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REVIEW

The cellular basis of tissue separation

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ABSTRACT

The subdivision of the embryo into physically distinct regions is one of the most fundamental processes in development. General hypotheses for tissue separation based on differential adhesion or tension have been proposed in the past, but with little experimental support. During the last decade, the field has experienced a strong revival, largely driven by renewed interest in biophysical modeling of development. Here, I will discuss the various models of boundary formation and summarize recent studies that have shifted our understanding of the process from the simple juxtaposition of global tissue properties to the characterization of local cellular reactions. Current evidence favors a model whereby separation is controlled by cell surface cues, which, upon cell-cell contact, generate acute changes in cytoskeletal and adhesive properties to inhibit cell mixing, and whereby the integration of multiple local cues may dictate both the global morphogenetic properties of a tissue and its separation from adjacent cell populations.

KEY WORDS: Cell-cell adhesion, Differential adhesion hypothesis, Embryonic boundaries, Ephrin/Eph signaling, Morphogenesis

Introduction

A system purely based on gene regulatory networks connected by intercellular signals may be sufficient to define and organize cell lineages *in silico* (Oliveri et al., 2008), but would fall short in mimicking embryonic development, where cells must not only acquire a particular fate, but also divide, change shape, adhere to each other and the extracellular matrix (ECM), migrate, disperse or cluster – thus forming tissues that eventually give rise to the adult organism. To complete any of these processes, cells must modulate their physical properties and adapt to those of their environment, starting with basic parameters such as cell shape, mass, viscosity, rigidity, position, motility and strength of adhesion.

That development is an intricate mixture of patterning/differentiation and morphogenesis has been recognized since the early times of experimental embryology. However, for much of the 20th century, the two fields evolved largely in isolation: with geneticists decoding signaling cascades and mechanisms of transcriptional regulation, while pioneers of modern morphogenesis were identifying and characterizing basic morphogenetic processes. Technical advances on both sides have provided increasingly refined and versatile ways to manipulate gene function and to study cell behavior and measure physical properties. In recent years, equipped with all this conceptual and technical knowledge, it has become possible to tackle the core of 'Entwicklungsmecanik' (mechanics of development; Roux, 1905).

One of the fundamental concepts of morphogenesis is tissue separation (see Glossary Box 1): the process that physically segregates two cell populations (Batlle and Wilkinson, 2012; Dahmann et al., 2011; Tepass et al., 2002; Wacker et al., 2000; Winklbauer, 2009). This process is essential to maintain the coherence of embryonic tissues despite the strong propensity of cells to mix, as augmented by extensive cell division and migration during development. The separation of embryonic tissues is generally detectable before any sign of tissue differentiation, by the appearance of a sharp partition between cell populations that are often morphologically undistinguishable. Such partitions have been named embryonic 'boundaries' (see Glossary Box 1 and Fig. 1 for key examples). Note that boundary formation is not restricted to the separation of future tissues and organs, but boundaries are also found between different regions of a single tissue; some are transient and may not even be reflected in the final organization of the embryo. Their importance goes beyond the simple need to divide the embryo into regions. For instance, they play an important role in tissue patterning and can organize other morphogenetic movements.

Box 1. Glossary

Boundary (embryonic). Physical frontier that prevents mixing between two cell populations. It generally corresponds to a sharp and smooth delimitation.

Cell affinity. Concept proposed by Townes and Holtfreter (Townes and Holtfreter, 1955), referring to the propensity to interact preferentially with cells of the same type.

Compartments. Subdivisions of an embryonic tissue delimited by stably inherited boundaries. Originally described for insect imaginal discs and embryonic epidermis, but later also identified in vertebrates (e.g. brain, limb buds).

Contact inhibition (of cell motility). Cell behavior triggered by direct cell-cell contact and involving local inhibition of protrusive activity and/or cell retraction.

Cortex. Actin-based structure coating the inner surface of the plasma membrane. Provides rigidity/mechanical resistance and counterbalances cytoplasmic hydrostatic pressure.

Differential adhesion hypothesis (DAH). Model based on analogy with the physics of immiscible liquids, which postulates that differences in cell-cell adhesion dictate the sorting of cell populations.

Differential interfacial tension hypothesis (DITH). Model whereby cell sorting is dictated by the balance between antagonistic forces produced by cell cortex contractility and cell-cell adhesion.

Homophilic/heterophilic binding. Interaction between identical/different CAMs or cell surface receptors.

Homotypic/heterotypic interaction. Contact between cells of the same/different type.

Interfacial tension. The result of tensile and adhesive forces at the interface between two cells or tissues (see surface tension).

Sorting. The process during which individual cells exchange neighbors, increasing the number of homotypic contacts and decreasing the number of heterotypic contacts.

Surface tension. Concept introduced to account for the tendency of cells and tissue explants to minimize their surface exposed to the medium, by analogy with the surface tension of liquids.

Tissue separation. General process that leads to the establishment of a physically isolated embryonic cell population.

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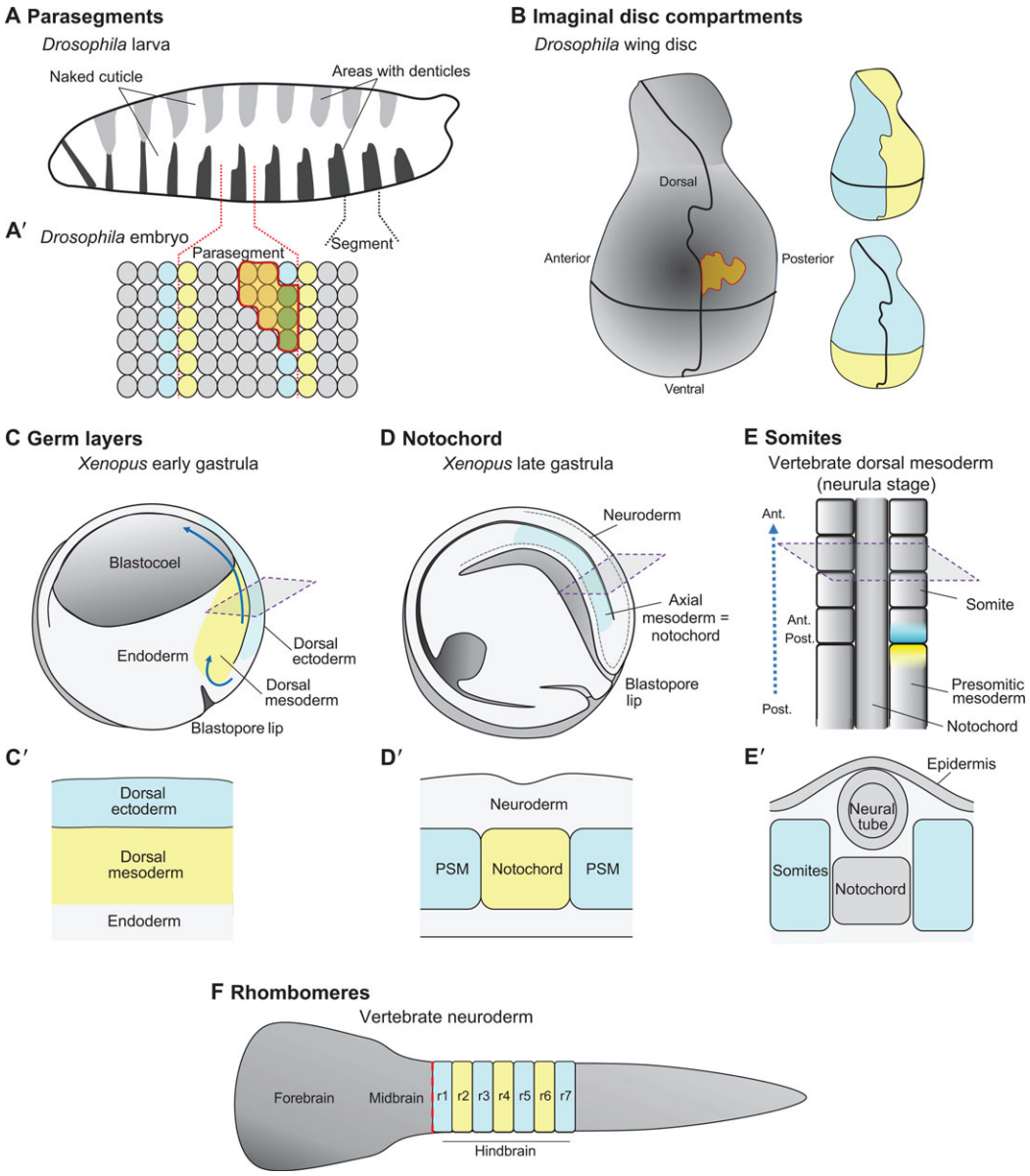

Fig. 1. Examples of boundaries. (A,B) *Drosophila* compartments. (A) Embryonic parasegmental boundary. The larval cuticle displays rows of hairs (denticles) that reflect the internal segmental organization of the organism. (A′) The initial embryonic segmental units (parasegments) comprise six rows of cells. These compartments were identified by the restriction of clonal expansion (orange area with red outline). Specific genes, such as *wg* (yellow) and *engrailed* (blue) are precisely expressed in single rows abutting the boundary. (B) Imaginal discs. The wing imaginal discs are sharply partitioned into anteroposterior and dorsoventral compartments. The corresponding boundaries were also discovered by clonal analysis (orange area) and are preserved during the massive growth of these structures during development. (C-F) Vertebrate boundaries. (C) Ectoderm-mesoderm boundary. In frog (and fish, not shown) embryos, the first visible boundary is formed between the involuting mesoderm and the overlying ectoderm. The mesoderm uses the ectoderm as a substrate for migration as it progresses toward the future anterior region. Directions of cell movements are marked by blue arrows. (D) Notochord-presomitic mesoderm (PSM) boundary. The axial region of the involuted trunk mesoderm forms the notochord, which is separated on both sides from the PSM. These boundaries have been proposed to organize convergence extension of both tissues (Keller et al., 2000). (E) Somite segmentation. The PSM is progressively segmented into somites. Each somite is polarized, with distinct genes expressed in the anterior and posterior halves. Boundaries form at the interface between the posterior cells of the last formed somite and the anterior cells of the unsegmented mesoderm. Ant., anterior; Post., posterior. (C′-E′) Transverse sections at the positions marked by the boxes in C-E. (F) Rhombomeres. Once separated from the midbrain, the hindbrain undergoes segmentation into seven rhombomeres (r1-r7). Unlike somites, rhombomeres do not appear internally polarized. Their separation is instead controlled by alternate gene expression in even and odd segments.

