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Abstract

This chapter discusses our current knowledge on the major segregation events that lead to the individualization of the building blocks of vertebrate organisms, starting with the segregation between "outer" and "inner" cells, the separation of the germ layers and the maintenance of their boundaries during gastrulation, and finally the emergence of the primary axial structure, the notochord. The amphibian embryo is used as the prototypical model, to which fish and mouse development are compared. This comparison highlights a striking conservation of the basic processes. It suggests that simple principles may account for the formation of divergent structures. One of them is based on the non-adhesive nature of the apical domain of epithelial cells, exploited to segregate superficial and deep cell populations as a result of asymmetric division. The other principle involves differential expression of contact cues, such as ephrins and protocadherins, to build up high tension along adhesive interfaces, which efficiently creates sharp boundaries.

Keywords

Morphogenesis, vertebrate development, gastrulation, cell sorting, embryonic boundaries, germ layers, ectoderm, mesoderm, tissue separation, Xenopus embryo, zebrafish embryo, mouse embryo, cell adhesion, cell migration, cadherin, asymmetric division, ephrins, Eph receptors, actomyosin contractility, cell cortex, cortical tension, contact inhibition, differential adhesion, interfacial tension, tissue surface tension, protocadherin, PAPC, pcdh8

1. Introduction

Early animal development follows a stereotypical sequence of events: The fertilized egg first undergoes a series of repeated cleavages to produce a multicellular structure called the blastula. A combination of patterning signals subdivides the blastula into different regions, determining the germ layers and creating "organizing" centres that further pattern the germ layers along the dorsal-ventral and anterior-posterior axes. Gastrulation then extensively remodels this prepatterned blastula, repositioning the various regions in order to build the general body plan. This remodelling requires a tight coordination between acquisition of cell fate and regulation of cellular properties responsible for morphogenesis, such as cell division, polarity, adhesion and motility.

While the general principles underlying morphogenetic processes are valid for all metazoans, the vertebrates have acquired some major distinctive features that directly impact early development. One of them is a multi-layered organization (Fig.1). This organization has two major consequences on morphogenesis of the vertebrate early embryo: Firstly, it creates two categories of cells, superficial cells exposed to the "outside", and deep cells confined to the inside (Fig.1B). We will see how this has been exploited by evolution to produce distinct cell types. Secondly, gastrulation now involves internalization of massive groups of cells "flowing" inside the embryo. Directly related to this multi-layered organization, the vertebrate embryo has also come up with new ways to segregate tissues and maintain their integrity.

Another characteristic of vertebrates, shared with our remote prochordate relatives, is the notochord, a rod-like structure that arises from the dorsal mesodermal midline and sets the primitive body axis. The notochord eventually degenerates in adult vertebrates, but it plays a central role during embryonic development in patterning the dorsal structures, in particular the nervous system and the paraxial mesoderm, also called presomitic mesoderm (PSM). The development of these two tissues is covered in two other chapters of this issue (Pujades, Naganathan & Oates).

In this chapter, I will summarize our current understanding of the early segregation processes. I will first focus on the amphibian *Xenopus* embryo, where these processes are best understood, and which may be considered as the archetypical model of early vertebrate development. I will start with the segregation between inner and outer layers of the blastula. We will see that this special type of segregation results is a consequence of asymmetric division of epithelial cells. I will then review the process of separation of the germ layers during gastrulation, followed by individualization of the notochord. In both cases, tissue boundaries are formed by a local increase in cortical tension and decrease of cell adhesion, controlled by the same cell-cell contact cues. I will then discuss the similarities and divergences observed in the fish and mouse embryos, and highlight some key questions that remain unsolved.

2. A short introduction to cell sorting

2.1 Pioneering experiments

The ability of cell populations to segregate from each other is an amazing property that has fascinated early embryologists (e.g. [1, 2]). The process has been most extensively studied by Johannes Holtfreter using amphibian embryos [3, 4]. By systematically mixing cells dissociated from various tissues, in all possible combinations, he discovered fundamental features of cell sorting: Firstly, combining cells from any two tissues would create compact aggregates, demonstrating the existence of a common adhesive mechanism. However, cells would eventually gather according to their origin and form discrete structures. This sorting phenomenon revealed that cells had an identity that could be maintained autonomously even within a mixed aggregate. As a result, cells were able to distinguish between self and non-self, preferentially associating with the former and segregating from the latter [3, 4]. In addition of sorting out, the different cell groups positioned themselves relative to each other in very reproducibly patterns. For instance, the ectoderm would systematically move out and segregate at the periphery of endoderm, as if the result of a "negative affinity" or "disaffinity". However, the mesoderm displayed a "positive affinity" for both ectoderm and mesoderm. When the three cell types were combined, the mesoderm would end up sandwiched between the ectoderm and the endoderm, "bridging" in a way the two tissues. The resulting structures strikingly resembled the native organization in the embryo. Townes and Holtfreter [3, 4] thus proposed the existence of different types of cell surface molecules, which would promote both non-specific and tissue-specific adhesion. Holtfreter's interpretation of these experiments was extraordinarily lucid and farsighted, and continues to impact our perception of morphogenesis.

2.2. Biophysical description: Tissue surface tension and adhesiveness

Holtfreter had already noted that adherent cells showed the "tendency to establish maximal mutual contact and to reduce their interface with the ambient aqueous medium to a minimum" [4]. In his footsteps, Malcolm Steinberg proposed a revolutionary description of tissue behaviour based on the analogy with liquids, introducing the concept of "Tissue Surface Tension" [5]. For the first time, a quantitative model could be used to predict the self-organizing properties of tissues based on their own physical properties. Steinberg's "Differential Adhesion Hypothesis" (DAH) proposed that cell sorting was controlled by quantitative differences in cell-cell adhesion [5]. The model, based on the analogy with the physics of liquids, postulated that groups of cells

were subjected to a "tissue surface tension". By maximizing the energy put into cell-cell adhesion, cells would spontaneously adopt a configuration characterized by a low "free energy". In agreement with this model, the cells with the strongest adhesiveness would typically gather in the centre of the cell mass, while the least adhesive cells would end up at its periphery (Box 1). While the initial experiments by Steinberg combined heterogeneous differentiated tissues, which was somewhat artificial, the same principles could be applied to the physiological situation of amphibian embryonic tissues, thus providing a theoretical framework to Holtfreter's observations [6-8].

The model was later corrected by including the contribution of the contractility of the actomyosin-based cell cortex [9-12]. In the "Differential Interfacial Tension Hypothesis" (DITH), the "surface tension" at a free cell surface is dictated by the contractility of the cortex, while a cell contact is subjected to an "interfacial tension" (more recently renamed "contact tension" [13]). Contact tension results from the contractility of the cortices at the contact interface combined with the negative tension resulting from the adhesive energy [10, 11, 13]. As the contact tension is lower than the sum of the two free surface tensions, the contact will spread until the forces equilibrate (Box1). The reduction of tension at contacts relative to the free surface defines the "adhesiveness" of the system [14] (Box 1). It turns out that it is the cortical contractility that plays a dominant role in setting contact tension [12, 13, 15]. Thus, adhesiveness is largely due to a repression of cortical contractility at the cell contact, the contribution of the adhesive energy being apparently rather marginal. One of the key properties of cadherins is precisely the ability to recruit signalling cytoplasmic components that downregulate myosin activity in the vicinity of the adhesive contacts [13, 16].

Besides cadherin-based mechanisms, other cell-contact receptors can also influence, positively or negatively, contact tension. The "repulsive" reaction generated by the interaction between ephrins and Eph receptors is an example of a contact-dependent process impinging on cortical contractility that can dramatically increase contact tension. Ephrins and Eph receptors constitute a major molecular mechanism used to drive sorting and build tissue boundaries in vertebrates (see below and chapters by Pujades and by Naganathan & Oates). Other surface proteins, such as Toll-like receptors, seem to fulfil the same function in Insects (Sharrock & Sanson).

2.3. The puzzle of the inverted embryo and its solution

Despite their success, the models of tissue self-organization based on DAH, and later on DITH, remained for many years in apparent contradiction with the normal organization in embryos. As we will see, this contradiction originated from an unfortunate omission. As a reminder, once gastrulation completed, the ectoderm surrounds the mesoderm, which in turn surrounds the endoderm. This typical configuration was perfectly reconstituted in Holtfreter's original experiments [4]. Importantly, Holtfreter had included the superficial layer of the ectoderm [4]. He had already noticed that this superficial layer was special, since it had a non-adhesive apical domain [17]. In later attempts to validate DAH, however, this layer was omitted, because it did not behave as predicted for "liquids" [8]. Under these conditions, the rest of the ectoderm, i.e. the deep layer, robustly sorted to the centre of the aggregate, surrounded by mesoderm, while the endoderm ended up peripheral [8] (Box 1). This configuration fitted so well with the prediction of DAH (and later DITH), that, even though it was blatantly inverted relative to the natural organization of the embryo, it was accepted without further ado. As this riddle remained ignored or forgotten for many years, inaccurate models of tissue segregation were perpetuated until recently. As explained below, the puzzle was definitevely solved by Ninomiya and Winklbauer [18](Box 1) by a series of elegant experiments, which demonstrated the importance of the superficial layer and its non-adherent apical surface, and could reconcile the normal tissue positioning with the theory of tissue surface tensions (Box 1).

2.4. High heterotypic interfacial tension, a unifying concept for tissue boundaries

Another key issue that was only recently conclusively solved is the principle responsible for the sharp segregation at embryonic boundaries. Here the field has been partly misled by an inaccurate analogy drawn between DAH/DITH-based models and the properties of immiscible liquids. In the case of oil and water, oil droplets tend to fuse to minimize to the oil-water interface, because oil-oil and water-water interactions are much stronger than oil-water interactions. In cellular terms, homotypic contacts would be more favourable than heterotypic contacts, or, translated in contact tensions, heterotypic tension would be lower than the homotypic tensions. The DAH/DITH models postulated a very different situation (Box 1). Here sorting would be driven by differences between the two homotypic contact tensions, high for one cell population, low for the other, while the heterotypic tension would be intermediate. We have recently demonstrated that under such conditions, cells fail to segregate, forming in the best of cases coarse clusters [19]. On the contrary, actual boundaries are straight limits that do resemble oil-water interfaces. Consistently, tension at the heterotypic tissue interface is higher than homotypic tension, thus fulfilling the analogy with immiscible liquids. Higher actomyosin contractility has been detected at all boundaries examined so far, and is unambiguously required for cell sorting and boundary formation (see below and the other reviews of this issue). One may conclude that high heterotypic interfacial tension (HIT) [19] is the actual driving force for boundary formation.

3. Early segregation and boundaries in Xenopus

3.1. Segregating inside from outside

3.1.1. The polarity switch of the egg membrane

The oocyte grows in the comfortable ambient of the uterus, where it is provided with plentiful of nutrients. In oviparous animals, the laid egg is abruptly exposed to the external hostile environment, and must resist desiccation when exposed to air, or the extreme osmotic pressures of fresh or sea water. Embryonic development then relies on internal supplies and exchanges with the outer medium are restricted to few components, the most essential being oxygen. During oocyte maturation and fertilization, the egg plasma membrane undergoes a major change, switching from an "absorptive" basolateral-like membrane to a "protective, apical-like" membrane adapted to the new environment. In *Xenopus*, this drastic shift is well illustrated by the quick and complete removal of Na/K-ATPase by endocytosis [20]. This process is highly symptomatic, since this ionic pump is the key basolateral determinant of polarized transport in epithelia. The newly-acquired apical nature of the egg membrane is reflected by the sequestration of atypical PKC (aPKC), a key component of the apical Par complex [21]. During the subsequent cell divisions, the original egg membrane will remain at the contact-free surface of the blastomeres (Fig.2A-D). The original cortex is tightly connected to the egg membrane. In the animal hemisphere of amphibian embryos, it can be easily tracked through the associated pigment granules, and remains localized at the surface [22, 23]. At each cleavage cycle, new basolateral membrane will be created *de novo* inside the embryo by direct delivery of vesicles to the cleavage furrow. Fast developing embryos such as *Xenopus* rely on a large intracellular vesicular pool of maternal origin to keep pace in assembling this new membrane [23-25]. Part of it is recycled from the endocytosis of the original oocyte membrane [26], contributing to insert the Na/K-ATPase to the new basolateral domain. Cadherins are other major components delivered to the furrows and thus directly targeted to the new cell-cell contact [27]. The boundary between the apical and basolateral domains is delimited by tight junctions [28], marked by cingulin and ZO1 [29] (Fig.2A-D). Intriguingly, the segregation of the apical domain seems to be somehow autonomous, as cingulin and ZO1 are found precisely at the limit between old and new membrane even in the absence of cell-cell contacts [29] (Fig.2A).

3.1.2. Asymmetric cleavage and formation of superficial and deep layers

The apical nature of the pre-existing egg membrane and the basolateral properties of the newly added membrane are general features of metazoan development [25, 30, 31]. In most invertebrates, the orientation of cleavage planes insures that all daughter cells inherit a portion of the egg membrane, accounting for the monolayered organization of the blastula (Fig.1A). Note that this is true even for compact blastulae composed of few cells, such as in ascidians or in c. elegans. However, in vertebrates, after a few divisions, some of the cells become buried as a result of asymmetric cleavage, eventually forming the deep cells of a multi-layered blastula (Fig.1B). The apical characteristics of the egg membrane appears to be a key determinant to this first segregation. This has been well documented in *Xenopus*, where, starting at the 6th cleavage, divisions occur along various planes, which could be directly related to the fate of the daughter cells [21] (Fig.2C): As long as both cells inherit of even a small portion of the aPKC-positive apical membrane, they both will remain superficial. If the domain is unilaterally inherited, the cell deprived of it will become a deep cell [21]. Importantly, inheritance of the apical domain is fully cell-autonomous, as it can be observed for single isolated blastomeres [21], and it dominantly imposes apico-basolateral polarity, as showed by Müller and Hausen in their 1995 paper [23](Box 2). Furthermore, this cell-autonomous polarity precedes the polarised cell junctional organization of the superficial layer.

