomesoderm vs. ectoderm is clear in most invertebrate models and seems ancestral, such a role remains more elusive in vertebrates (Schneider and Bowerman, 2013), perhaps obscured by the co-option of maternal nβ-cat in dorsal specification pathway. However, In *Xenopus* blastulae, nβ-cat accumulates in a ring coinciding with the prospective mesoderm (Schohl and Fagotto, 2002). Although nβ-cat is insufficient to induce mesoderm in ectodermal explants, both maternal and zygotic β-cat are required for proper *tbxt* expression in the marginal zone and for mesoderm formation (Vonica and Gumbiner, 2002; Schohl and Fagotto, 2003). Notably, nβ-cat was shown to accumulate by mechanical forces generated during epiboly in zebrafish, promoting mesoderm specification (Brunet et al., 2013). The emerging picture shows that nβ-cat is necessary for neurectoderm vs. endomesoderm fate choices in mammals, thus suggesting a conserved role for nβ-cat in endomesoderm vs. ectoderm decisions in metazoans. Notch has the opposite role in comparison to nβ-cat, promoting neuroectoderm vs. endomesoderm decisions both in mammals and amphibians. Thus, an antagonistic balance between these pathways seems to establish the boundary between these germ layers in these vertebrate models, whereas in Camarodonta sea urchins, where the prospective endoderm is adjacent to the prospective ectoderm, Notch places the boundary between ectoderm and endoderm. Interestingly, resembling mammals and amphibians, Notch is also required for the establishment of the ectoderm/mesoderm boundary in arthropods. In *Drosophila*, Notch is involved in positioning the limit between the neurectoderm and the mesoderm, by promoting mesectoderm specification in a single row of cells at the border, from which the fly glia and some additional neurons will arise (Martín-Bermudo et al., 1995; Morel and Schweisguth, 2000). In the spider, the posterior half of the body develops as the result of growth of the domain surrounding the blastopore through progressive activation of the Delta/Notch pathway. Delta is expressed in some prospective mesodermal cells and activates Notch in neighboring cells, which are prevented from adopting a mesodermal fate while being instructed towards a caudal ectoderm fate (Oda et al., 2007). Interesting analogies were proposed between posterior growth in spiders and axial elongation in vertebrates (Oda and Akiyama-Oda, 2008). Thus, it will be interesting to investigate whether Delta/Notch signaling has an analogous role in neuroectoderm vs. mesoderm segregation during axial elongation.

In invertebrates, different combinations of a binary switch ON/OFF involving Notch and nβ-cat were proposed to underlie endomesoderm specification and segregation (McCauley et al., 2015). Whereas nβ-cat specifies endomesoderm in the first step, the output of β-cat and Notch signaling in the second step (segregation) varies among taxa. This is summarized in Fig. 7, where we add other models to compare. The most striking example is the difference between echinoderm models. While during segregation, Camarodonta sea urchins employ Delta/Notch to specify mesoderm and nβ-cat to specify endoderm, the opposite occurs in the sea stars. A similar situation emerges when comparing amphibians and teleosts: while Notch favors endoderm vs. mesoderm in *Xenopus*, it seems to do the opposite in zebrafish, although this remains to be confirmed for the latter. Down-regulation of endodermal markers by N^{act} was observed on the second half of gastrulation in zebrafish (Kikuchi et al., 2004), whereas expansion of endodermal precursors was observed during the first half of gastrulation in *Xenopus* (Revinski et al., 2010), and it is known that Notch signaling can elicit opposite effects on different germ layers derivatives depending on time (Glavic et al., 2004; Contakos et al., 2005; Revinski et al., 2010).

Further investigation is needed to understand if Notch and nβ-cat pathways interact in the segregation of germ layers in vertebrates. The relationship between Notch and nβ-cat seems to be conserved in these taxa. For example, a ventral Notch activity restricts the dorsal center in the *Xenopus* blastula by destabilizing β-cat in a non-canonical fashion, independent of β-catenin phosphorylation by gsk3β (Acosta et al., 2011) and Notch1 protein and mRNA are enriched in the ventral region from the beginning of embryogenesis (Castro Colabianchi et al., 2018). Moreover, it was shown that Notch inhibits mesoderm specification by this non-canonical mechanism in mESc (discussed above) (Kwon et al., 2011). However, it is not known if such mechanism contributes to the strong inverse correlation between Notch and nβ-cat accumulation that underlies the key steps in the delimitation of germ layers in invertebrates.

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