In this Review, I will provide a short summary of the history of the field and revisit some basic concepts and definitions. I will then introduce the major molecular players that have been implicated in the process of boundary formation and discuss the principal current models in the light of recent experimental data. I will finally attempt to draw an updated picture of tissue separation viewed from a cellular perspective, highlighting the key role of local reactions.

Historical overview of tissue separation

Pioneer morphogeneticists, such as Holtfreter, Moscona and Steinberg, showed that dissociated cells taken from different embryonic tissues would initially form a mixed aggregate, but would later progressively sort to re-form well-segregated populations (Fig. 2C) (Wilson, 1907; Moscona and Moscona, 1952; Townes and Holtfreter, 1955). These experiments revealed that single cells, once

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determined, maintained their identity in isolation, even when surrounded by other cell types. Cells were able to recognize the identity of their neighbors, gathering with cells of the same type and ultimately resegregating. Holtfreter named this basic property of embryonic cells 'cell affinity' (see Glossary Box 1) (Holtfreter, 1939).

Another fundamental discovery was that of 'compartments' (see Glossary Box 1). *Drosophila* geneticists observed that clones produced in imaginal discs did not always expand randomly, but were instead restricted to one or other side of virtual lines that partitioned the tissue (Fig. 1A,B) (Garcia-Bellido et al., 1973; reviewed by Dahmann and Basler, 1999). The notion of a 'compartment boundary' was subsequently expanded to the vertebrate embryo (Altabef et al., 1997; Dahmann et al., 2011; Fraser et al., 1990; Zervas et al., 2004).

With the characterization of the cell junctions and of the actin cytoskeleton, the concept of cell affinity was translated at the cellular level into the notion of cell-cell adhesion, and Steinberg had the revolutionary idea to couple this cell biological concept with the physical principle of surface tension (see Glossary Box 1), proposing the differential adhesion hypothesis (DAH; see Glossary Box 1) to explain cell sorting (Steinberg, 1970). The discovery of cell-cell adhesion molecules (CAMs) that bind specifically to themselves (homophilic binding, Fig. 2A; see Glossary Box 1) (Nose et al., 1988) suggested a molecular explanation for cell-cell affinity and tissue segregation (Steinberg and Takeichi, 1994). Differential cortical tension was put forward as an alternative to DAH as a mechanism to explain tissue separation (Harris, 1976). Brodland (2002) integrated both adhesion and tension in a single differential interfacial tension hypothesis (DITH; see Glossary Box 1), and the relative importance of these two parameters is still a matter of debate. Moreover, we now have a much more comprehensive view of the molecules involved in tissue separation, which include not only CAMs but also cell-cell repulsive factors, such as the ephrin/Eph pairs, and numerous intracellular molecules that regulate cytoskeletal activity. These are discussed further below.

Examples of boundary formation

A number of classic examples in both vertebrate and invertebrate systems have served as paradigms for our analysis of tissue separation. In *Drosophila*, key patterning processes subdivide early embryonic blastoderm into stripes corresponding to the segmented organization of the future larva (Sanson, 2001). Patterning is translated into physical boundaries, called parasegment boundaries (Fig. 1A). The early segmentation produces an extremely refined pattern of gene expression, which may be restricted to single rows of cells (Sanson, 2001), and the parasegment boundaries are important to prevent dividing cells from blurring this fine pattern (Monier et al., 2010). Larval imaginal discs face another challenge: intense cell proliferation and tissue growth through larval life. In order to maintain patterning in these expanding tissues, two orthogonal dorsoventral and anteroposterior boundaries bisect the imaginal discs (Fig. 1B). These were discovered by the analysis of genetically produced mosaic embryos, in which clones of cells were found to be restricted to one side of a straight 'line' that defined a compartment (Garcia-Bellido et al., 1973; Martinez-Arias and Lawrence, 1985) (Fig. 1A,B); although the dorsoventral boundary of the fly wing disc is retained as the wing margin in the adult, neither the parasegment

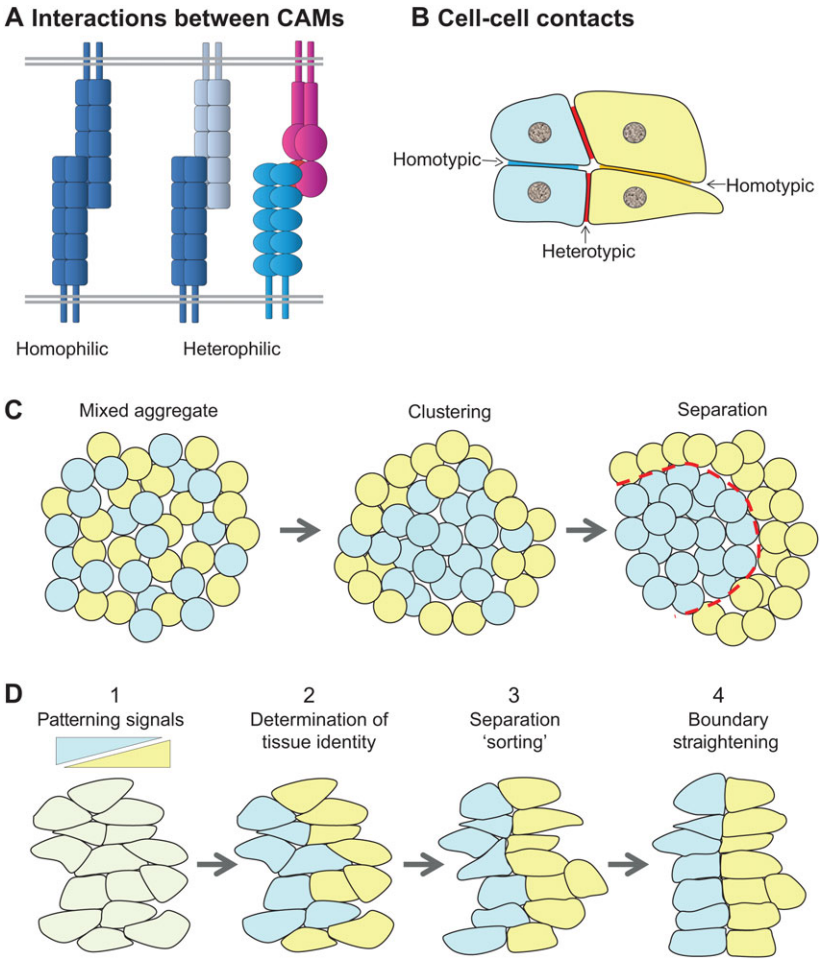

Fig. 2. Cell sorting at boundaries and in cell aggregates. (A) Types of cell-cell adhesive interaction. Cell-cell adhesion can be mediated by homophilic interactions between identical cell-cell adhesion molecules (CAMs), or heterophilic interactions between either different isoforms from the same CAM family or between different CAMs. (B) Types of cell-cell contact. Contacts between cells of the same type are called homotypic, whereas those between different cell types are heterotypic. Note that this definition is based purely on cell identity: in both cases, adhesions may be homophilic or heterophilic in nature, and heterotypic contacts may also be non-adhesive. (C) Cell sorting in mixed aggregates. Cell sorting is typically assayed *in vitro* by mixing two populations of dissociated cells. They may then progressively sort, producing initially coarse clusters, which may eventually be separated by clear-cut boundaries (red dashed line). (D) Typical cell rearrangements during boundary formation. In an originally homogenous cell mass (1), localized patterning signals (blue and yellow triangles) determine two cell types (2). The position/geometry/properties of the signals roughly delineate the two regions, but at single-cell resolution the fringes of the two populations are intermingled. As cells acquire 'separation behavior' (3), they sort out and the interface sharpens, eventually forming a straight boundary (4).

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nor the anteroposterior imaginal disc boundaries leave direct anatomical traces of their initial position.

Although boundaries in vertebrate embryos also often start as inconspicuous lines delimiting otherwise indistinguishable cell populations, they generally develop into actual physical separations of embryonic tissues. Examples of vertebrate boundaries include the early separation of ectoderm and mesoderm (Fig. 1C), and the subsequent longitudinal isolation of the axial mesoderm, which produces the notochord, from the adjacent paraxial mesoderm [also called presomitic mesoderm (PSM)] (Fig. 1D). This region in turn becomes segmented into somites (Fig. 1E). Boundaries also appear in the neuroderm, first separating the midbrain from the hindbrain, then segmenting the latter into seven rhombomeres (Fig. 1F). These boundaries all become anatomically visible at some point, although they do not necessarily correspond to the final contour of an adult structure: ectoderm and mesoderm obviously differentiate and separate into many different tissues and organs. The notochord will regress and disappear, while different parts of the somites give rise to structures as distinct as the vertebrae or muscles. However, some boundaries do persist in the adult; for example, that which separates the stomach into two halves (San Roman and Shivdasani, 2011).

In addition to these classical cases, many other segregation events occur in developing embryos, most of which have hardly been studied. Among them are the placodes that go on to form various structures such as eyes and ears (Baker and Bronner-Fraser, 2001), or the multiple domains of neural crest cells, which populate many different but very specific regions of the vertebrate embryo (Theveneau and Mayor, 2012; McKeown et al., 2013). Whether these systems involve bona fide boundaries is an unexplored and very exciting question.

General principles of tissue separation and boundary formation

Before getting into the heart of the discussion, it is useful to consider some concepts and definitions, in particular what should be considered a boundary and whether tissue separation may be equated to the phenomenon of cell sorting (see Glossary Box 1).