These asymmetric cleavages result in a blastula composed of an outer layer of epithelial cells firmly held together by adherens and tight junctions, covering an inner mass of non-polarized cells (Fig.2D). The complete segregation of the two cell groups is particularly spectacular in the ectodermal animal hemisphere, where the superficial layer can be manually peeled off from the inner layer. Its cell-cell contacts are so tight that they resist dissociation. During gastrulation, the superficial and deep layers remain separated. They contribute to epiboly through different morphogenetic processes (Fig.2E-F) [32, 33], and will acquire distinct fates: In the ventral ectodermal region (prospective epidermis of the tadpole), the superficial cells will produce so-called goblet cells, while the deep cells will give rise to three cell types, including the multi-ciliated cells [34]. Interestingly, the inner cells will eventually intercalate between the goblet cells, will acquire *de novo* an apical domain, and will contribute to build the "mosaic" monolayered mucociliary epidermis of the tadpole [35]. In the dorsal ectodermal region, which forms the nervous system, the deep cells will undergo an early differentiation into primary neurons, while the cells originating from the superficial layer will be maintained in an undifferentiated proneuronal state. Here again, superficial and deep cells will eventually intercalate to produce a monolayered neural tube [36]. As for the dorsal marginal zone, while the deep cells will form the dorsal mesoderm structures, the superficial layer will form the dorsal wall of the digestive tract (Fig.2F). In this case, the two layers will remain definitively separated. Similarly, the superficial layer of the endoderm maintains a polarized organization during gastrulation [37] and will build the ventral part of the digestive epithelium (Fig.2E,F).

In summary, the multi-layered organization of the blastula has important implications for amphibian development. The original egg membrane, together with the associated cortical domain, constitutes a first determinant that is inherited by the superficial cells, and will maintain them segregated from the deeper cells. The non-adhesive nature of the apical membrane [17, 26] is key for cell sorting by imposing a superficial position (Fig.2 and Box 1).

Intriguing questions remain unanswered, starting with the molecular basis for the sturdiness and apparent autonomy of the apical domain. What is the nature of this inheritable polarity? Is there a "determinant" associated with the membrane or with the cell cortex? Is it constituted by stably localized components, or resulting from a dynamic equilibrium similar to the interplay between the Par complexes of the *C. Elegans* egg? The nature and properties of the early cell-cell junctions also remain mysterious: While the concentration of cingulin and ZO1 at the apical-basolateral

interface is a hallmark of tight junctions [29], the transmembrane occludin distributes all along the basolateral membrane [38]. Similarly, adhesive junctions are diffuse along the whole lateral membrane, and will only later concentrate in a more typical zonula adherens [39]. Finally, the later intercalation of deep cells in the superficial epithelial layer is a fascinating puzzle: What makes them moving toward the surface? How are they able to break through the existing superficial junctions to expose their own new apical domain? Note that in urodeles, the multi-layered organization is already lost during gastrulation, as deep cells intercalate into the superficial layer to form a pseudostratified epithelium [40]. Could there be general mechanism(s) controlling intercalation, which could be differentially regulated at different stages and in different species? As we will see below, asymmetric division leading to a multi-layered organization and its subsequent remodelling occur in other vertebrates, and extending our investigations to additional species should tremendously help our understanding of this fundamental developmental process.

3.2. Individualization of the germ layers

3.2.1. Patterning and gastrulation movements

The *Xenopus* blastula is patterned by the combined action of maternal and early zygotic Wnt, TGFβ/Nodal, FGF, NF-kB and BMP signals. This complex inductive network results in the determination of the three germ layers, ectoderm, mesoderm, and endoderm, and their further regionalization under the influence of "dorso-anteriorizing" and "ventro-posteriorizing" activities [41, 42](Fig.2E). One example of regionalization is the subdivision of the ectoderm into prospective neuroderm ("dorsal") and epidermis ("ventral"). A particularly relevant example for the present topic is the patterning of the dorsal mesoderm along the future antero-posterior axis into the mesendoderm, the prechordal mesoderm and the posterior or trunk mesoderm. Note that despite large differences in topography, the molecular networks appear conserved throughout vertebrates [43].

Gastrulation involves several coordinated morphogenetic processes, which are driven both by the intrinsic movements of each tissue and by the forces exerted by the other tissues. The ectoderm undergoes epiboly to cover the whole embryo surface. To achieve this large surface expansion, the 3-4 cell-thick deep layer thins to a single cell layer through radial intercalation, while the superficial monolayer merely stretches [32, 33]. Mesoderm and endoderm are both internalized by active migration, with distinct modalities: The endoderm flows inward, powered by an ingression-like migration of its individual cells [37]. As for the mesoderm, it undergoes involution, as it rolls as a sheet of cells around the so-called blastopore lip, and crawls along the inner surface of the ectoderm (blastocoel roof) [44, 45]. This process starts dorsally, where different mesoderm subregions show distinct behaviours [45]: Following the "leading" mesendoderm, the anterior (prechordal) mesoderm spreads over the ectoderm surface through active migration combined with radial intercalation. Its cells are consistently highly motile and mesenchymal-like. The posterior (chordal or trunk) mesoderm involutes during the second half of gastrulation. Its cells are more tensile and much less motile. They pull on each other perpendicularly to the direction of involution, a process called mediolateral intercalation. As a result, the posterior mesoderm undergoes convergent extension, acquiring a narrow and elongated shape, and will give rise to the dorsal axial structures. Mesoderm involution spreads to the ventral side. In parallel, the blastopore lip forms a circular-tensile and multicellular structure that constricts to close the blastopore, which terminates gastrulation [46, 47].

3.2.2. The ectoderm-mesoderm boundary

During gastrulation, a boundary maintains the internalized mesoderm separated from the overlying ectoderm. This boundary has been the topic of previous reviews [16, 48, 49], and I will only summarize its major features. The boundary first appears at the onset of gastrulation, when

the mesendoderm is brought in contact with the ectoderm blastocoel roof, pushed by the rotating flow of the endodermal mass [50](Fig.2F). It is then expanded by the involution of the dorsal mesoderm and its translocation along the ectoderm roof (Fig.2G) and spreads to the ventral side [51].

This boundary has several distinctive features: First, it forms by "apposition" of pre-existing tissues, unlike the "planar" boundaries that bisect epithelial monolayers, such as the classical compartment boundaries, i.e. the boundaries that restrict cells of different lineages to their respective compartment [52]. Secondly, it separates two basal surfaces (Fig.2G). These surfaces are not yet physically separated by a lamina, but cells adhere directly across the boundary, the ectoderm surface acting as as adhesive substrate for mesoderm migration (Fig.2G). A sparse network of fibronectin is secreted by the ectoderm, which plays an instructive role for mesoderm migration [53-55], but does not represent a physical barrier for cell migration [56]. It is important to remember that the cells abutting the boundary, both on the ectoderm and on the mesoderm side, are deep cells without apical-basolateral polarity, their whole surface being equally capable of cell-cell adhesion. As we will see below, the properties of the boundary interface result from the heterotypic contact between the two cell types. Another remarkable feature is the perfect coordination between segregation and involution: The property to separate from the ectoderm is progressively implemented in the mesoderm as it transits through the blastopore lip, while the two tissues remain continuous in the non-involuted region [56](Fig.2G).

The *Xenopus* ectoderm-mesoderm boundary has provided a powerful experimental system study tissue separation (Box 3), in particular thanks to the ease of reconstituting the boundary *in vitro* using tissue explants [56]. The possibility to manipulate each tissue separately and to visualize the live boundary at high resolution has allowed us to perform a full characterization of the function of ephrin-Eph signalling in tissue separation[57, 58]. Ephrin and Eph receptors are cell membrane proteins that function as reciprocal ligand and receptor, thus capable to generate signals in both the ephrin-expressing and the Eph-expressing cells (Box 4). They are mostly known to induce contraction of the actin cytoskeleton, responsible for repulsive behaviour, in particular the so-called growth cone collapse during neuronal pathfinding. They are however capable of various other cellular activities, including stimulation of cell-cell adhesion under some conditions [59]. Ephrins and Eph receptors are also widely expressed in early embryos, in complex but highly specific patterns. They had been involved in the case of the hindbrain and somitic boundaries (see chapters by C. Pujadas and A. Oates in this issue), strongly suggesting a role of cell-cell repulsion at these interfaces [60, 61], although alternative models had been proposed [62]. The Xenopus ectoderm-mesoderm system allowed the first direct visualization of repulsive reactions, and the unambiguous demonstration that this process was indeed due to ephrin-Eph interactions across the boundary.

The ephrin-Eph system turned out to be rather complex. Multiple ephrins AND Eph receptors are expressed in both germ layers [57, 58, 63](Box 4). According to the traditional view of promiscuous Ephrin-Eph binding (Box 4), repulsion should occur at all cell contacts. Why then was repulsion exclusively observed at the ectoderm-mesoderm interface? We demonstrated that ephrin-Eph pairs were highly selective [58](Box 4). This selectivity had been observed *in vitro* [64], but neglected until recently [65]. As a result, homotypic contacts only generate basal levels of ephrin-Eph signalling, while expression of key ephrin-Eph pairs in partial complementary patterns boost the signal at heterotypic contacts, resulting in high HIT, visible as overt repulsion (Fig.2 and Box 4). The same principle applies at later stages at the ventral ectoderm-mesoderm boundary, then again at the notochord boundary (see below), which led us to postulate that ephrins and Eph receptors act as a tissue "identity code" [58].

Further analysis by Winklbauer and colleagues [66] showed that ectoderm-mesoderm separation also required a second pathway, acting in parallel with ephrin-Eph network. This mechanism

relies on the protocadherin 8, also named paraxial protocadherin or PAPC. This protocadherin forms homophilic interactions, yet it does not function as a cell adhesion molecule, but rather as a regulator cadherin-mediated cell-cell adhesion through intercellular signalling [66-68]. At the gastrula stage, PAPC is specifically expressed in the mesoderm. Although the downstream mechanisms are still not fully elucidated, in the model proposed by the authors, the pool of non-ligated PAPC exposed at the boundary interface indirectly downregulates cadherin-based cell-cell adhesion (Fig.2H,I). Within the mesoderm, however, this activity is blocked by homotypic PAPC-PAPC interaction [66]. The result of these two opposite activities results in increased HIT, accounting for PAPC contribution to tissue segregation (Fig.2H,I). At later stages, PAPC is used again at least twice: At the end of gastrulation, it becomes asymmetrically restricted expressed to the presomitic mesoderm (see below), then during somitogenesis to the anterior half of the forming somites (see review by Naganathan & Oates in this issue). In the latter case, PAPC is also responsible for cadherin downregulation at the boundary [69], confirming a general role of PAPC at boosting contact tension at tissue interfaces.

Note that the endoderm also efficiently sorts from the ectoderm and from the mesoderm, but the mechanism of separation has yet to be elucidated [16].

3.2.3. Ectoderm-mesoderm separation and the principle of high heterotypic interfacial tension

The *Xenopus* ectoderm-mesoderm system has also contributed to solve the old debate about the role of global physical tissue properties. The existence of obvious adhesive and/or tensile differences between tissues had long led astray the search for the cellular basis of separation, seemingly supporting DITH [11, 15, 70-72]). In *Xenopus*, ectoderm is both significantly stiffer and more cohesive than mesoderm, but systematic manipulation of each parameter demonstrated that neither adhesive nor tensile differences contributed to ectoderm-mesoderm separation [19]. This contrasted with the efficiency of ephrin-Eph signalling to drive cell sorting, and to produce and maintain sharp boundaries [19]. We showed that this efficiency was directly due to the capacity of ephrin-Eph signalling to increase local contact tension at the boundary interface [19]. The unilateral expression of PAPC has the same effect through a different pathway [66]. This principle, which we named "high heterotypic interfacial tension" or HIT [19], in contrast to DITH, is supported by biophysically-based computer simulations [19, 73, 74], and appears to account for boundary formation in both vertebrates and invertebrates (see other chapters of this issue).

3.2.4. Anterior-posterior organization of the dorsal mesoderm

The subdivision of the mesoderm along the anterior-posterior axis is an interesting case of self-organization through cell sorting [16, 18, 75]. Winklbauer and colleagues showed that posterior mesoderm has higher surface tension than anterior mesendoderm. When these explanted tissues were combined, the latter systematically engulfed the former, in perfect agreement with the analogy of the minimization of surface tension of liquid drops (Box 1). However, this radial topography was at odds with the *in situ* linear anterior-posterior organization of the mesoderm. The authors demonstrated that coating the mesoderm explants with the embryo epithelial layer had a spectacular effect: Inside the epithelial coat, the mesoderm regions adopted the correct configuration of a linear array (Box 1). The explanation for this remarkable action of the epithelial layers resides in the non-adhesive nature of the apical domain, which as such is exempt from "tissue surface tension". I mentioned above that the impact on the relative position of the germ layers had been previously noted [8]. The contribution of Ninomiya and Winklbauer [18] provided a rigorous analysis of the consequences of epithelial coating. Most importantly, it made explicit the fundamental principle that epithelial coating abolishes the impact of tissue to medium surface tension on deep layers, which dominates cell sorting in classical reaggregation experiments. This principle does not only explain why the stiffer ectoderm does not locate to the

centre of the embryo as predicted by DITH, but also how tissues are free to explore other configurations than radial. The authors further conclude that sorting of mesoderm cells into distinct regions under these physiological conditions cannot not be driven by DITH, but rather by more specific mechanisms, probably based on selective adhesion [18]. The need for specific sorting mechanisms was amply confirmed by the involvement of ephrin-Eph and PAPC systems in boundary formation [48].