Defining a boundary

The term ‘embryonic boundary’ has historically covered two different concepts: gene expression boundary and tissue boundary. The first type was subdivided into two categories based on lineage-tracing experiments: those boundaries that restricted clone expansion were named lineage boundaries (or compartment boundaries); when a clone could contribute to both sides of the boundary, the term non-lineage boundary was used. This distinction implied fundamental differences in the way that the identity of the two cell populations (as defined by gene expression patterns) was set: in the former case, the identity was stably inherited by each cell, whereas in the latter situation it had to be actively maintained by non-cell-autonomous patterning signals. Lineage boundaries are rather rare, but include the classic examples delimiting compartments in insects and the segments of the vertebrate brain (reviewed by Dahmann et al., 2011; Tepass et al., 2002; Fraser et al., 1990). Examples of non-lineage boundaries are the notum-wing boundary in *Drosophila* and the somite boundaries in vertebrates (Zecca and Struhl, 2002; Tepass et al., 2002).

The analysis of vertebrate boundaries has somewhat complicated this distinction, particularly because the parallel between lineage and stability of cell identity is not as absolute as originally thought. The notochord-PSM boundary is a good illustration of this point. According to the classical definition, this is a bona fide lineage boundary, since, once formed, it permanently isolates the two cell populations. However, when PSM cells are grafted into the notochord, they can adopt notochord fate and integrate into their new environment (Domingo and Keller, 2000). However, if single notochord cells are forced to adopt a stable cell-autonomous PSM fate, they will sort out and incorporate into the adjacent PSM, as expected from a lineage-dependent process (Reintsch et al., 2005). In other words, the ability of PSM cells to sort is cell-autonomous, but seems to depend on a genetic program that requires constant signaling from neighboring cells. Whether insect lineage boundaries always correspond to stable cell identity remains an open question: the anterior and posterior cells of imaginal discs fail to sort when isolated and mixed (Fausto-Sterling and Hsieh, 1987), indicating that the information required to maintain their separation is lost under these experimental conditions. Thus, despite its historical interest, the notion of lineage/non-lineage expression boundaries is not directly relevant to the mechanism of separation.

The concept of tissue boundary, defined as an absolute limit to cell mixing, focuses on the central process of physical separation (Tepass et al., 2002). The term is used for all types of physical boundaries – those that partition a cell mass into distinct regions (e.g. brain segmentation or notochord formation) as well as those that are formed by the apposition of two pre-existing cell populations (e.g. the involuting mesoderm sliding along the ectoderm in fish and frogs). Boundary formation may also involve the migration of individual cells, as in the cases of mesoderm ingression during gastrulation in amniotes and micromere migration in echinoids, which resemble cell sorting as reconstituted from mixed aggregates. Note that the apparent diversity of these modes of boundary formation might not necessarily reflect fundamental differences in terms of the actual mechanism that prevents cell mixing. In vertebrates, all these types of boundary occur, yet, as we will see below, they all rely on the same set of molecules and probably on very similar mechanisms.

It is notable that boundaries tend to coincide with the limits of domains of gene expression, indicating a tight link between patterning and the physical restriction of cell movement. However, gene expression boundaries do not necessarily require physical separation, and these two concepts should not be considered equivalent.

Relationship between cell sorting and boundary formation

The discovery that dissociated embryonic cells maintain their tissue identity and are able to sort back from mixed aggregates (Townes and Holtfreter, 1955) is at the foundation of our understanding of tissue separation. Note that the original experiments had the caveat of using cells isolated long after tissues had formed. However, since then the principle has been validated by direct observation of cell sorting within the time window of normal tissue separation (e.g. Wacker et al., 2000; Fagotto et al., 2013; Calzolari et al., 2014). The reconstitution of the separation process in mixed aggregates remains a simple and powerful assay with which to study the underlying mechanisms (e.g. Chen et al., 2009; Ninomiya et al., 2012). It is however crucial to remember that mere sorting into distinct clusters cannot be equated to separation (Fig. 2C). Ideally, the presence of a tight boundary preventing cell mixing should be experimentally verified, for instance by live microscopy, but a smooth interface is a reasonably good criterion for efficient boundary formation and tissue separation. As discussed later, this invariable feature of embryonic boundaries (e.g. Dahmann et al., 2011) reflects an abrupt discontinuity in adhesion/tension. The expression of a truncated

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paraxial protocadherin [PAPC; also known as protocadherin 8 (PCDH8)], for example, typically produces strikingly sharp interfaces (e.g. Chen et al., 2009), whereas a difference in cadherin levels does not (Foty and Steinberg, 2004; Ninomiya et al., 2012), suggesting that cells can cluster without necessarily forming a boundary.

How much sorting occurs in embryos?

Considering the high precision with which patterning processes can set positional information, it is legitimate to ask whether any actual sorting is required during boundary formation, or whether boundaries simply stabilize separation between already distinct cell populations. So far, the detailed movements of single cells during the process of boundary formation have only been studied for the *Xenopus* notochord boundary (Keller et al., 1989; Wilson and Keller, 1991; Shih and Keller, 1992a, b; Fagotto et al., 2013). The observations showed that very small displacements of less than one cell diameter toward one side or the other are sufficient to straighten the nascent boundary (Fig. 2D). The situation is probably similar in most other systems. For instance, the compartment boundaries of the *Drosophila* wing imaginal disc exist, and are relatively smooth, from very early stages (Landsberg et al., 2009). These data suggest that cells do not actively sort out in order to form the boundaries in these contexts. The ingression of individual mesoderm cells in amniotes, which generates a boundary between ectoderm and mesoderm layers, more closely resembles the classical picture of cell sorting, although it is difficult to distinguish the actual mechanism of separation from simple exclusion of mesoderm cells from the ectodermal epithelial layer upon loss of apical-basolateral polarity. In general, however, we have yet to achieve a comprehensive picture, in any species, of the degree to which active cell sorting contributes to tissue separation during development.

Cell adhesion and contractility in tissue separation

Any description of tissue separation must take into account two key cellular components: the actin cytoskeleton and cell-cell adhesion complexes. The cortex (see Glossary Box 1) underlying the plasma membrane constitutes a complex and versatile actin-based structure that can rapidly change between two types of configuration (Fig. 3A): it can be enriched in actomyosin fibers, which confer high tension, rigidity and stability, or it can be composed of polymerizing/depolymerizing actin filaments, which produce dynamic outward-expanding protrusions. Protrusive and contractile activities can be viewed as antagonistic, their balance determining specific cell cortex properties.

Among the many CAMs expressed in metazoans, a dominant role for cadherins in cell-cell adhesion and morphogenesis, and the requirement for a connection with the actin cytoskeleton, were concepts that emerged early on (Gumbiner and McCrea, 1993; Kemler, 1993; Takeichi, 1995; Gumbiner, 1996). Classical cadherins, such as the epithelial E-cadherin or the neural and mesenchymal N-cadherin, are traditionally viewed as selective homophilic adhesion molecules (although, as discussed later, there might in fact be significant binding between different cadherins). They are characterized by a highly conserved C-terminal tail, which recruits a set of cytoplasmic proteins called catenins (Fig. 3A) (Gumbiner, 2005). Association with β-catenin and α-catenin is required for adhesion (Nagafuchi et al., 1994), and the classical model (now somewhat controversial) considers that catenins act as a physical connection to actin fibers (discussed by Ratheesh and Yap, 2012). Cadherins can form large adhesive clusters, interacting with

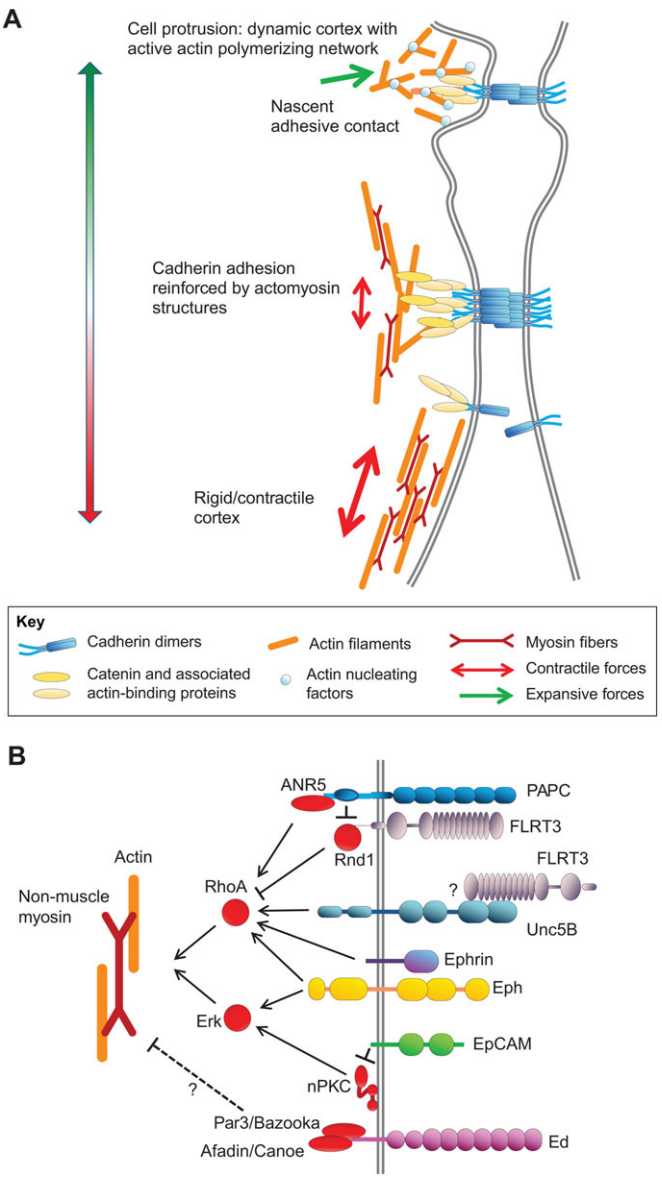

Fig. 3. Key molecules implicated at embryonic boundaries. (A) Cell cortex and cadherin adhesion. The actin cytoskeleton that underlies the plasma membrane can adopt two major configurations: a dynamically polymerizing network typical of expanding protrusions (top, green arrow), or a rigid system comprising bundles of contractile actomyosin fibers (bottom, red arrows). Protrusive activity (top) favors the formation of cadherin contacts. In turn, cadherin-catenin complexes recruit various actin regulatory proteins, including actin nucleating factors, which promote actin dynamics and protrusive activity, thus establishing a dynamic feedback at nascent cell-cell contacts. By contrast, strong cortical contractility (bottom) is antagonistic to cell adhesion, preventing the formation of new contacts and destabilizing/disrupting existing contacts. However, contractility is not necessarily opposed to adhesion: actomyosin structures are recruited to maturating adhesions, where they are thought to anchor cadherin clusters and reinforce adhesion (center). The relationship between these various actin pools is unclear. (B) Cell surface cues. A number of cell surface molecules regulating tissue separation have been identified. Cytoplasmic effectors are represented in red; arrows do not necessarily represent direct interactions. All components impinge on the regulation of myosin activity, generally either via RhoA or Erk. FLRT3 can interact in *cis* with PAPC and in *trans* with Unc5B. Ephrins and Eph are receptors and ligands for each other (not shown). Alone, PAPC tends to activate RhoA via ANR5 (Ankef1), but its association with FLRT3 relieves Rnd1-mediated Rho inhibition. The pathway downstream of Ed is not yet elucidated. EpCAM directly inhibits novel PKCs (nPKC).