3.3. Notochord formation

During late gastrulation, two parallel clefts progress through the dorsal mesoderm to isolate the axial notochord from the PSM (Fig.3C,D). The formation of these two "notochord boundaries" has been the first process of boundary formation studied in detail at the cellular level by Ray Keller and colleagues [76, 77]. Determination of these two tissues has taken place earlier, in the upper dorsal marginal zone of the early gastrula, under the control of zygotic Wnt and BMP signals, counteracted in the dorsal side by secreted inhibitors (Fig.3A). The Wnt pathway works as a switch between PSM fate, triggered by its activation, and notochord fate, which requires its repression [78]. The presumptive notochord field is marked by specific transcription factors, such as Not and FoxA4, while the PSM field is positive for the myogeneic factors MyoD and myf5 [43]. Despite this clear-cut inductive pattern, the fate of these cells remains surprisingly labile: Domingo and Keller showed through heterotypic grafts that the cellular environment of the gastrula can very effectively redirect cell fate. This capacity is strikingly high for the notochord, and persists even at late stages, once the notochord has already separated [79]. Consistently, we failed in our attempts to adapt the *in vitro* ectoderm-mesoderm separation assay for the notochord boundary, as notochord and presomitic explants tend to fuse (unpublished observation). Presumably, Townes and Holtfreter might have made a similar observation: Among all the tissue combinations that they presented, this one is prominently missing, although one finds notochord-endoderm and PSM-endoderm combinations [4]. This plasticity contrasts with the segregation of the germ layers, which is already determined cell autonomously at the early gastrula stage [4, 19, 56]. Note that animal cap explants induced into dorsal mesoderm by activin-treatment do form a typical notochord, separated from PSM by clear boundaries [80], indicating a strong self-organizing capacity of these embryoid bodies. How high plasticity and tissue segregation can be conciliated in this system is a fascinating unanswered question.

This plasticity can be experimentally circumvented by forced cell-autonomous activation or repression of the zygotic Wnt pathway [81, 82] (Box 5). This approach allowed to study in detail the process of cell sorting, which relies on HIT: We could show that both sorting of isolated cells as well as formation of the endogenous boundary require ephrin-Eph signalling and myosin activity [82]. Specific ephrin-Eph pairs were involved, similar to the ectoderm-mesoderm boundary. Thus, despite different expression patterns (Fig.3E), the system relies on the same ephrin-Eph code (Box 4). PAPC, which at this stage is strongly expressed in the presomitic mesoderm but is now repressed in the notochord [83], probably acts in parallel, as suggested in zebrafish [84], but direct evidence is missing. Another protocadherin, Axial Protocadherin or protocadherin 1, is expressed exclusively in the notochord, where it probably plays a complementary role [85]. Despite the high similarities with the ectoderm-mesoderm boundary, the notochord boundary forms a fully non-adhesive interface [82, 86] (Fig.3F). The difference is consistent with the need for the notochord and the presomitic mesoderm to slide along each other to accommodate for changes in geometry as they independently undergo convergent extension [76, 87]. At the cellular level, the non-adhesive nature of the boundary could be explained by the inability of cadherins to form stable adhesive clusters at heterotypic contacts [82]. This effect appeared to be a direct consequence of the extreme cortical contractility along these contacts, since clusters would form within minutes after addition of the myosin inhibitor blebbistatin. Presumably, high contractility produces a shear stress that destabilizes cadherin trans interactions, as reported during Drosophila embryo elongation [88]. Importantly, tension

affected specifically cadherin clustering, not cadherin turnover, as the non-adhesive heterotypic membraes displayed high levels of unclustered cadherin [82]. Interestingly, live imaging indicated a progressive "maturation" of the boundary, which, before reaching the final non-adherent state, goes through a transient adhesive state similar to the ectoderm-mesoderm boundary [82]. These observations suggest that different types of boundaries can be set by tuning contractility [48, 49].

4. Segregation in the zebrafish

4.1. Segregation of the EVL and its equivalence to *Xenopus* superficial layer

Compared to prochordates or *Xenopus*, the geometry of the Zebrafish egg is strongly distorted due to the large volume of the yolk, now confined to a huge single syncytial cell. As a consequence of the meroblastic mode of cleavage, the entire organism derives from the animally located blastoderm. Note however that a purely "superficial" view of fish development would be inaccurate: Indeed, as result of incomplete cleavage, a number of nuclei end up within the superficial region of the yolk, the yolk syncytial later (YSL). The YSL shows a dynamic behaviour and has important functions in early development, both as source of inducing signals and as active protagonist of morphogenetic movements [89-94]. The yolk thus deserves to be considered an embryonic layer on its own.

Despite these morphological differences, the cleaving zebrafish embryo undergoes essentially the same early separation processes as in *Xenopus*. Starting at the 32-cell stage, some of the blastoderm cells end up buried under the superficial cells. These latter form the so-called "epithelial enveloping layer" or EVL, while the deep cells form the "epiblast" (Fig.4). Segregation of the EVL and its general properties are virtually identical to that of the superficial layer in *Xenopus*. Segregation results from asymmetric cleavage: Cells that inherit the apical domain form the EVL, cells that lack it become deep cells. The parallel between the EVL and the *Xenopus* superficial layer goes well beyond the process of segregation: Both fulfil the same protective function, forming a tight barrier that buffers the harsh differences in osmotic pressure [94]. Interestingly, deep cells also intercalate back in the EVL at later stages [95], again similar to *Xenopus*. Thus, the description of the EVL as a separate "extraembryonic" tissue may be partly misleading, as it obscures its intimate relationship with the epiblast. This latter should not be considered as the equivalent of the whole *Xenopus* ectoderm, but rather of its deep layer, at least during early developmental stages. This has immediate consequences on cell sorting, as the EVL is bound to have a coating effect on the deep cells (Box 1). There are nevertheless clear differences in the later development of the two layers. The fate of the EVL appears to be restricted to epidermis. It does not participate to neuroderm development [96]. It remains entirely superficial, does not invaginate during gastrulation, and does not participate to formation of the digestive track. This justifies to consider it primarily as a protective layer.

What remains unknown in the fish is the origin of the "apical polarity determinant". The EVL expresses specific cytokeratins, which are already present maternally and are specifically sequestered in the prospective EVL [96-98]. Once again, this is reminiscent of *Xenopus*, where epidermal cytokeratins accumulate at the apical side of the superficial ectoderm [99]. Although there is no direct evidence, it is reasonable to assume that in fish the apical domain of superficial cells also corresponds to the original egg membrane.

4.2. Fish germ layer separation

During gastrulation, the EVL and the epiblast undergo epiboly, crawling on the surface of the yolk (Fig.4). Both mesoderm and endoderm cells originate from the epiblast (thus the deep cells). Mesoderm cells involute within the so-called shield, the fish equivalent of the blastopore "lip" [100, 101]. Endoderm cells internalize individually by an ingression-like mechanism, and migrate to cover the surface of the YSL [102], paving the way for the involuting mesoderm, which will fill the space between the epiblast and endoderm layers. Though fish mesoderm and endoderm

cells form looser layers and tend to move more individually, their modes of internalization are strikingly similar to amphibian gastrulation.

What ensures stable separation of the internalized mesoderm and endoderm cells from the overlying epiblast layer? Heisenberg and colleagues argued for a DITH-based mechanism [15, 72]. This was based on measurements of cell adhesion and cortical stiffness for the three germ layers, and on the reaggregation experiments. Indeed, the relative position of the layers were in agreement with the relative stiffness, but not adhesion: The ectoderm, which was the stiffest but the least adhesive, always sorted to the centre. The endoderm, which was the softest but the most adherent, engulfed both ectoderm and mesoderm [11]. These data were consistent with the demonstration that cortical contractility controlled most of the contact tension, while the adhesive energy represented a marginal contribution [15]. However, the claim that germ layer separation was controlled by differences in tissue tension had several caveats. The model was tested by inhibiting the Rock-myosin pathway in ectoderm cells, with the intent of lowering the difference in cortical contractility with mesoderm. The fact that the two cell types failed to sort under these conditions was interpreted as decisive evidence for DITH [72]. Yet, these experiments only showed that myosin was involved: They did not address the actual role of differences in tissue tension. This distinction is essential, since myosin is not only involved in global tissue tension and adhesion, but in multiple other processes, including cell migration and ephrin-Eph repulsion, which are directly relevant here. Consistently, we showed in *Xenopus* that ectoderm-mesoderm separation required the Rock-myosin pathway in both tissues [57]. Along the same lines, Rho activation rescued separation upon loss of ephrin signalling in either of the two tissues [57]. Another major issue pertained to the tension at heterotypic contacts, which is a key parameter to discriminate between DITH and HIT mechanisms [19, 74] (Box 1). Unfortunately, the studies in zebrafish only measured homotypic contact tensions [15, 72]. Heterotypic contact tension was assumed to be intermediate, based on DITH, trivially creating a circular argument. Furthermore, it was later realized that the *in vitro* experiments had been performed in a medium of exceedingly high osmolarity, which had led to incorrect estimates of cortical tension [103]. Most importantly, the sorting experiments were performed in the absence of the natural epithelial coating, and the model was based on the principle that sorting would be dictated by differences in cell to surface tension. As expected, the sorting pattern was inverted [72]. As mentioned above, a major impact of the superficial epithelium is to damped tissue tensile differences [18], which thus cannot account for tissue separation under physiological conditions.

While additional experiments are needed, the available information strongly suggest that the principle and molecular mechanisms of ectoderm-mesoderm separation in fish is similar to *Xenopus*: The relative smoothness of the boundary interface suggests high heterotypic tension, in agreement with the HIT model [19, 73, 74]. In fact, Winklbauer and colleagues have shown that the role of PAPC is conserved in the fish [66]. The involvement of the ephrin code in fish awaits confirmation, but would be fully consistent with the high expression of multiple ephrinBs and of their corresponding Eph receptors at the onset of gastrulation (Expression Atlas database, https://www.ebi.ac.uk/gxa/home).

5. Segregation in the mouse

5.1. Early segregation of the extraembryonic and embryonic tissues

From fish and amphibians to mammals, the vertebrate embryo went through two major innovations, firstly development of extraembryonic tissues, common to all amniotes, then, unique to mammals, implantation in the uterine wall through one of the extraembryonic layers, the trophectoderm (TE). With this switch to a developmental mode that relies on continuous maternal supplies, the formation of the extraembryonic layers became a priority, and indeed the two first segregation events deal with the sequential appearance of the TE and the primitive

endoderm (PrE). I will here summarize these processes in the mouse, which is by far the best understood mammalian model.

The early cleaving mammalian embryo (2 to 8 cells) is recognizable by its grape-like appearance (Fig.5A). The blastomeres are poorly adherent, lack tight junctions and show no apparent sign of epithelial polarity. They were long thought to be identical, equally totipotent cells, although this notion is now being revised [104-106]. Since zygotic transcription is already detectable before the first cleavage and fully active at the 2-cell stage, mammalian development is generally viewed as controlled exclusively by zygotic programs. We will see that this is also not entirely correct.

At the 8-cell stage, the embryo undergoes a transition to a more classical epithelial organization. The process, called compaction (Fig.5B), involves expansion of cell-cell contacts and acquisition of apico-basolateral polarization, marked by accumulation of typical apical components, such as aPKC and Ezrin at the surface of the embryo [105, 107]. Note, however, that at this stage, the apical domains are not yet sealed. Indeed, the tight junctions assemble in a stepwise sequence that is only completed at the blastocyst stage [108]. Formation and segregation of superficial and deep cells occurs during the next two rounds of division immediately following compaction (Fig.5C). The resulting superficial layer will become the first extraembryonic layer, i.e. the TE. The deep cells are called the inner cell mass (IM) (Fig.5D), the development of which will be discussed below.

The origin of the apical polarity remains intriguing. Isolated blastomeres do polarize "spontaneously", a property that can be tracked back to the early 8-cell stage, before compaction [107]. These blastomeres divide asymmetrically, display differences in cortical tension, and form "miniblastocysts", reconstituting a smaller scale of the TE-IM sorting of whole embryos [109, 110]. Because of the apparent lack of pre-existing asymmetry, the search for symmetry-braking processes has focused on topography and/or stochastic variations, but conclusive data are still missing [105, 111-113]. Interestingly, a "subcortical maternal complex" has been identified, which localizes exclusively along the outer cell membrane, controls cortical cytoskeletal organization, and is essential for progression past the 2-cell stage [104, 106, 114, 115]. These observations reveal a previously unexpected contribution of maternal components to mammalian development. They suggest that the egg membrane may also provide an apical determinant in the mouse embryo. Note, however, that this potential cue is not sufficient to build a polarized epithelium before compaction. How maternal cues, mechanisms driving compaction [116, 117] and other potential symmetry breaking mechanisms may cooperate is an exciting outstanding question.

The mechanisms determining the TE-IM segregation have long been a matter of debate, and the issue is still not definitively solved. Predetermined oriented division has been excluded based on the variability of cleavage planes [113]. Clearly, the polarized organization resulting from compaction plays an important role in the outcome of blastomere cleavage: Those blastomeres that have inherited an apical, aPKC-positive cortex remain superficial, cells lacking it sink in and form the IM [109, 113, 118, 119]. Kokotkevich at al [118] directly demonstrated the role of the apical cortex through grafting experiment [118]. The orientation of the cleavage plane depends partly on determinants localized in the apical domain itself and partly on additional mechanical/shape constraints [120]. Mechanically, the process of internalization of the prospective IM cells is viewed as a case of sorting based on DITH [110]. Direct measurements by pipette aspiration showed that asymmetric divisions produced cells with differences in cortical tension, which were predictive of the sorting outcome, as the stiffest cells systematically ended inside the cell mass. Consistently, experimental manipulation of myosin contractility affected sorting as predicted by DITH [110]. Another process has been proposed to contribute to segregation using the microtubules of the cytokinetic bridge for direct delivery of E-cadherin positive membranes to prospective IM cells [111, 121]. What remains unclear is how to integrate

the non-adhesive nature of the apical domain in a DITH-based model, which, at least in its classical form, assumes entirely adhesive cell surfaces. One possible answer could be that this apical domain is still plastic and capable of adhesion at this stage. Alternatively, the non-adhesive nature of the apical surface could contribute to cell positioning, as proposed in other systems.

5.2. Trophectoderm development

Once segregated, the TE will form the first sealed epithelium. The TE inner basolateral surface expresses Na+/K+ATPase, which is instrumental in creating the blastocyst cavity, similar to the blastocoel of other embryos. The TE will later play a central role in embryo implantation. The complexity of its development is beyond the scope of this review. I will only mention that the TE region facing the cavity will become the mural TE, while the region covering the embryonic cell mass become the polar TE. A recent study has discovered that the two regions are separated by a sharp planar boundary, prominently enriched in actin [122]. The formation of this new boundary remains to be investigated.

5.3. Sorting within the inner cell mass

The next step in structuring the mammalian embryo is the separation of the IM into two populations, the presumptive epiblast (Epi), which will give rise to the future embryonic tissues, covered on one side by a monolayered epithelium, and the primary endoderm (PE), which separates it from the blastocyst cavity (Fig.5F). This step is a nice physiological case of single cell sorting. The IM is first a "salt and pepper" mixed aggregate of Epi- and PE- fated cells. The cell fate decision between Epi and PE has been the subject of many studies and has been covered very nicely by recent reviews [119, 123]. The two prevailing models put forward either stochastic fluctuations or local heterogeneities. In both cases, small initial differences are predicted to get amplified and eventually stabilized. The actual sorting mechanism is unclear. Experiments manipulating E-cadherin argue against a role of differential adhesion [124]. The current favoured mechanism involves differential "anchoring" of outer cells through their apical domain. However, this model hardly explains how some cells may travel more than two cell diameters to reach their final position, nor the variety of behaviours observed by live imaging: A number of PE-fated cells are already at the surface and do not migrate, while some Epi cells always remain inside the IM mass. Other cells, however, move from inside to outside, from outside to inside, or go back and forth [125]. Intriguingly, the "salt and pepper" distribution of Epi and PE precursors appears to be mirrored by heterogeneous levels of cortical aPKC, indicative of a "pre-polarized" state of PE cells [126]. How would this drive "long distance" sorting? A more comprehensive characterization of adhesion and polarity properties during this process is clearly needed. By analogy with cell sorting in *Xenopus* mosaic notochords (Box 5), one could hypothesize that PE cells with high aPKC may lose the ability to form stable contacts with Epi cells. Heterotypic contact instability coupled with "capture" of PE cells at the blastocyst interface could be sufficient to drive their efficient segregation.

5.4. Mouse gastrulation and germ layer separation

Mouse gastrulation follows the typical model of amniote embryos. The internalized cells originate from the pseudostratified epiblast, which has developed by epithelization of the Epi cell mass. The blastopore equivalent is an elongated groove, the "primitive streak" (Fig.5). Endoderm and mesoderm-fated cells undergo on-site epithelial to mesenchymal transition and "ingress" as a stream of loosely interacting cells. The sequence of ingression is stereotypical, starting with disruption of the basal lamina of the epiblast, followed by apical constriction, bulging of the cell soma and translocation in the basal direction [127]. Interestingly, cells appear to ingress individually and asynchronously, while their immediate neighbours temporarily maintain their polarity, ensuring continuous integrity of the epiblast during the whole process [127]. Thus, ingression through the primitive streak can be mechanistically viewed as analogous

to other processes involving "basal extrusion" from an epithelium, such as the TE-IM segregation.

To the best of my knowledge, what keeps germ layers separated after ingression has never been directly interrogated. It has been implicitly assumed that the basal lamina of the epiblast acts as a physical barrier, but this assumption is not as trivial as it may seem. By electron microscopy the lamina appears extremely thin (30-50nm) [128]. It is generally continuous outside of the streak, but gaps were observed, coinciding with blebbing of ectoderm cells [128]. These observations suggested that a simple increase in cell tension is sufficient to disrupt this layer and establish contacts between the two germ layers. This would be consistent with studies of cell behaviour in the streak, which showed that ingressing cells could force their way through the lamina, without the need to degrade it [129, 130]. One should remember that the thin matrix that lines the *Xenopus* ectoderm-mesoderm and notochord boundaries does not seem to offer any observable resistance to cell migration [56, 82]. Surely the amniotic epiblast lamina appears more continuous, and crossing it must require more robust intrusion. Yet, one may question whether the lamina represents the sole component responsible for separation in the amniote models, or whether additional mechanisms exist to back up this fragile barrier. The mouse mesoderm has conserved the expression of both EphA4 and PAPC [131]. While these molecules have other functions during gastrulation [67, 68, 83, 132-134], it is tempting to speculate that they also contribute to maintain germ layer separation in mammals.

6. Conclusion

We have seen that the earliest process of segregation in amphibian, fish and mouse occurs during cleavage and is based on asymmetric inheritance of the apical egg membrane. The appearance of a separate protective superficial layer had a huge impact on vertebrate development. Firstly, the deep layer, now freed of the rigid constrains of the epithelial organization, could explore complex three-dimensional morphogenetic movements. Furthermore, the deep cells, when coated by the superficial layer, were also freed from the otherwise dominating cell to medium surface tension, and could then organize into linear structures [16, 18]. Note that in mammals, the superficial layer was commuted from a protective to an extraembryonic "invasive" function, yet the switch only occurs at the implantation stage, and the original polarity is still observed during the early stages. Exploiting the non-adhesiveness of the apical domain also contributes to the phenomenon of ingression, for instance in the primitive streak of amniotes.

While asymmetric cleavage has been extensively studied in other models, such as the first cleavage of the C. elegans egg, much remains to be done to characterize it in vertebrate embryos. For instance, it will be quite important to better define the determinants localized in the egg membrane and/or the underlying cortex, the cross-talk between the two structures, and the mechanisms responsible for their stable inheritance. Another important question is the plasticity of this polarity, in particular during early segregation in the mouse embryo.

The second major mechanism of segregation in the early vertebrate embryo is the formation of a boundary by building tension at heterotypic contacts. The general biophysical principle is the same as for most of the other boundaries covered in this issue, although here it involves formation of a visible cleft between deep non-polarized cells. The molecular mechanisms have been best characterized in the case of the *Xenopus* ectoderm-mesoderm boundary. The ephrin-Eph and PAPC systems can both be viewed as cell identity "codes". While the ephrin code relies on a complex network, PAPC is unilaterally expressed and seems to work more like a binary code, increasing adhesion at homotypic contacts and decreasing it at heterotypic contacts. This ephrin-Eph and PAPC "double code" most certainly controls ectoderm-mesoderm, notochord as well as somitic boundaries (Naganathan & Oates, this issue). The mechanisms responsible for segregation of the endoderm are still unknown. As for germ layer separation in mammals, the underlying mechanisms have not yet been directly addressed. One needs to determine the

potential contributions of basal extrusion, directional migration, and the possible involvement of ephrin and/or PAPC-based repulsive reactions and high heterotypic interfacial tension. In order to be able to link the mechanisms of germ layer separation in *Xenopus* and in amniotes, it will be essential to understand the transition between the two modes of gastrulation. The answer is likely to be found in other amphibian embryos, specifically urodeles such as *Cynops*, which have a pseudostratified, single-layered, epiblast and where gastrulation proceeds via a primitive streak [135, 136].

I have highlighted here some of the traits of early tissue formation that are strikingly similar between vertebrates. The knowledge gathered in one species should be of great help to fill in the gaps in the others. The models have too often been studied in isolation, and the field would certainly benefit from more systematic cross-species comparisons, and from revisiting the older literature, which contains a mine of precious observations.

Abbreviations

DAH, Differential Adhesion Hypothesis; DITH, Differential Interfacial Tension Hypothesis; Epi, epiblast; EVL,: epithelial enveloping layer; HIT, high heterotypic interfacial tension; IM, the inner cell mass; PE, primary endoderm; PrE, primitive endoderm; PSM, presomitic mesoderm; TE, trophectoderm; YSL, yolk syncytial later.

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Competing interests

The author declares that no competing interests exist.

Figure legends

Figure 1. Monolayered versus multi-layered development. **A)** The typical invertebrate blastula consists of a hollow ball made of an epithelial monolayer surrounding a cavity called the blastocoel. Gastrulation usually occurs by simple invagination of a portion of the monolayer. **B)** The cleavage pattern of vertebrate embryos has been modified by the introduction of asymmetric divisions that produce superficial and deep cells, resulting in a multi-layered blastula. Gastrulation typically involves an inward flow of cell mass.

Figure 2. Segregation in early Xenopus development. A-D) Cleavage and formation of the blastula. **A)** The egg membrane has apical properties (dark red). During cleavage, the furrows (blue) are formed by delivery of new membrane through exocytosis. This membrane has basolateral characteristics. The boundary between the old apical membrane and the new basolateral membrane is delimited by accumulation of tight junction components (orange spots). **B)** The morula (32 cells) is composed of a single polarized epithelium sealed by tight junctions (orange spots). **C)** The two subsequent divisions display variable cleavage planes. Division planes perpendicular to the apical surface (dark green arrows) produce two identical daughter cells. Division planes parallel to the apical surface (purple arrows) produce two dissimilar daughter cells, one superficial and one internal. The latter is not polarized (light blue). In the case of oblique planes, the result depends on whether the egg membrane is inherited by both cells (light green arrows), or only one cell (light purple arrows). The latter case yields a superficial and a deep cell. **D)** The early blastula (128 cells) is composed of a polarized outer layer enclosing non-polarized deep cells. **E-F)** Organization of the late blastula (E) and early gastrula (F), with the position of the germ layers and regionalization of the mesoderm. The dorsal mesoderm is subdivided into leading mesendoderm (ME), anterior (AM) and posterior (PM) mesoderm. The red line highlights the boundary between the involuting mesoderm and the ectoderm. The interface between the endoderm and the mesoderm does not form a visible boundary (dotted red line). af, archenteron floor; ar, archenteron roof; bc, bottle cells; bp, blastopore; sf, superficial layer. **G)** Enlargement of the dorsal region of the early gastrula, highlighting the ectoderm-mesoderm boundary. The mesendoderm and the mesoderm form a single unit in terms of separation behaviour. At the blastopore lip (bl), the mesoderm progressively acquires its separation capacity (hatched), its posterior end remaining continuous with the ectoderm. Dashed arrows: direction of involution. G') Detail showing the superficial ectoderm and the deep layers of the ectoderm, mesoderm and endoderm. **H)** Molecular control of ectoderm-mesoderm separation. Top: Simplified diagram of the ephrin-Eph network. The red double arrows symbolize repulsive signals. The asymmetric expression of the ephrinB3-EphA4 and the ephrinB2-EphB4 pairs are crucial to produce a stronger repulsive signal across the boundary. Weaker activity is detected in the mesoderm. In the ectoderm, the weak ephrin-Eph interactions positively impact on cell adhesion (green double arrow). Bottom: Differential action of the protocadherin PAPC, which favours adhesion at homotypic contacts in the mesoderm but decreases it at the heterotypic contacts across the boundary. **I)** Separation behaviour at the ectoderm-mesoderm boundary. Heterotypic contacts undergo cycles of repulsion due to ephrin-Eph-induced contractility (red lines) and re-adhesion (cadherins in green). This mechanism is well-suited to maintain separation, while the mesoderm crawls using the ectoderm as an adhesive substrate. Arrow: direction of mesoderm migration.