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multiple factors that regulate the cytoskeleton, such as vinculin, α-actinin, actin filament nucleating factors and Rho GTPase regulators (Baum and Georgiou, 2011; Ratheesh and Yap, 2012; Brieher and Yap, 2013). An alternative to the classical model proposes that the cadherin complex represents a hub for regulation of the actin cytoskeleton, rather than a direct physical connection, and that α-catenin can regulate the local cytoskeleton independently of its interaction with cadherins and β-catenin (Yamada et al., 2005; Halbleib and Nelson, 2006).

Many studies have provided direct molecular evidence for a substantial cross-talk between cadherins and the cytoskeleton, yet the picture is currently far from clear (Baum and Georgiou, 2011; Brieher and Yap, 2013; Ratheesh et al., 2012). For example, Rho-regulated myosin activity is antagonistic to Rac-dependent protrusive activity, and thus to the establishment of adhesive contacts, yet at the same time it appears to be involved in strengthening adhesive contacts and in coupling of the cytoskeleton to adhesive structures (Cavey and Lecuit, 2009; Yamada and Nelson, 2007). The relationship between the different actin pools is also unclear, although they are all likely, in one way or another, to be interconnected.

Cell-cell contact molecules in tissue separation

In addition to cadherins, a number of cell surface molecules have been implicated in regulating boundary formation (see Table 1 and Fig. 3B), most notable among which being the ephrin/Eph signaling system.

Ephrins and Eph receptors

Ephrins and Eph receptors are best known for their essential role in the development of the nervous system and its correct wiring, but are expressed in all stages and tissues during vertebrate embryonic development, and, as discussed later, there is ample evidence for their importance in tissue separation as well as in other developmental processes. Ephrin/Eph activation requires lateral clustering, and a peculiarity of the ephrin/Eph systems is the ability of both types of molecules to function as reciprocal ligands and receptors (Lisabeth et al., 2013), with Ephs mediating 'forward signaling' and ephrins mediating 'reverse signaling'. In both cases, ephrin/Eph activation leads, among many other effects, to the upregulation of Rho and Rac, along with cytoskeleton remodeling, typically resulting in the retraction of cell protrusions (Fig. 3B) (Lisabeth et al., 2013).

Table 1. Cell surface contact molecules implicated in separation at tissue boundaries

Cell surface molecule	Ligand	Heterophilic *cis* interactions	Downstream signaling	Effect on contractility	Boundary (species)	Distribution	References
Ephrins/Eph receptors	Ephrins/Eph receptors	Ephrins (–), Eph receptors (–)	RhoA, Erk	Activation (low signal can promote cell adhesion)	Ectoderm-mesoderm (X), notochord-PSM (X), somites (C, Z), rhombomeres (C, Z), midbrain-hindbrain (Z)	Multiple ephrins and Ephs in all tissues, (partial) complementary expression	Barrios et al., 2003; Cooke et al., 2005; Durbin et al., 1998; Fagotto et al., 2013; Kemp et al., 2009; Mellitzer et al., 1999; Oates et al., 1999; Park et al., 2011; Rohani et al., 2011; Sela-Donenfeld and Wilkinson, 2005; Watanabe et al., 2009; Xu et al., 1999
Ed	Homophilic		?	? Activation at contacts where Ed is absent	Follicular epithelium (formation of appendices), epidermis (dorsal closure) (D)	Boundary forms at interface between Ed-expressing and Ed-negative cell populations	Chang et al., 2011; Laplante and Nilson, 2006, 2011; Lin et al., 2007
PAPC	Homophilic	FLRT3 (–), Fz7 (+?)	ANR5 (d), RhoA	Activation	Ectoderm-mesoderm (X), somites (X, M) (DN)	Mesoderm (early gastrula), PSM (neurula), anterior half of somites	Barrios et al., 2003; Chen and Gumbiner, 2006; Kim et al., 2000, 1998; Kraft et al., 2012; Medina et al., 2004; Rhee et al., 2003
FLRT3	Unc5B (?)	PAPC (–)	Rnd1 (d), RhoA	Inhibition	Ectoderm-mesoderm (X)	Mesoderm	Chen et al., 2009; Karaulanov et al., 2009
Unc5B	FLRT3 (?)		RhoA	Activation	Ectoderm-mesoderm (X)	Ubiquitous at gastrulation	Karaulanov et al., 2009; Yamagishi et al., 2011
EpCAM	Homophilic		PKC (d), Erk	Inhibition	Ectoderm-mesoderm (X) (GOF)	Ubiquitous at gastrulation	Maghzal et al., 2013, 2010

This table is restricted to molecules establishing direct cell-cell contact, with the exception of the netrin receptor Unc5B, which is included for its putative interaction with FLRT3. The properties of FLRT3 are unclear. *Cis* interactions have been reported to positively (+) or negatively (–) influence the functions of some of these proteins. All activities appear to convergence toward regulation of myosin activity via Rho and/or Erk. d, direct interactors, with experimental evidence for role in separation. Listed boundaries correspond to cases demonstrated by loss-of-function mutant or depletion, except for PAPC in somites (dominant negative, DN) and EpCAM (gain-of-function, GOF). D, *Drosophila*; C, chicken; M, mouse; X, *Xenopus*; Z, zebrafish.

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Most bilaterian metazoans (except insects) have several ephrins and Ephs, and although the classical textbook picture represents one cell type expressing one ephrin and the second cell type expressing the cognate Eph receptor, in reality most cells co-express several ephrins and several Ephs. This makes the final signaling output difficult to predict, as it might depend not only on relative abundance, binding affinities and kinetics, but possibly also on *cis* and *trans* interactions at the membrane, and on complex intracellular cross-talk (Pasquale, 2010). A particularly interesting observation is that a relatively strong signal seems to be required to trigger cell repulsion. When ligand/receptor clustering and activation is limited, ephrins and Ephs remain bound at the cell surface and effectively act as adhesion molecules (Murai and Pasquale, 2003).

Biophysical representation of adhesion and contractility

Tissue biophysical properties are generally described by a simplified model that was originally inspired by an analogy with the physics of fluids (Steinberg, 1970; Brodland, 2002). In this model, cortical actin contractility and cell-cell adhesion bonds are viewed as the two major components, acting antagonistically: cortical contractility tends to minimize the cell surface (conceptually equating to surface tension), whereas cell adhesion favors cell spreading, either on an ECM substrate or on another cell (Fig. 4A) (Brodland, 2002; Lecuit and Lenne, 2007). This antagonism is represented by two opposing forces, the resulting force corresponding to a cell's interfacial tension (see Glossary Box 1; Fig. 4A). This simple physical description is quite successful at simulating experimental data and provides clear predictions for cell behavior and morphology. Most relevant to the problem of tissue boundaries is the fact that the cell shape and, in particular, the angles formed between adjacent contacts reflect the adhesive and contractile state of these interfaces: a near hexagonal cell shape with ~120° angles corresponds to a situation in which forces are well balanced, whereas angles closer to 90° indicate that one side has high interfacial tension due to either lower adhesion or higher cortical tension (Fig. 4D) (Dahmann et al., 2011; see also Manning et al., 2010). At the larger scale of the tissue, this imbalance translates into a smooth line partitioning the tissue, which is a typical landmark for many boundaries.

Of course, the comparison with fluids is only an analogy and, as readily pointed out by Harris (1976), cells are not only much more complex, but also, and most importantly, 'active' systems. For instance, one of the immediate consequences of the cross-talk between cadherin adhesions and the actin cytoskeleton is the striking reduction of the cortex along cell-cell contacts (Fig. 4B,C) (Yamada and Nelson, 2007).

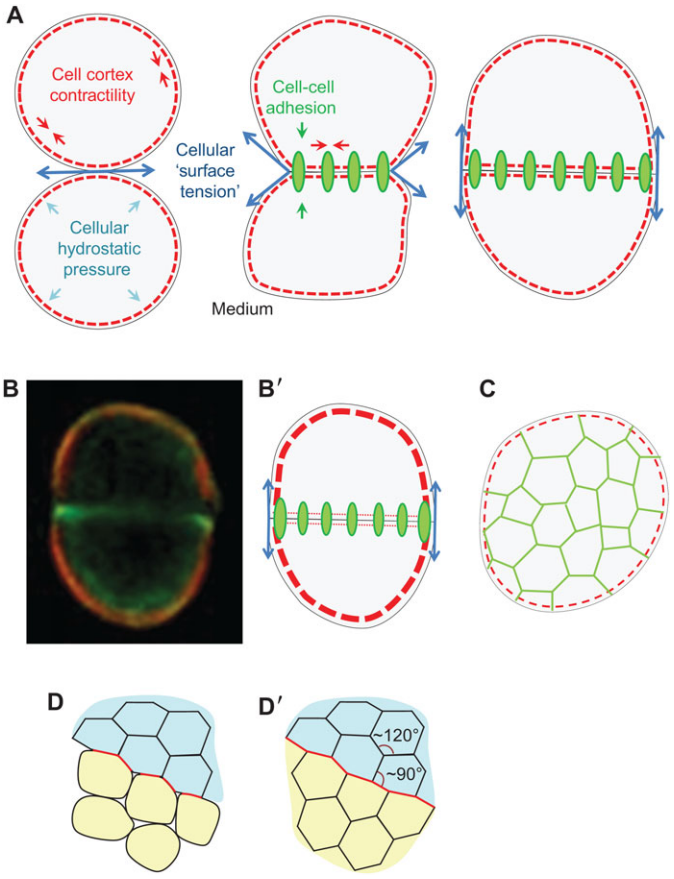

Fig. 4. Cell 'surface tension' and boundary geometry. (A) Cell surface tension, adhesion and interfacial tension. Forces exerted on adhering cells are classically modeled using the concept of cellular surface tension, by analogy with the surface tension of liquids. For single cells, the surface tension (blue arrows) is dictated by the cortical contractility, which tends to minimize the cell surface area (cell rounding). In a system with two or more cells, cell-cell adhesion produces an opposing force that increases cell-cell contact. Interfacial tension results from the balance of these two forces. Three examples are represented with increasing adhesion (green) to contractility (cortical actin in red) ratios. (B,B′) A more realistic representation of multicellular systems should consider the cross-talk between cell adhesion and contractility. (B) Two live *Xenopus* ectoderm cells expressing cadherin-GFP accumulating at cell-cell contacts and RedFP-Utrophin, a marker for actin, showing the thick cortex that borders free cell edges (author's unpublished image). Note the comparatively weak actin signal at cell-cell contacts. (B′) Schematic representation of B. (C) Aggregates of embryonic cells tend to adopt a compact organization that minimizes the edges (dashed red line) exposed to the medium. (D,D′) Cell geometry reflects forces. The shape of the cells and the angles at cell vertices reflect the underlying forces. In a perfectly equilibrated, tightly packed epithelium, cell shapes should be hexagonal, with 120° angles at cell vertices (D′, blue tissue). Loosely adhering cells should be circular/spherical (D, yellow tissue). Heterotypic contacts at tissue boundaries are represented by red lines. Even when both tissues appear similar in terms of adhesion and tension, boundary interfaces tend to be straight, with vertex angles approaching 90° (D′). This is indicative of high interfacial tension, implying unique properties of the boundary, something that is incompatible with classic DAH/DITH models.

Classical models of tissue separation

Various models have been proposed to explain tissue separation. Some have been based on physical considerations (DAH and DITH), whereas others were inspired by the observation of cell behavior (contact inhibition; see Glossary Box 1) or by the discovery of important molecular components, such as cadherins (differential CAM expression) or ephrins (contact inhibition). Ultimately, they all aim to provide a mechanistic explanation for the absence of mixing across the tissue interface. In this section, I will outline each model, and discuss the evidence for and against it.

Differential CAM expression

Model

This model states that different cell populations are sorted based on the spectrum of adhesion molecules expressed at their surface, with cells expressing the same CAM(s) clustering together. The hypothesis relies on the homophilic binding property of most CAMs, particularly cadherins.

Experimental evidence

The model appeared with the discovery of the first CAMs and of their homophilic binding properties (Edelman, 1986; Nose et al.,

1988; Matsuzaki et al., 1990; Inuzuka et al., 1991; Steinberg and Takeichi, 1994), and was lent significant plausibility by the expression patterns of adhesion molecules, which appeared to be restricted to a subset of tissues (reviewed by Oda and Takeichi, 2011). However, there have been surprisingly few experimental validations of this model. I am aware of only two studies that directly addressed the role of cadherin differential expression in cell sorting. Both dealt with the organization of brain structures: Inoue et al. provided evidence for a role of R-cadherin (cadherin 4) and cadherin 6 in the sorting of two populations of neurons in the mouse telencephalon (Inoue et al., 2001), while Price et al. demonstrated that type II MN-cadherin controlled the sorting of motor neurons in the chick spinal cord (Price et al., 2002). Other CAMs, such as PAPC, have been implicated in the sorting of embryonic cell populations (Kim et al., 2000; Medina et al., 2004), but these molecules seem to act more as cell surface signaling 'sensors' than adhesion molecules mediating mechanical coupling (Table 1).

Critique

The very basis of the model – the selectivity of cadherin homophilic binding – has been questioned: classical cadherins were reported to be able to interact extensively via heterophilic interactions (Shan et al., 2000; Shimoyama et al., 2000; Niessen and Gumbiner, 2002; Duguay et al., 2003; Patel et al., 2006; Prakasam et al., 2006; Ounkomol et al., 2010), and heterophilic adhesions have recently been demonstrated in a physiological context (Straub et al., 2011). Cells expressing similar levels of two different cadherins completely failed to sort in classical aggregates, and it was suggested that the previously reported cell sorting results could have been due to differences in cadherin levels. It was also argued that re-aggregation assays used to measure cell-cell adhesion had often been performed under conditions favoring fast interaction kinetics that were irrelevant on the time-scale of establishment of contacts in real tissues (Shimoyama et al., 2000; Niessen and Gumbiner, 2002; Duguay et al., 2003). The debate remains open: some measurements of homophilic and heterophilic interactions have confirmed strong differences (Katsamba et al., 2009), whereas others have argued for promiscuity (Shi et al., 2008). As for *in vivo* experiments, the two published studies (Inoue et al., 2001; Price et al., 2002) are unfortunately not fully conclusive: in the case of MN-cadherin the results were based on the use of an incompletely characterized dominant-negative construct, whereas depletion experiments in the mouse did not produce a phenotype expected based on sorting by R-cadherin and cadherin 6. Thus, further *in vivo* analysis is essential to resolve this fundamental contention as to the specificity of cadherin interactions.

Differential adhesion

Model

The DAH states that cell populations can sort based purely on differences in the strength of cell-cell adhesion. This biophysical model is based on the tendency of tissues to minimize their surface, which is equivalent to minimization of surface tension in liquids (Fig. 2). Under these conditions, cells with highest adhesion will tend to cluster, effectively leading to sorting of the two cell populations at equilibrium.

Experimental evidence

This hypothesis was based on seminal work by Steinberg, who showed that in *in vitro* cell-sorting experiments, cell populations adopt configurations that correlate with their adhesive strengths (e.g. Steinberg, 1970). Under more controlled conditions, sorting could be obtained based purely on different levels of a single cadherin in culture cells (Duguay et al., 2003; Foty and Steinberg, 2004). There is, however, only one case in which cadherin levels were conclusively shown to be responsible for sorting in an *in vivo* context: the organization of follicle cells surrounding *Drosophila* oocytes (Godt and Tepass, 1998). There have been reports of differences in cadherin turnover/stability between ectoderm and mesoderm germ layers, which might explain separation (Ulrich et al., 2005; Kraft et al., 2012), although others have been unable to confirm these differences (Chen et al., 2009).

Critique

Major criticisms first came when Harris (1976) questioned the equivalence between actively rearranging cells and liquid molecules passively subjected to Brownian movements, noticed the very small areas of the membrane covered by cell-cell junctions (which in his view challenged the measurements of surface tension) and argued that cytoskeletal components, in particular of the cell cortex, should be incorporated in the model. His alternative theory was coined the differential surface tension hypothesis (DSTH), which was later reworked into DITH (Brodland, 2002).

Equating cadherin directly with cell adhesion is also problematic. Increasing or lowering cadherin levels does generally lead to a corresponding increase or decrease in cell-cell adhesion, but this is also likely to affect other cellular properties, in particular those of the cortical cytoskeleton. Because of their intimate interdependence, it has not been possible so far to design experimental conditions that satisfactorily dissociate adhesion and contractility.

The effect of manipulating cadherin levels and functions in normal embryonic tissues has been studied in the *Xenopus* gastrula, and the results did not support DAH: interference with cadherin adhesion did not affect normal tissue separation, while the artificial creation of adhesive differences failed to induce separation (Ninomiya et al., 2012). Sorting at the notochord boundary was found to be astonishingly resistant to the manipulation of cell adhesion (Reintsch et al., 2005; Fagotto et al., 2013).

Differential interfacial tension

Model

DITH is essentially complementary to DAH. It is based on the same physical principles, but introduces cortical contractility as the key force that must be balanced to reach equilibrium. Thus, the interfacial tension is the result of two antagonistic forces: cortical tension and adhesion (Brodland, 2002; Lecuit and Lenne, 2007). The models of Harris (1976) and Brodland (2002) were both rather general, and could explain sorting based on differences in adhesion or tension (or both). However, they have been most commonly used as a model, which I refer to as the DITH model, that is antagonistic to DAH.

Evidence

In the absence of tools to selectively modulate cortical tension, the current evidence is primarily correlative: Heisenberg and co-workers have measured cell adhesion and tension for cells of the zebrafish germ layers using atomic force microscopy or a dual aspiration pipette system (see Box 2) (Krieg et al., 2008; Maitre et al., 2012). They found that the values were more consistent with DITH than DAH: tensile forces appeared to largely dominate over cell adhesion and thus were more likely to control separation. The authors therefore argued that cortical contractility is the major motor for cell sorting in the embryo.

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Box 2. Experimental approaches to study tissue boundary properties

Measurements of tension and adhesion

- **Laser ablation (whole tissues).** Lesions of the cell cortex cause relaxation of the adjacent cell vertices, which can be used to estimate cortical tension. The method was used to demonstrate high tension along compartment boundaries (Landsberg et al., 2009).
- **Cell geometry (whole tissues).** Cell shape and angles formed between adjacent contact surfaces provide a readout for relative interfacial tension.
- **Atomic force microscopy/dual aspiration pipette (single cells and cell pairs).** Both methods can measure cortical tension by probing the resistance of the cell surface. Adhesion is measured by determining the force required to detach two adherent cells (Krieg et al., 2008; Maitre et al., 2012).