Figure 3. The notochord boundary. A) Patterning of the mesoderm takes place at the early gastrula stage. It involves both Wnt and BMP signalling and their differential repression in the dorsal side by secreted inhibitors (red crosses). **B)** The inhibition of both pathways in the dorsal-most region of the posterior mesoderm determines the notochord field (no), marked by the transcription factors Not and FoxA4. The adjacent lateral regions, where BMP signalling is inhibited, but Wnt signalling is active, form the paraxial or presomitic mesoderm (PSM),

expressing the myogenic transcription factors MyoD and myf5 [43]. The mesodermal structures are sandwiched between the neuroderm (ne) and the archenteron roof (ar). **C)** Cross-section of a late gastrula, showing the position of the notochord. **D)** Ventral view of the involuting trunk mesoderm of a late gastrula, preceded by the anterior/prechordal mesoderm. The two boundaries separating the notochord from the PSM form progressively as the mesoderm turns around the blastopore lip (curved dashed arrows). The interfaces are first jagged, then straighten and become visible clefts. In parallel, the movements of convergent extension are initiated on either side of the boundary (double arrows). **E)** Molecular cues at the notochord boundary. Top: The ephrin-Eph network. Bottom: PAPC is expressed exclusively in the PSM. It likely acts similarly as earlier at the ectoderm-mesoderm boundary. AxP: Axial protocadherin, potentially fulfilling the same function on the notochord side. **F)** The notochord boundary is characterized by strong local contractility, which prevents formation of cadherin adhesions across the interface.

Figure 4. Cell sorting during early zebrafish development. **A)** Sagittal section of the early cleaving embryo. Some divisions (purple double arrows) lead to asymmetric distribution of the apical membrane (dark red), separating superficial cells from deep cells. Symmetric divisions (green double arrows) produce two superficial cells. YSL: Yolk syncytial layer. **B)** Same view of the blastula stage. The outer cells form the superficial enveloping layer (EVL), the deep cells the epiblast (Epi). Both the EVL and epiblast undergo epiboly to cover the yolk mass (dark red arrows). **C)** Detail of the dorsal side (rotated 90°) of an early gastrula. The endoderm forms by ingression of single cells from the epiblast (yellow) that cover the YSL [102]. Deep cells with a mesoderm fate (pale red) involute through the shield (~ blastopore lip) and migrate between the endoderm and the ectodermal epiblast [100]. Epiboly of the EVL and the epiblast continues until the EVL covers the whole cell mass. Whether the yolk membrane has apical or basolateral characteristics is unknown (question mark). The red line represents the ectoderm-mesoderm boundary, which remains poorly characterized.

Figure 5. Cell sorting during early mouse development. **A)** The early morula is made of eight morphologically identical blastomeres, which are weakly adherent. Maternal polarity determinants are sequestered in the outer portion of the membrane/cortex, which is named here "pre-apical" membrane (purple). **B)** The morula undergoes compaction, involving maximal expansion of lateral cell-cell contacts and segregation of the apical membrane and cortex (dark red line). The first components of the tight junctions start to appear (grey dots). **C-D)** Segregation of the inner cell mass (IM) from the superficial trophectoderm layer (TE) through asymmetric inheritance of the apical domain. Note that few cleavages directly isolate deep cells (purple double arrows). Most asymmetric cleavages produce two unequal superficial cells (orange double arrows). The cell inheriting little or no apical determinant will then move inside the embryo in a myosin-dependent process (black arrows), engulfed by the superficial layer (red arrows). Tight junctions assemble in a stepwise sequence (grey to orange) and become functional at the blastocyst stage. The detail of their biogenesis and distribution, in particular during partial asymmetric divisions, is not well characterized. **E)** Formation of the blastocyst cavity and determination of epiblast and hypoblast cells (light blue and yellow) in the IM. Cavitation requires the full maturation of tight junctions (orange spots). **F)** Sorting of the epiblast and hypoblast cells, the latter forming an epithelial layer with a new apical domain (dark red). **G)** Detail of the primitive streak showing ingression of individual cells, which involves a temporary epithelial to mesenchymal transition. The dark blue line represents the epiblast basal lamina. Ingression starts with disruption of the basal lamina (orange cell, broken line), followed by detachment from the apical domain (red cells) and migration (arrows).

Box legends

Box 1. Cortical tension, contact tension, tissue surface tension and epithelial coating. A,B) Cortical and contact tensions. A) Cell and tissue geometry reflect underlying forces. For a simple cell doublet, the angles at vertices are determined by the equilibrium between cortical tensions Ct and contact tension T. Contact tension is mainly dictated by the local cortical contractility along the contact, which is reduced under the influence of adhesive interactions relative to the free surface tension. This reduction defines the relative adhesiveness $\alpha = (T-Ct)/Ct$, and is directly related to the angle θ by the relation $\alpha = 1-\cos(\theta)$ [14, 137]. **A')** Doublet asymmetry indicates a difference in Ct: The cell with the lowest Ct, here B, tends to engulf the other cell. The same relationship applies to groups of cells and tissues, replacing Ct and T with "tissue surface tensions" γ [13, 16]. **B)** Example of two doublets with high and low θ, indicative of high and low adhesiveness, respectively. **C,D) Heterotypic contacts and boundary formation. C)** For cell types with different homotypic tension $T_{AA} < T_{BB}$, heterotypic tension T_{AB} is predicted to be intermediate, consistent with the interfacial tension hypothesis (DITH). Under these conditions, cells can cluster but do not efficiently segregate. **D)** Higher contact tension can be triggered by local stimulation of contractility and/or decrease in adhesion. High heterotypic interfacial tension (HIT) creates a smooth boundary. **E,F) Tissue positioning and epithelial coating. E)** In reaggregation or tissue fusion experiments, tissues with the highest tissue surface tension γ are surrounded by cells with lower γ. Thus, ectoderm typically ends up at the centre, surrounded by mesoderm and endoderm. Coating the tissue aggregate with the superficial epithelial layer (ep) reverses the position of the tissues, reproducing the normal organization in the embryo. This is due to the non-adhesive apical surface (dark red), which forces the superficial position of polarized cells, which in turn preferentially attract the deep ectoderm cells [16]. **F)** Epithelial coating also explains tissue positioning in linear patterns, for instance for anterior "A" and posterior "B" mesoderm. In vitro, "A" engulfs "B" due to its lower γ. Upon inclusion of the ectoderm epithelial layer, which mimics the natural situation, the system is only subjected to the tissue to tissue tensions, which are much weaker than the surface tensions of the tissues exposed to the medium. This allows the mesoderm regions to sort based on other parameters, such as specific adhesion [13, 16].

Box 2. Experimental demonstration of cell autonomous inheritance of the apical domain. A) A single blastomere dissociated from a 64-cell stage embryo can divide along various cleavage planes. Daughter cells that inherit the apical egg membrane (light purple) will remain superficial cells (dark green), and develop into spheroids expressing the bHLH gene ESR6e, while those lacking the apical domain will become deep cells (light green), and develop into ESR6e-negative spheroids [21]. **B)** A single blastomere from a labelled embryo is grafted inside the blastocoel of a host embryo. Descendant blastomeres that have inherited of the original egg membrane form an ectopic, inverted, polarized layer with the apical domain facing the inside of the blastula. Other descendants lacking this apical domain integrate into the deep layer [23].

Box 3. Ectoderm-mesoderm separation as an experimental model for cell sorting at embryonic boundaries. **A)** The classical sorting assay [4] involves dissection of tissue explants and their dissociation in an alkaline, calcium-free buffer. Cell populations are mixed and left to reaggregate in a physiological buffer. Wild type ectoderm and mesoderm cells sort into small clusters that progressively merge into larger groups delimited by smooth boundaries [19]. Sorting is quantified in terms of clustering and interface smoothness. **B)** The tissue separation assay [56] uses a large piece of ectoderm as cellular substrate, on which tissue aggregates are laid. Wild type ectoderm aggregates sink and mix within the ectoderm substrate, while mesoderm aggregates remain separated, thus reconstituting the endogenous boundary. Both assays can be used to test any recombination of tissues.

Box 4. The ephrin code. Ephrin and Eph receptors are cell surface molecules involved in repulsive reactions. They have been extensively studied in the nervous system, where they function as contact guidance cues, but are also widely expressed in other tissues, in adults and embryos. Ephrin-Eph interaction can trigger signalling both in the Eph-expressing cell (forward signalling) and in the ephrin-expressing cell (reverse signalling). Their activation leads to local remodelling of the actin cytoskeleton, mainly via Rho-dependent actomyosin contractility. **A)** Ephrins and Eph receptors are classified in A and B subfamilies. Traditionally, ephrin-Eph interactions are considered to be promiscuous within each subfamily, ephrinAs reacting with all EphAs, and ephrinBs with all EphBs (top panel). One exception is EphA4, which can interact with ephrins of both subfamilies. However, this assumed promiscuity is at odds with widely different binding affinities, even within the same subfamilies [64, 65, 138]. Furthermore, it could not account for the case of the early vertebrate gastrula, where multiple ephrins and Eph receptors are expressed in all tissues, yet overt repulsion is restricted at the tissue boundaries. We demonstrated a strong functional selectivity for ephrinB1,2,3 and EphB2, B3 and A4 receptors in the physiological context for early embryonic boundaries, resulting in a network of interactions (bottom panel): Some members bind multiple partners (e.g. ephrinB2 binds all three receptors), while others can only interact with a single partner (e.g. ephrinB3 with EphA4, ephrinB1 with EphB2). **B)** Simplified diagram presenting the major ephrin and Eph receptors expressed in the dorsal ectoderm and mesoderm (ephrins and Ephs presented in separate cells for clarity's sake). The output of these complex systems relies on three simple principles, selectivity, complementary expression, and balance with adhesive forces (cadherins in green): Repulsion at a given contact results from the combined action of multiple ephrin-Eph pairs (red double arrows, thickness symbolizes the relative intensity). The partially complementary expression of some key ephrin-Eph pairs serves as a "code" that discriminates between homotypic contacts, where repulsion is weak (ectoderm) to moderate (mesoderm), and heterotypic contacts, where it is sufficiently strong to overcome adhesion. In addition to the dorsal ectoderm-mesoderm boundary, this code also accounts for ventral ectoderm-mesoderm separation (not shown), as well as for the notochord boundary (Fig.3). The existence of weaker repulsive signals at homotypic contacts likely contributes to dynamic adhesion within the tissues[37]. Similar systems with multiple ephrins and Eph receptors are frequently observed, which may be explained based on the same principles [63, 65, 139, 140].

Box 5. The notochord as an experimental model for cell sorting. The individualization of the notochord from the PSM is a powerful system to study cell sorting in a physiological context [58, 81, 82]. **A)** Single cells can be manipulated and tracked in mosaic embryos, produced by simple injection of plasmid DNA targeted to the prospective dorsal side. DNA is not transcribed until mid-blastula (A'), leaving early developmental processes unaffected. Expression in the early gastrula is highly mosaic (A", green cells). Sorting is scored at neurula stage, once the notochord is fully individualized (A'"). **B)** Manipulation of the Wnt zygotic pathway is used as fate switch: Its constitutive activation (β-catenin or LEF1-VP16 chimera) induces cell-autonomous PSM fate. Among manipulated cells, those mis-localized in the notochord will all sort laterally to the PSM, leaving the notochord "empty" (B'). Conversely, repression of the pathway (β-catenin-engrailed repressor chimera) causes opposite sorting to the notochord (B"). (B'") Mosaic ephrin/Eph depletion with morpholinos (MO) perturbs sorting and disrupts the boundary. **C-D)** The sorting process of single cells can be studied by live imaging of dorsal mesoderm explants [81, 82]. **C)** mRNA co-injection is used for broad expression of any marker of interest (e.g. cadherin-GFP). This example shows a plasmid designed for co-expression of the LEF1-VP16 activator and a membrane Cherry fluorescent protein (C'). This strategy allows to track single PSM-fated cells live (orange cells with red outline) with high spatial and temporal resolution, while monitoring cadherin-GFP-positive adherent structures. **D)** Typical contact-dependent sorting behaviour of a PSM cell mis-localized in the notochord. As a result of high heterotypic contact tension, the PSM cell fails to establish stable cadherin contacts (green dots) with notochord cells (1) and is

randomly "pushed around" (2). Contact with another PSM cell across the boundary (3) is immediately stabilized (4, green line). The imbalance between this adhesive contact and the non-adhesive heterotypic interfaces (red line) drives rapid and irreversible crossing toward the PSM tissue (5). The cell becomes fully integrated and the boundary straightens (6). Computer simulations support the generalization of this HIT-dependent sorting behaviour [19]. Directional migration [102, 141] may increase the speed of sorting. It is important to note that formation of endogenous boundaries usually involves minimal cell movement, limited to slight displacements that smoothen the interface [48]. Artificial single cell sorting in a mosaic embryo or in mixed aggregates exaggerates properties that are otherwise difficult to detect during normal boundary formation. Experimental data showed a perfect consistency of the molecular and cellular mechanisms [19, 82], validating the relevance of cell sorting assays.