Functional dissection of tissue boundaries

- **Chromophore-assisted laser inactivation (CALI) (whole tissues).** This technique allows local inactivation of a fluorescently tagged protein by targeted irradiation with high-intensity laser light. CALI was used to demonstrate a requirement for myosin II at the parasegment boundary (Monier et al., 2010). Note that controlled inactivation at different cell contacts would be required to discriminate between local and global effects on cell contractility. Even finer tools based on photo-activation or photo-uncaging are becoming available (Wu et al., 2009; Goguen et al., 2011; Morckel et al., 2012).
- **Cell sorting in mosaic embryos (whole tissues).** When single cells are misplaced in the adjacent tissue they establish heterotypic contacts, which mimic a normal boundary and can be studied at high resolution (Fagotto et al., 2013).
- **Reconstitution of tissue separation (tissue explants).** A boundary can be reconstituted by the apposition of two tissue explants. The power of this approach is the ease of manipulating each tissue separately and the direct access to the boundary interface, which can be selectively manipulated, for instance with soluble inhibitors/activators. This approach was essential in demonstrating that ectoderm-mesoderm separation is dependent on ephrin/Eph signaling across the boundary (as opposed to global changes in tissue properties (Rohani et al., 2011).
- **Reconstitution of tissue separation (single cells).** Dissociated cells seeded on a dish promptly re-establish homotypic and heterotypic contacts, which can be analyzed by live microscopy (e.g. Carmona-Fontaine et al., 2008).

Critique

As discussed below, the model assumes that interfacial tension between two tissues can be inferred from the tensile properties of each tissue taken separately. Yet this assumption has not been validated, and interfacial tension between two different cell types has not been investigated. The model also lacks direct experimental support. Inhibition of ROK and myosin have been used to decrease contractility (Krieg et al., 2008; Maitre et al., 2012), but these experiments are difficult to interpret because the targeted molecules are involved in many processes, including ephrin/Eph-induced repulsion.

General critique of DAH and DITH

Assumption of system at equilibrium and of energy minimization

Phenomenologically, tissues and single cells adopt shapes that strikingly resemble the behavior of liquids and bubbles (Fig. 2), and this analogy has been exploited in DAH and DITH. It is however legitimate to question whether an open cellular system consuming energy would need to minimize energy, or would even reach equilibrium. One issue that has been rarely addressed is the time-scale involved (Voss-Bohme and Deutsch, 2010). Systems based on DAH and DITH are slow to reach stable configurations (hours to days; e.g. Foty and Steinberg, 2005; Krens et al., 2011), and it will be important to test whether they can account for morphogenetic processes that typically follow each other at fast pace and which might never reach true equilibrium.

Assumption of adhesive/tensile differences

The fundamental assumption of DAH and DITH is that two cell types must express global differences in either adhesion or cortical tension (or both) that would be sufficiently strong to drive sorting under physiological conditions. Recent publications advocating DITH have focused on the zebrafish germ layers, which indeed show differences in both parameters. Differences were also found between *Xenopus* ectoderm and mesoderm (Luu et al., 2011). However, at other boundaries studied in some detail, such as *Drosophila* compartments (Landsberg et al., 2009; Monier et al., 2010; Aliee et al., 2012) and *Xenopus* notochord and PSM (Reintsch et al., 2005; Fagotto et al., 2013), cell shape, cadherin and myosin/phospho-myosin levels appeared similar on either side of the boundaries.

Assumption of 'intermediate' interfacial properties

Both DAH and DITH assign to heterotypic (see Glossary Box 1) contacts adhesive or tensile values that are intermediate between those of the two tissues. Experimental data argue, on the contrary, that the properties of the boundary interface cannot be explained by the simple juxtaposition of two cell populations with different adhesive/tensile properties. Heterotypic adhesion between zebrafish germ layers was found to be equal or lower than the lowest homotypic adhesion in each tested pair (Krieg et al., 2008). As for cortical tension, in all cases thus far examined, it appears to be highest at the boundary, arguing that the conditions at the interface are not quantitatively intermediate but qualitatively different. Evidence for this includes results from laser ablation in the imaginal disc (Aliee et al., 2012), myosin accumulation at parasegmental and imaginal disc boundaries in *Drosophila* (Monier et al., 2010; Aliee et al., 2012), in the hindbrain (Calzolari et al., 2014) and at the notochord boundary in *Xenopus* (Fagotto et al., 2013). Published images also indicate boundary myosin enrichment between ectoderm and mesoderm in zebrafish (figure 4B in Maitre et al., 2012). The same conclusion can be drawn from cell shapes and angles at cell vertices and the characteristic smoothness of the boundaries, which are indicative of higher contractility and/or lower adhesion at the boundary than within each of the tissues (e.g. Monier et al., 2010; Dahmann et al., 2011).

Assumption of 'independent' tension and adhesion

Finally, another caveat in the current versions of DAH/DITH is that tension and adhesion are considered to be fixed independent parameters. As noted by Amack and Manning (2012), this assumption can hardly be held valid considering the abundant evidence for intense cross-talk between the cadherin-based adhesive complexes and the actin cytoskeleton (Fig. 4B) (Ratheesh and Yap, 2012).

Local contractility

Model

This model may be considered as an adaptation of the principles of DITH, with the fundamental difference that separation does not rely on global properties but on a local increase in contractility along the

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boundary interface. This produces high interfacial tension between the two cell populations independently of global tissue tension and adhesiveness (Dahmann et al., 2011). Contractility is predicted to be regulated at the boundary interface by interaction between heterophilic cell contact molecules (Dahmann et al., 2011; Landsberg et al., 2009).

Experimental evidence

As mentioned above, there is ample correlative evidence for qualitatively different tensile properties at the boundary. In particular, insect boundaries are typically marked by strong actomyosin structures, which seem to be connected via cadherin adhesions to form extended supracellular 'chains' (Fig. 5A′). Actin structures also prominently mark vertebrate boundaries (Fagotto et al., 2013; Calzolari et al., 2014). High interfacial tension was confirmed by laser ablation experiments on the *Drosophila* wing anteroposterior boundary (see Box 2) (Landsberg et al., 2009). Global interference with myosin II function disrupted boundary alignment in all three models of insect boundaries (Landsberg et al., 2009; Major and Irvine, 2006; Monier et al., 2010), and the targeted inactivation of myosin II at the parasegment boundary using chromophore-assisted laser inactivation (CALI; see Box 2) demonstrated that its activity was required to maintain boundary function (Monier et al., 2010).

Critique

The implicit assumption of this model is that the different cell identities on either side of the boundary are not so much defined by their adhesive or tensile properties as by the expression of specific cell surface molecules. As we will see, ephrins and Ephs seem to play this role in vertebrates. In the case of the insect compartment boundaries the source of such local signals is unknown. Those signals that are known to position the boundary and provide compartment identity [Wnt/Wingless (Wg) for the parasegments, Notch for the dorsoventral wing boundary, Hedgehog and DPP/BMP for the anteroposterior boundary (reviewed by Dahmann et al., 2011)] currently show no obvious direct connection with actomyosin contractility.

The current model presented for insect compartment boundaries states that tensile forces parallel to the interface are the motor of separation (Dahmann et al., 2011; Aliee et al., 2012). Yet, although differences in adhesion were considered as potential regulators of

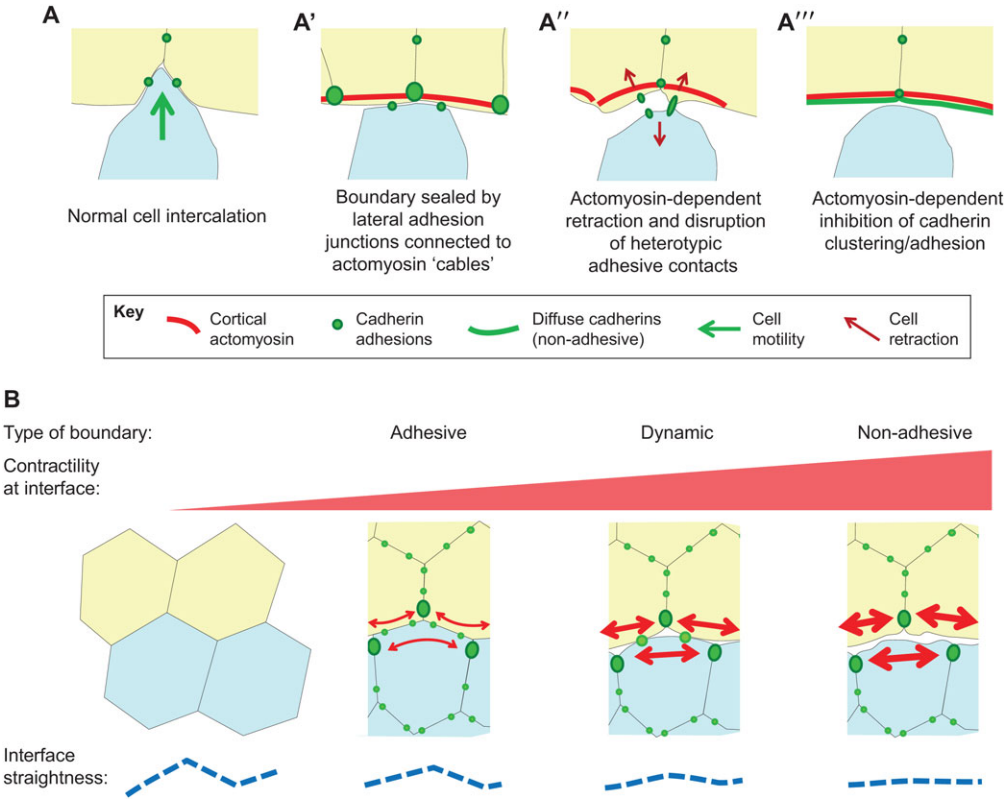

Fig. 5. Cortical contractility and mechanisms of separation. (A) Mechanisms of inhibition of cell mixing. Cell intercalation may be inhibited by different mechanisms, all of which are dependent on actomyosin contractility. (A′) Actomyosin structures connect and reinforce cadherin junctions, building supracellular 'cables' that seal the boundary. (A″) Actomyosin contractility leads to cell retraction and disruption of cell contacts. (A‴) Contractility prevents cadherin clustering and the establishment of heterotypic adhesive contacts. (B) Levels of contractility may account for the different types of boundaries. The schematic, which is based on live observation of the formation of the *Xenopus* notochord boundary (Fagotto et al., 2013), shows the progression from the initially uniform tissue to the final boundary. The successive behaviors seem to correspond to the mechanisms presented in A-A‴ and may recapitulate different boundary types. The process appears to be driven by the progressive increase in contractility of the actin cortex along the boundary (red double-headed arrows), triggered by ephrin/Eph signaling. The earliest signs of separation include cortex thickening, increased cadherin clusters at contacts abutting the future boundary and some flattening of the boundary interface. This boundary is equivalent to the 'adhesive boundary' that is seen, for example, at insect parasegments. The second intermediate phase is characterized by stronger cell contractions and by repeated formation and disappearance of cadherin clusters across the boundary in an attempt to reinforce cell adhesion in reaction to tension. The interface has significantly straightened. This boundary resembles the 'dynamic boundary' found between ectoderm and mesoderm. Finally, as tension further increases, cadherin clusters cannot be maintained and adhesion is disrupted. The final boundary is characterized by low adhesion and high tension. This represents a 'non-adhesive' boundary.

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tension (Dahmann et al., 2011), the reciprocal action of increased tension on cell-cell adhesion along and/or across the boundary has not been explicitly addressed. DE-cadherin appears homogenously distributed in the wing disc (Landsberg et al., 2009), but low along parasegment boundaries (see figures 1e and 2d in Monier et al., 2010). As discussed below, cadherin adhesion is sensitive to local tension, a parameter that should be included in the model. Note that additional factors, such as anisotropic stress and oriented cell division, should also be considered (Aliee et al., 2012).

Repulsive cues/contact inhibition

Model

In this model, separation is controlled by local repulsive reactions. Each tissue expresses a set of membrane-associated cues that trigger retraction at heterotypic interfaces, thus inhibiting migration and adhesion. No global differences in adhesion/tension between the two adjacent tissues are required, although the model is not incompatible with an additional contribution from DAH/DITH. This model may be considered a particular case of the hypothesis of local contractility, since one major effect of repulsive cues, such as ephrin/Eph signaling, is the stimulation of myosin-driven contractility.

Experimental evidence

So far the strongest evidence concerns ephrins and Eph receptors in vertebrates. These molecules are expressed in specific patterns and loss-of-function experiments have demonstrated their requirement at all vertebrate boundaries tested thus far. This is notably the case in the hindbrain for EphA4 and ephrin B2a, which show complementary expression in rhombomeres r3/r5 and also in r2/r4 (Xu et al., 1995; Xu et al., 1999; Cooke et al., 2005; Xu and Wilkinson, 2013). The same two molecules were also found to be required for somite segmentation: in this case, ephrin B2a is enriched posteriorly and EphA4 anteriorly in each segment (Durbin et al., 1998; Barrios et al., 2003; Watanabe et al., 2009; Watanabe and Takahashi, 2010). The situation is more complex at the *Xenopus* ectoderm-mesoderm separation, with each tissue expressing several ephrins and Eph receptors. Here, separation requires the function of multiple ephrin/Eph pairs, with ectodermal ephrins reacting with mesodermal Eph receptors and, conversely, mesodermal ephrins reacting with ectodermal Eph receptors (Rohani et al., 2011). We recently showed that the notochord boundary is controlled by a similar ephrin/Eph-dependent mechanism (Fagotto et al., 2013).

Ephrin/Eph signaling specifically activates Rho GTPases at the boundary (Rohani et al., 2011), leads to actomyosin accumulation (Fagotto et al., 2013; Calzolari et al., 2014), and is responsible for robust repulsive reactions at heterotypic (non-self) contacts (Rohani et al., 2011; Fagotto et al., 2013).

Thus, similar to neurons, early embryonic tissues appear to use ephrins and Eph receptors to recognize heterotypic contacts. Evidence for this function includes sorting of transplanted cells in mosaic rhombomeres (Cooke et al., 2005; Cooke and Moens, 2002) and the observation that ephrin or Eph misexpression or ectopic expression is sufficient to redirect the sorting behavior of the manipulated cell, effectively switching its identity (Cooke and Moens, 2002; Watanabe et al., 2009; Rohani et al., 2011; Fagotto et al., 2013; Calzolari et al., 2014).

Critique

Although ephrins and Ephs are clearly essential cues controlling separation in vertebrates, there is currently no evidence for a similar role of repulsive cues at insect boundaries. Even in vertebrates, repulsive reactions have thus far only been directly observed at the *Xenopus* ectoderm-mesoderm and the notochord-PSM boundaries. Applying high-resolution imaging to other boundaries is likely to show a similar behavior, but the latest model for hindbrain segmentation favors the generation of an actin cable (Calzolari et al., 2014).

Ephrins and Ephs have complex properties, including that of promoting cell adhesion under some circumstances. They positively influence the cohesion of zebrafish rhombomeres and *Xenopus* ectoderm (Cooke et al., 2005; Rohani et al., 2011). Thus, a model was proposed that combines contact inhibition and DAH (Cooke et al., 2005; Sela-Donenfeld and Wilkinson, 2005; Xu and Wilkinson, 2013).

Furthermore, a model based exclusively on ephrins and Eph receptors would fail to explain the role of several other molecules known to be involved in separation (Fig. 3B). I will later discuss how repulsive cues may be integrated in a general model whereby a strong tensile/adhesive discontinuity is created at the boundary by a combination of several parameters. Regardless of this additional complexity, ephrins and Eph receptors emerge as major determinants of separation in vertebrates, and they show all the properties expected of the long sought 'tissue affinities' postulated by Holtfreter.

Revisiting the concept of a boundary from a cell-based perspective

Altogether, there is a strong case for a general model of separation in which local high actomyosin contractility, controlled by cell contact-dependent signals, plays a major role (Landsberg et al., 2009; Aliee et al., 2012; Dahmann et al., 2011; Fagotto et al., 2013; Calzolari et al., 2014). From the point of view of the classical analogy with liquids, this localized contractility corresponds to a high interfacial tension, thus satisfying the condition for efficient separation. How might local contractility and high interfacial tension be achieved at a cellular and molecular level? Here, I discuss models in which the boundary is considered either as a coordinated supracellular structure or as the product of individual cell-autonomous reactions (Fig. 5A).

The boundary as a supracellular seal

Adhesive junctions and the actin cortex are known to form an integrated tensile system that can physically couple cells across a tissue (Cavey and Lecuit, 2009). Increased tension is expected to strengthen adhesion (Yonemura et al., 2010; le Duc et al., 2010; Ladoux et al., 2010; Ratheesh and Yap, 2012), which could here consolidate lateral homophilic junctions, thus 'sealing' the boundary (Fig. 5A′). At the macroscopic level, this type of boundary can be seen as a continuous 'cable' made of alternating actin bundles and robust cadherin junctions. This configuration is indeed observed at insect boundaries (Landsberg et al., 2009; Monier et al., 2010) and in the zebrafish hindbrain (Calzolari et al., 2014). Whether these cables actually function as seals is more difficult to demonstrate, at least *in vivo*: the tight integration of adhesive and cytoskeletal structures throughout the tissue makes it hard to isolate the specific properties of the boundary, even using local perturbations (laser ablation or CALI; see Box 2).

The boundary as a contact-inhibited interface

A different way to view the boundary is to consider it as the product of individual reactions at heterotypic contacts. High contractility would antagonize cell adhesion (Fig. 3A) and could disrupt (Fig. 5A″) or even prevent (Fig. 5A‴) heterotypic bonds, which are essential for cell intercalation (Fig. 5A). The *Xenopus*

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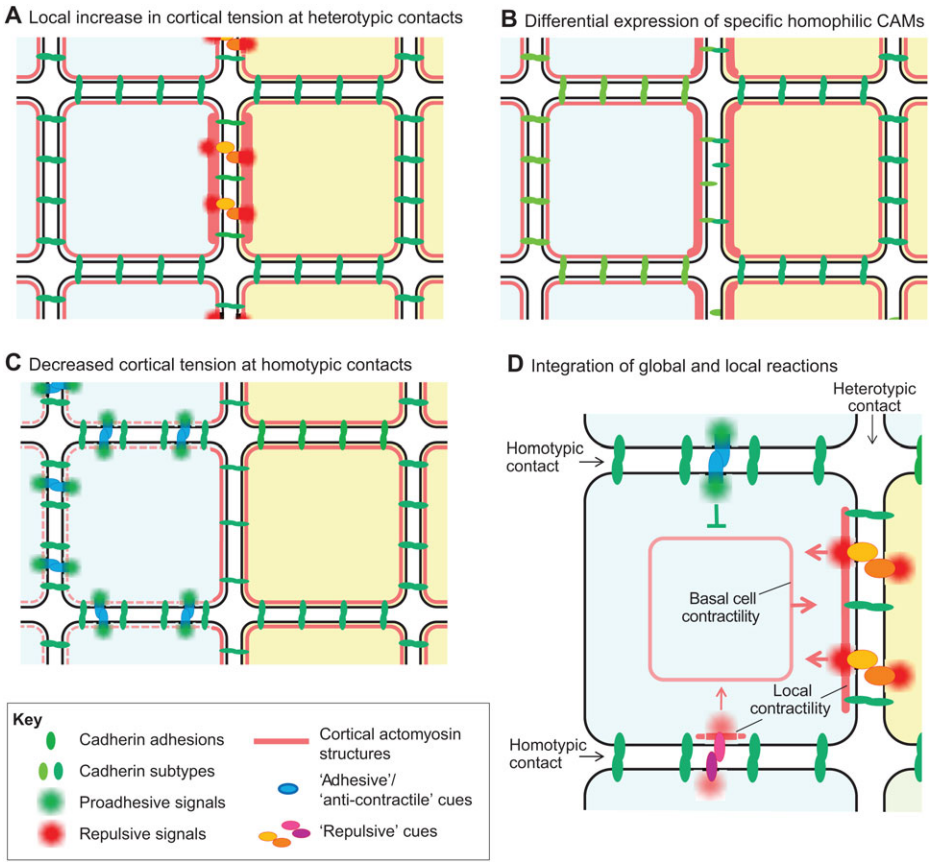