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Figure 1

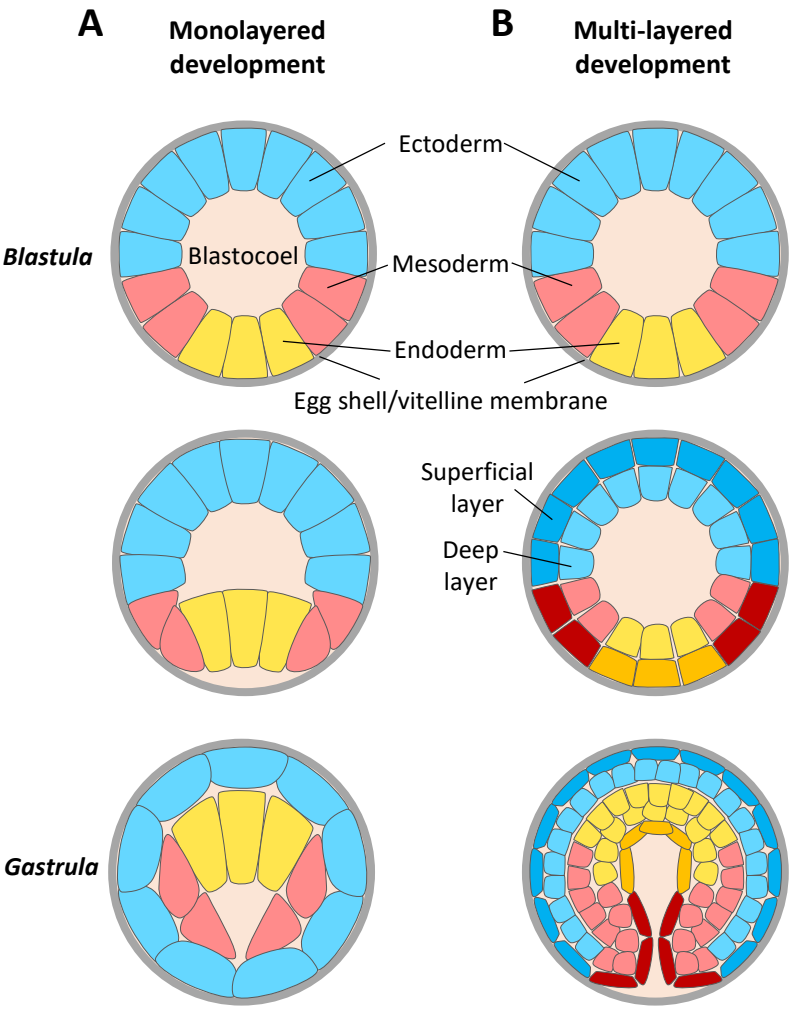

A
Monolayered development
B
Multi-layered development
Ectoderm
Blastocoel
Mesoderm
Blastula
Endoderm
Egg shell/vitelline membrane
Superficial layer
Deep layer
Gastrula

Figure 2

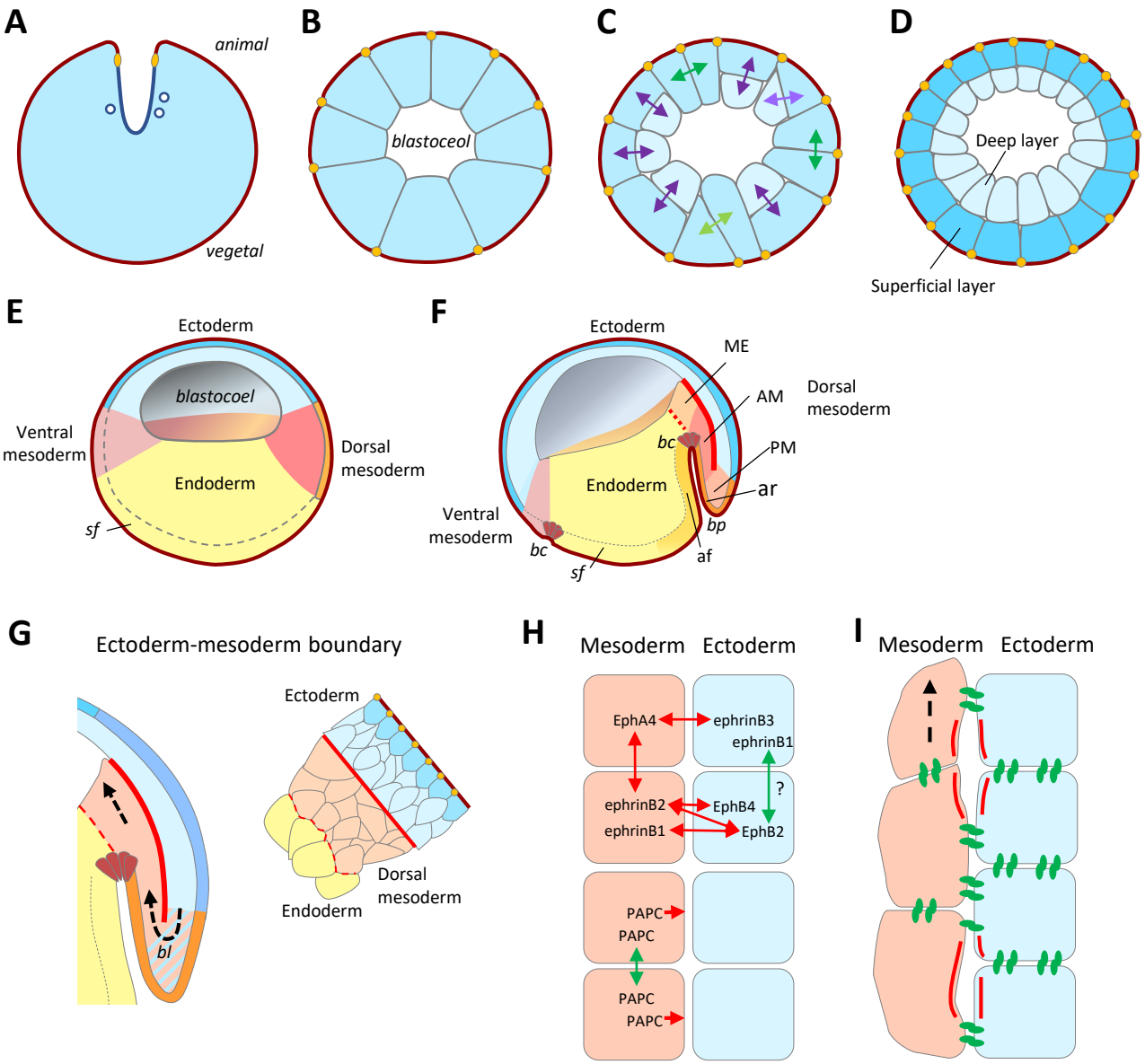

A
animal
vegetal
B
blastoceol
C
D
Deep layer
Superficial layer
E
Ectoderm
blastocoel
Ventral mesoderm
Dorsal mesoderm
Endoderm
sf
F
Ectoderm
ME
AM
Dorsal mesoderm
bc
PM
Endoderm
ar
Ventral mesoderm
bp
bc
af
sf
G
Ectoderm-mesoderm boundary
bl
Ectoderm
Dorsal mesoderm
Endoderm
H
Mesoderm
Ectoderm
EphA4
ephrinB3
ephrinB1
ephrinB2
EphB4
?
ephrinB1
EphB2
PAPC
PAPC
PAPC
PAPC
I
Mesoderm
Ectoderm

Figure 3

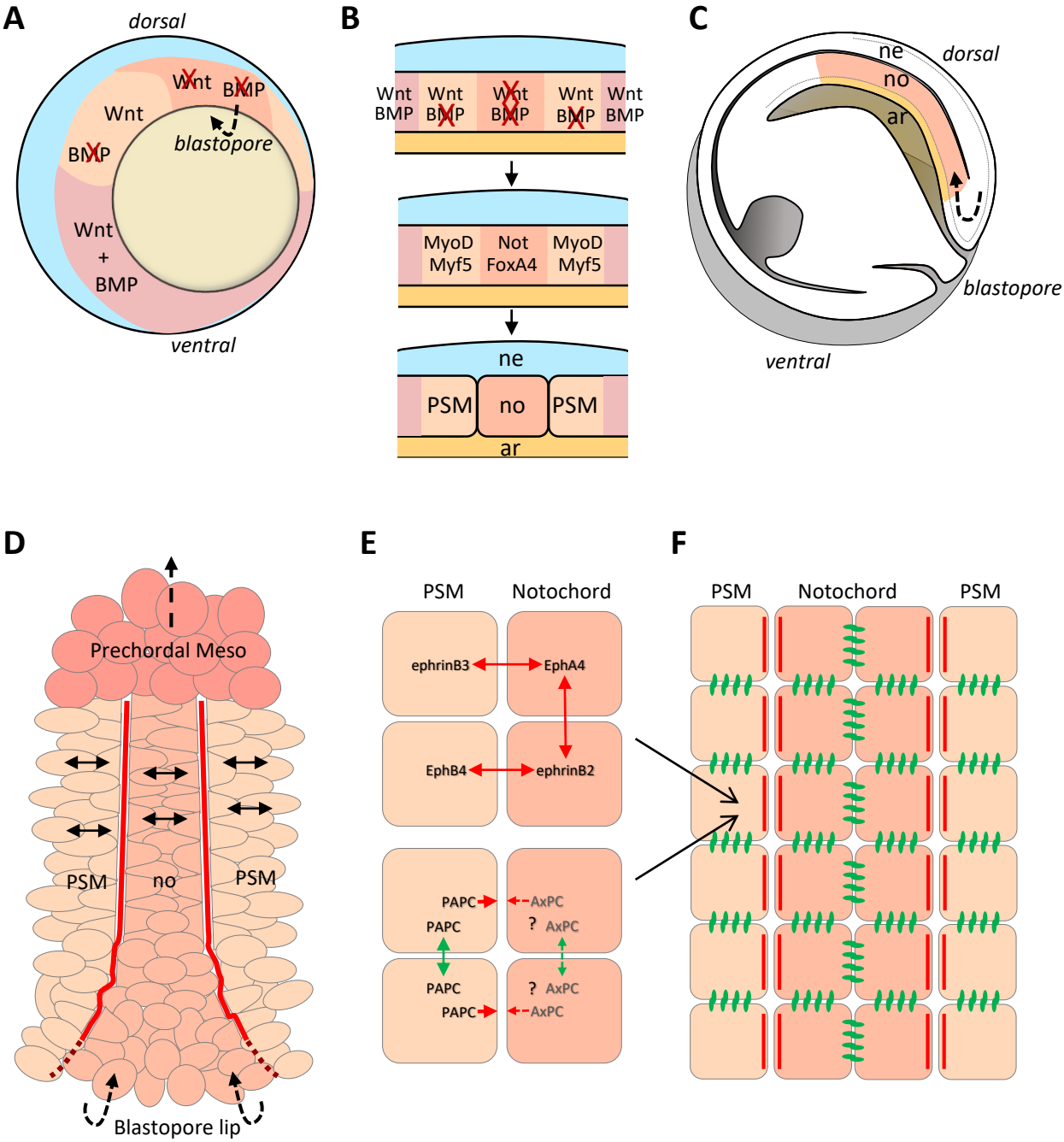

A
dorsal
Wnt
BMP
Wnt
BMP
blastopore
Wnt
+
BMP
ventral
B
Wnt
BMP
MyoD
Myf5
Not
FoxA4
MyoD
Myf5
ne
PSM
no
PSM
ar
C
ne
dorsal
no
ar
blastopore
ventral
D
Prechordal Meso
PSM
no
PSM
Blastopore lip
E
PSM
Notochord
ephrinB3
EphA4
EphB4
ephrinB2
PAPC
AxPC
PAPC
? AxPC
PAPC
? AxPC
PAPC
AxPC
F
PSM
Notochord
PSM

Figure 4

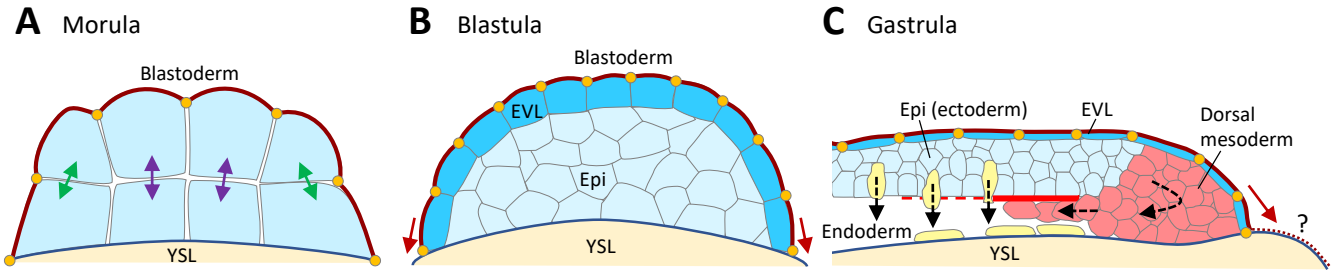
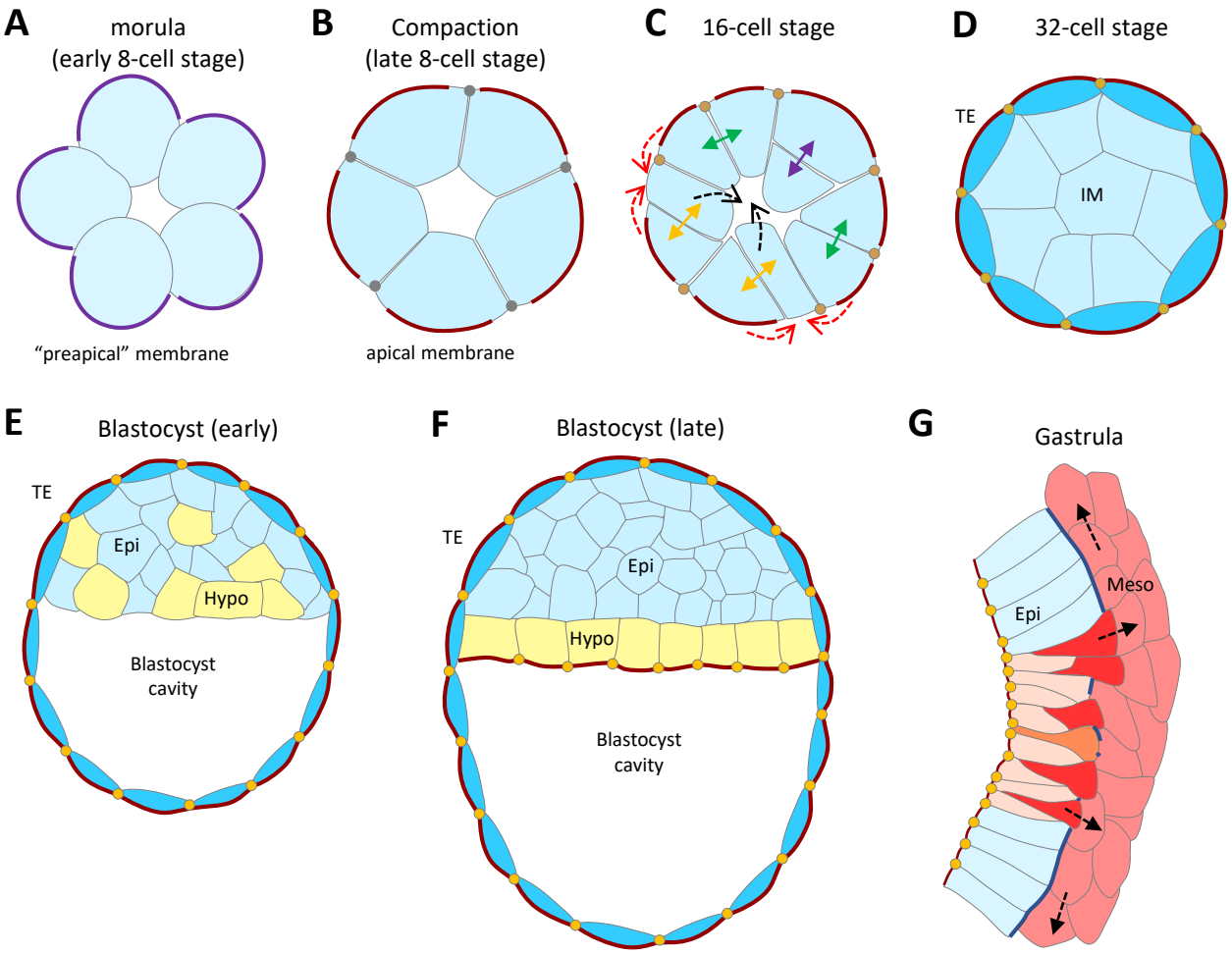
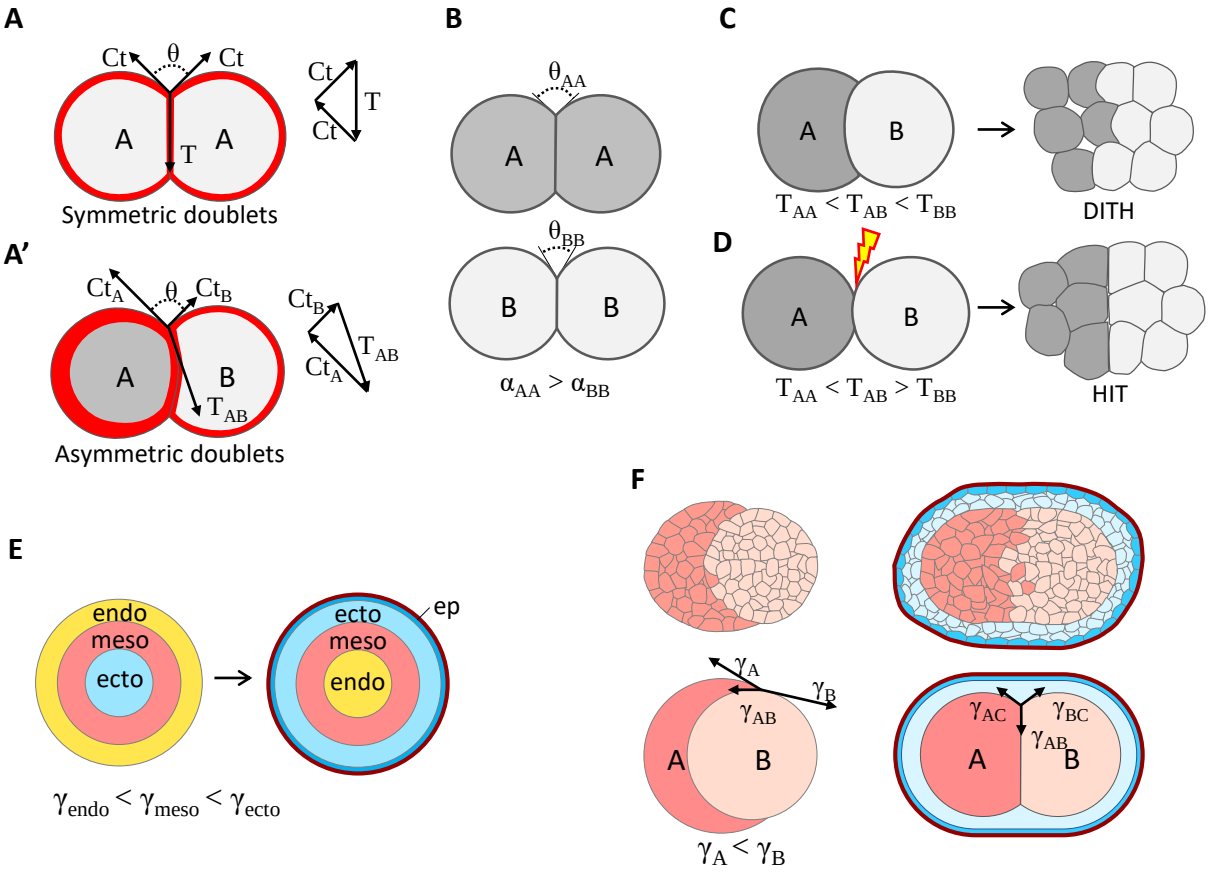

A Morula
Blastoderm
YSL
B Blastula
Blastoderm
EVL
Epi
YSL
C Gastrula
Epi (ectoderm)
EVL
Dorsal mesoderm
Endoderm
YSL
?

Figure 5

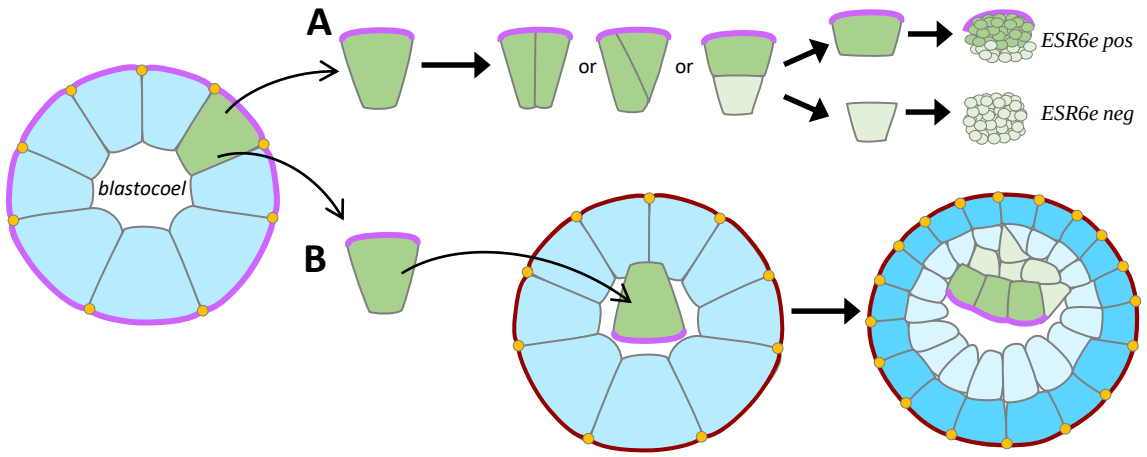

A
morula
(early 8-cell stage)
"preapical" membrane
B
Compaction
(late 8-cell stage)
apical membrane
C
16-cell stage
D
32-cell stage
TE
IM
E
Blastocyst (early)
TE
Epi
Hypo
Blastocyst
cavity
F
Blastocyst (late)
TE
Epi
Hypo
Blastocyst
cavity
G
Gastrula
Meso
Epi

Box 1



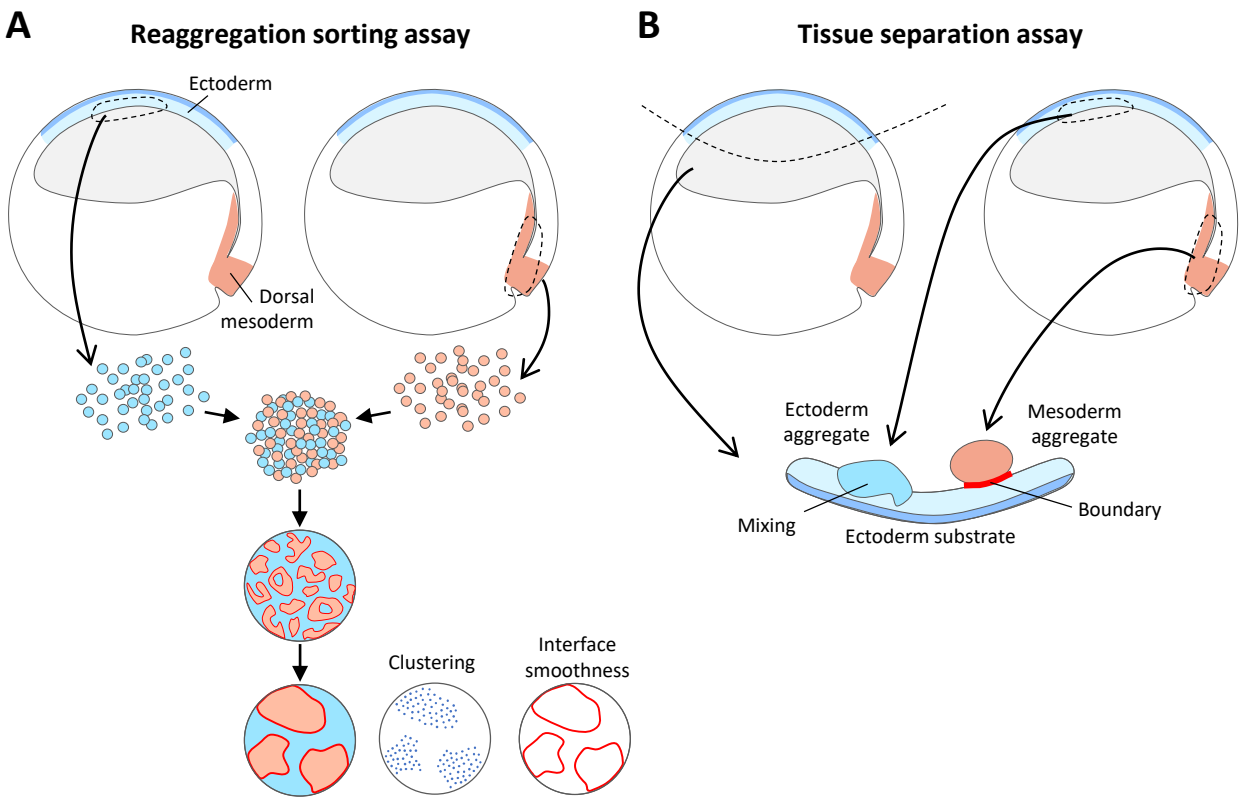
Box 1. Cortical tension, contact tension, tissue surface tension and epithelial coating.
A,B) Cortical and contact tensions. A) Cell and tissue geometry reflect underlying forces. For a simple cell doublet, the angles at vertices are determined by the equilibrium between cortical tensions Ct and contact tension T. Contact tension is mainly dictated by the local cortical contractility along the contact, which is reduced under the influence of adhesive interactions relative to the free surface tension. This reduction defines the relative adhesiveness $\alpha = (T-Ct)/Ct$, and is directly related to the angle θ by the relation $\alpha = 1-\cos(\theta)$ [14, 20]. **A')** Doublet asymmetry indicates a difference in Ct: The cell with the lowest Ct, here B, tends to engulf the other cell. The same relationship applies to groups of cells and tissues, replacing Ct and T with "tissue surface tensions" γ [13, 16]. **B)** Example of two doublets with high and low θ, indicative of high and low adhesiveness, respectively. **C,D) Heterotypic contacts and boundary formation. C)** For cell types with different homotypic tension $T_{AA} < T_{BB}$, heterotypic tension T_{AB} is predicted to be intermediate, consistent with the interfacial tension hypothesis (DITH). Under these conditions, cells can cluster but do not efficiently segregate. **D)** Higher contact tension can be triggered by local stimulation of contractility and/or decrease in adhesion. High heterotypic interfacial tension (HIT) creates a smooth boundary. **E,F) Tissue positioning and epithelial coating. E)** In reaggregation or tissue fusion experiments, tissues with the highest tissue surface tension γ are surrounded by cells with lower γ. Thus, ectoderm typically ends up at the centre, surrounded by mesoderm and endoderm. Coating the tissue aggregate with the superficial epithelial layer (ep) reverses the position of the tissues, reproducing the normal organization in the embryo. This is due to the non-adhesive apical surface (dark red), which forces the superficial position of polarized cells, which in turn preferentially attract the deep ectoderm cells [16]. **F)** Epithelial coating also explains tissue positioning in linear patterns, for instance for anterior "A" and posterior "B" mesoderm. In vitro, "A" engulfs "B" due to its lower γ. Upon inclusion of the ectoderm epithelial layer, which mimics the natural situation, the system is only subjected to the tissue to tissue tensions, which are much weaker than the surface tensions of the tissues exposed to the medium. This allows the mesoderm regions to sort based on other parameters, such as specific adhesion [13, 16].

Box 2



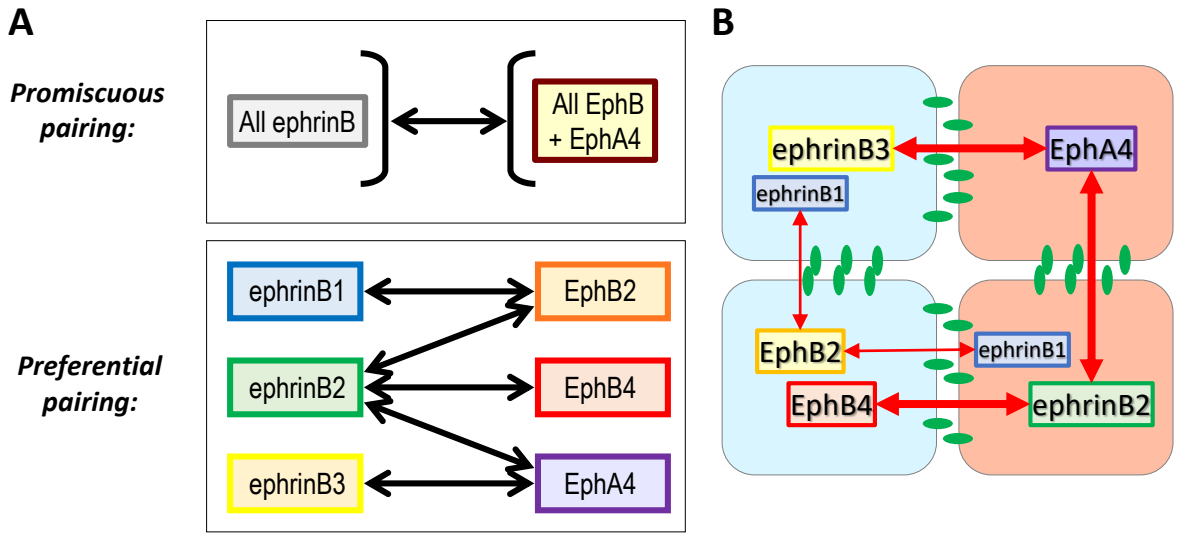
Box 2. Experimental demonstration of cell autonomous inheritance of the apical domain.
A) A single blastomere dissociated from a 64-cell stage embryo can divide along various cleavage planes. Daughter cells that inherit the apical egg membrane (light purple) will remain superficial cells (dark green), and develop into spheroids expressing the bHLH gene ESR6e, while those lacking the apical domain will become deep cells (light green), and develop into ESR6e-negative spheroids [22]. **B)** A single blastomere from a labelled embryo is grafted inside the blastocoel of a host embryo. Descendant blastomeres that have inherited of the original egg membrane form an ectopic, inverted, polarized layer with the apical domain facing the inside of the blastula. Other descendants lacking this apical domain integrate into the deep layer [24].

Box 3



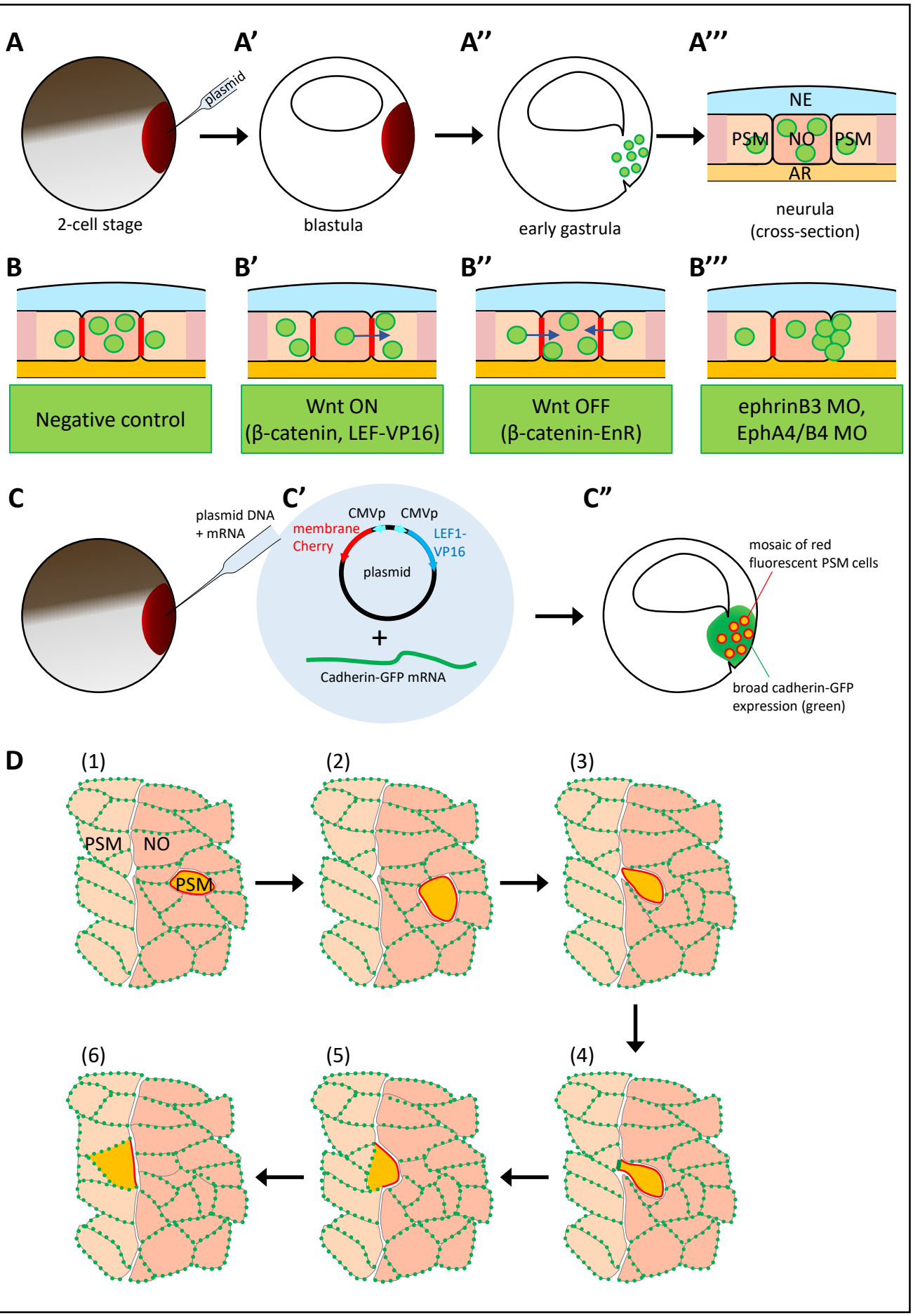
Box 3. Ectoderm-mesoderm separation as an experimental model for cell sorting at embryonic boundaries. **A)** The classical sorting assay [4] involves dissection of tissue explants and their dissociation in an alkaline, calcium-free buffer. Cell populations are mixed and left to reaggregate in a physiological buffer. Wild type ectoderm and mesoderm cells sort into small clusters that progressively merge into larger groups delimited by smooth boundaries [19]. Sorting is quantified in terms of clustering and interface smoothness. **B)** The tissue separation assay [54] uses a large piece of ectoderm as cellular substrate, on which tissue aggregates are laid. Wild type ectoderm aggregates sink and mix within the ectoderm substrate, while mesoderm aggregates remain separated, thus reconstituting the endogenous boundary. Both assays can be used to test any recombination of tissues.

Box 4



Box 4. The ephrin code. Ephrin and Eph receptors are cell surface molecules involved in repulsive reactions. They have been extensively studied in the nervous system, where they function as contact guidance cues, but are also widely expressed in other tissues, in adults and embryos. Ephrin-Eph interaction can trigger signalling both in the Eph-expressing cell (forward signalling) and in the ephrin-expressing cell (reverse signalling). Their activation leads to local remodelling of the actin cytoskeleton, mainly via Rho-dependent actomyosin contractility. **A)** Ephrins and Eph receptors are classified in A and B subfamilies. Traditionally, ephrin-Eph interactions are considered to be promiscuous within each subfamily, ephrinAs reacting with all EphAs, and ephrinBs with all EphBs (top panel). One exception is EphA4, which can interact with ephrins of both subfamilies. However, this assumed promiscuity is at odds with widely different binding affinities, even within the same subfamilies [62-64]. Furthermore, it could not account for the case of the early vertebrate gastrula, where multiple ephrins and Eph receptors are expressed in all tissues, yet overt repulsion is restricted at the tissue boundaries. We demonstrated a strong functional selectivity for ephrinB1,2,3 and EphB2, B3 and A4 receptors in the physiological context for early embryonic boundaries, resulting in a network of interactions (bottom panel): Some members bind multiple partners (e.g. ephrinB2 binds all three receptors), while others can only interact with a single partner (e.g. ephrinB3 with EphA4, ephrinB1 with EphB2). **B)** Simplified diagram presenting the major ephrin and Eph receptors expressed in the dorsal ectoderm and mesoderm (ephrins and Ephs presented in separate cells for clarity's sake). The output of these complex systems relies on three simple principles, selectivity, complementary expression, and balance with adhesive forces (cadherins in green): Repulsion at a given contact results from the combined action of multiple ephrin-Eph pairs (red double arrows, thickness symbolizes the relative intensity). The partially complementary expression of some key ephrin-Eph pairs serves as a "code" that discriminates between homotypic contacts, where repulsion is weak (ectoderm) to moderate (mesoderm), and heterotypic contacts, where it is sufficiently strong to overcome adhesion. In addition to the dorsal ectoderm-mesoderm boundary, this code also accounts for ventral ectoderm-mesoderm separation (not shown), as well as for the notochord boundary (Fig.3). The existence of weaker repulsive signals at homotypic contacts likely contributes to dynamic adhesion within the tissues [36]. Similar systems with multiple ephrins and Eph receptors are frequently observed, which may be explained based on the same principles [63, 65, 139, 140].

Box 5


A
A'
A''
A'''
plasmid
2-cell stage
blastula
early gastrula
NE
PSM
NO
PSM
AR
neurula
(cross-section)
B
B'
B''
B'''
Negative control
Wnt ON
(β-catenin, LEF-VP16)
Wnt OFF
(β-catenin-EnR)
ephrinB3 MO,
EphA4/B4 MO
C
C'
C''
plasmid DNA
+ mRNA
CMVp
CMVp
membrane
Cherry
LEF1-
VP16
plasmid
+
Cadherin-GFP mRNA
mosaic of red
fluorescent PSM cells
broad cadherin-GFP
expression (green)
D
(1)
(2)
(3)
(4)
(5)
(6)
PSM
NO
PSM

Box 5. The notochord as an experimental model for cell sorting. The individualization of the notochord from the PSM is a powerful system to study cell sorting in a physiological context [56, 78, 79]. **A)** Single cells can be manipulated and tracked in mosaic embryos, produced by simple injection of plasmid DNA targeted to the prospective dorsal side. DNA is not transcribed until mid-blastula (A'), leaving early developmental processes unaffected. Expression in the early gastrula is highly mosaic (A", green cells). Sorting is scored at neurula stage, once the notochord is fully individualized (A'"). **B)** Manipulation of the Wnt zygotic pathway is used as fate switch: Its constitutive activation (β-catenin or LEF1-VP16 chimera) induces cell-autonomous PSM fate. Among manipulated cells, those mis-localized in the notochord will all sort laterally to the PSM, leaving the notochord "empty" (B'). Conversely, repression of the pathway (β-catenin-engrailed repressor chimera) causes opposite sorting to the notochord (B"). (B'") Mosaic ephrin/Eph depletion with morpholinos (MO) perturbs sorting and disrupts the boundary. **C-D)** The sorting process of single cells can be studied by live imaging of dorsal mesoderm explants [78, 79]. **C)** mRNA co-injection is used for broad expression of any marker of interest (e.g. cadherin-GFP). This example shows a plasmid designed for co-expression of the LEF1-VP16 activator and a membrane Cherry fluorescent protein (C'). This strategy allows to track single PSM-fated cells live (orange cells with red outline) with high spatial and temporal resolution, while monitoring cadherin-GFP-positive adherent structures. **D)** Typical contact-dependent sorting behaviour of a PSM cell mis-localized in the notochord. As a result of high heterotypic contact tension, the PSM cell fails to establish stable cadherin contacts (green dots) with notochord cells (1) and is randomly "pushed around" (2). Contact with another PSM cell across the boundary (3) is immediately stabilized (4, green line). The imbalance between this adhesive contact and the non-adhesive heterotypic interfaces (red line) drives rapid and irreversible crossing toward the PSM tissue (5). The cell becomes fully integrated and the boundary straightens (6). Computer simulations support the generalization of this HIT-dependent sorting behaviour [19]. Directional migration [95, 122] may increase the speed of sorting. It is important to note that formation of endogenous boundaries usually involves minimal cell movement, limited to slight displacements that smoothen the interface [47]. Artificial single cell sorting in a mosaic embryo or in mixed aggregates exaggerates properties that are otherwise difficult to detect during normal boundary formation. Experimental data showed a perfect consistency of the molecular and cellular mechanisms [19, 79], validating the relevance of cell sorting assays.