Fig. 6. Possible mechanisms for the generation of local contractility at the boundary. Contractility and adhesion are intimately interrelated, thus modulating one of them is expected to affect the other. Heterotypic contacts at the boundary are characterized by increased contractility and lower or more dynamic adhesion compared with homotypic contacts, which could be produced by different mechanisms. (A) Repulsive cues such as ephrins/Ephs boost contractility locally. (B) Assuming high specificity of homophilic binding of CAMs, tissues expressing two different CAMs would poorly adhere to each other. Lower adhesion would be expected to increase contractility along the boundary. (C) Asymmetry between homotypic and heterotypic contacts could also be achieved by increasing adhesion/decreasing contractility at homotypic contacts through 'pro-adhesive/anti-contractile' cues, such as EpCAM or Ed. For simplicity, only one cell population is modified in C, although the processes could occur independently on both sides of the boundary. Different mechanisms might cooperate to produce robust tissue separation. (D) Overall representation of the integration of intrinsic cellular parameters (basal cell contractility and cadherin levels) and pro-adhesive and repulsive signals generated at cell-cell contacts. Adhesive and repulsive cues affect the immediate, local environment (adjacent cortex and cell-cell adhesions), but also feed into the global cellular tension (represented in the center of the cell for clarity), which in turn contributes to the local outputs at different contacts. Note that cues stimulating contractility can also be found at homotypic contacts (e.g. ephrins/Ephs and PAPC) and, even though they might not act as acutely as those controlling heterotypic contacts, they are expected to contribute to the overall basal cell contractility.

ectoderm-mesoderm and notochord-PSM boundaries seem to fit with this model. In the first case, ephrin/Eph-mediated signals establish cycles of attachment-detachment: direct contact triggers repulsion, which in turn disrupts adhesions. Once cells separate, the repulsive signal decays, new protrusions are formed and contacts are re-established, initiating a new phase of repulsion (Rohani et al., 2011). Separation at the *Xenopus* notochord-PSM boundary is also based on ephrin/Eph-mediated high contractility at the boundary, but with distinct consequences for heterotypic contacts (Fagotto et al., 2013): tension appears here to be particularly exacerbated, causing intense blebbing activity, while cadherins fail to cluster across the boundary, producing a largely non-adhesive interface (Fig. 5A″) (Fagotto et al., 2013).

These observations show that the same function of preventing cell intercalation may be achieved through different mechanisms, all relying on localized contractility. Note that these mechanisms might not necessarily be mutually exclusive but, on the contrary, co-operate: cortical tension is expected to impact on both homotypic lateral adhesions and heterotypic adhesion, and might well under some conditions simultaneously strengthen the former and weaken the latter. It will be interesting to investigate cadherin adhesion at other boundaries controlled by ephrin/Eph signaling, such as in the hindbrain (Calzolari et al., 2014).

Distinguishing boundary types

Beyond the common property of a barrier to cell mixing, boundaries differ widely in the degree to which the two tissues adhere to each other, which is likely to reflect both the properties of each tissue and the specific requirements for the function of that boundary. Different types of boundary can be distinguished in terms of their physical properties, each of which may be explained based on one of the cellular mechanisms described above (Fig. 5A-A″).

Insect compartment boundaries partition epithelial monolayers, the integrity of which as a single tissue must be maintained, and indeed typical apical adherens junctions are consistently present at the boundary (Lawrence and Green, 1975; Herszterg et al., 2013). Since these epithelial cells are not very motile (Monier et al., 2010; Dahmann et al., 2011), a relatively 'mild' mechanism might be sufficient in this case to ensure separation. Although the strength

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and dynamics of adhesion have not been explicitly studied, this type of boundary may be defined as 'adhesive' (Fig. 5B) and tentatively explained based on a 'sealing' mechanism (Fig. 5A′).

At the other extreme, the notochord-PSM boundary is the prototype of a 'non-adhesive' boundary (Fig. 5B). Dorsal mesoderm cells are extremely motile, hence the need for a strong mechanism of separation. There is no mechanical requirement to keep the notochord physically connected to the PSM, as both tissues are restrained by the surrounding neuroderm and endoderm layers (Fig. 1D). Here, the lack of adhesion across the boundary offers the possibility for each tissue to adjust its length independently. The *Xenopus* ectoderm-mesoderm boundary is somewhat intermediate: there is significant adhesion between the two tissues, but this adhesion is dynamic (Fig. 5B) due to the cycles of detachment-reattachment (Rohani et al., 2011). These latter two cases rely largely on cell-autonomous reactions at heterotypic contacts (Fig. 5A″,A‴).

These various types of boundary might not necessarily require fundamentally different mechanisms (Fig. 5B). Our recent data on the nascent notochord-PSM boundary suggested that the boundary matured through a sequence that recapitulated the three scenarios presented above (Fagotto et al., 2013), appearing to involve a progressive increase in tension paralleled by characteristic changes in the organization and dynamics of cadherin adhesions. Two intermediate stages could be distinguished that resembled an adhesive and then a dynamic boundary (Fig. 5). Eventually, tension seemed to reach levels that could no longer be compensated by adhesion, producing the mature non-adhesive boundary. The hypothesis that tension, or more accurately the balance between tension and adhesion, may dictate the boundary behavior is consistent with the fact that RhoA overactivation turned the ectoderm-mesoderm boundary from a dynamic into a non-adherent interface (Rohani et al., 2011).

Common principles of boundary formation

Despite the diversity of systems and the many remaining unknowns, it is possible to extract some general principles that may apply to all boundaries – specifically, the unique properties of boundaries, the central role of actomyosin contractility, and the interplay between local signals and global properties.

First, available data strongly indicate that boundaries represent discontinuities with specific cellular properties that cannot be explained by models based on global differences in adhesion or tension and which are of crucial importance to achieve and maintain tissue separation.

The second principle is the central role that actomyosin contractility plays in boundary formation: signaling events implicated in tissue separation all converge on actomyosin (Table 1 and Fig. 3B), and there is increasing direct evidence for the importance of contractility in boundary formation (Landsberg et al., 2009; Monier et al., 2010; Rohani et al., 2011; Fagotto et al., 2013). Adhesion and contractility strongly influence each other and cannot be considered as independent opposing forces. More realistic models will need to take into account positive and negative cross-talk, including tension-induced reinforcement of adhesion (Yonemura et al., 2010; le Duc et al., 2010) – a reaction that, up to a certain level, will work against interfacial tension.

A major implication of the unique properties of boundaries is the need for highly localized signals. Contact inhibition by ephrin/Eph signaling seems to be ideal for this purpose, and indeed appears to be a major mechanism of separation in vertebrates (Fig. 6A). However, a similar local discontinuity may be created by alternative mechanisms, among which the 'old' model of differential CAM expression remains valid (Fig. 6B). Signals that would increase adhesion or decrease tension at homotypic contacts could also create a similar asymmetry (Fig. 6C). This type of mechanism could, for instance, explain the function of homotypic cell surface proteins such as Echinoid (Ed) in insects or PAPC in vertebrates (Table 1).

Reactions at the boundary cannot however be considered in isolation, but must be set within a broader integrated view of the cell (Fig. 6D). The effects of adhesion and tension propagate throughout the cell, influencing both homotypic and heterotypic contacts. Global parameters must also contribute to the balance: basal cortical contractility, cell adhesion and cell motility are set according to the

Box 3. Open questions

What are the local cues at insect compartment boundaries?

Despite intensive screens (Vegh and Basler, 2003), these cues have remained elusive. The single ephrin and Eph receptor are obvious candidates that should be tested. Classical signals, such as Wg and Notch, might also directly control cell contractility by as yet unidentified mechanisms.

The nature of insect boundaries: cell-autonomous or supracellular properties?

In vertebrates, separation by contact inhibition represents a cell-autonomous property that can be observed in mosaic embryos or reaggregation assays (e.g. Townes and Holtfreter, 1955; Cooke et al., 2005; Fagotto et al., 2013). In insects, boundaries are generally viewed as supracellular structures composed of adherens junctions connected by actomyosin cables (Landsberg et al., 2009; Monier et al., 2010) (Fig. 5A′). However, contact inhibition could provide superficially similar properties to these boundaries. High-resolution analysis of cell behavior at the boundary and of single cells in mosaic embryos, and *in vitro* reconstitution of heterotypic contacts, would help to distinguish between these mechanisms.

Integrating local and global reactions

Local signals such as ephrin/Eph signaling must act on top of more pervasive mechanisms that set basal tension and adhesion for each tissue. Signals at homotypic contacts must also be taken into account (Fig. 6C,D). The challenge is now to distinguish these different types of inputs and eventually determine their regulation and interplay. This will involve *in vivo* and *in vitro* manipulation of local signals (e.g. ephrin soluble ligands, photoactivatable reagents) and of global adhesion and contractility (e.g. cadherin overexpression/depletion, or global Rho/ROK activation/inhibition).

Is it all a matter of tension?

Most evidence is consistent with contractility being the major parameter regulated during tissue separation. Yet, other levels of regulation could be involved, affecting, for example, cadherin adhesion directly. It should now be possible to examine whether myosin activation is sufficient to account for all aspects of ephrin/Eph-dependent separation, or whether myosin-independent processes can be identified.

Can differences in tension account for the various types of boundaries?

Validation of this hypothesis requires determination of the actual tensile forces exerted at homotypic and heterotypic contacts. So far, this has only been achieved in insect wing imaginal discs (Landsberg et al., 2009). The model predicts that experimental manipulation of myosin activity and cadherin levels should be sufficient to switch between boundary types.

Other contact-dependent mechanisms

The classical model of differential expression of homophilic CAMs remains conceptually the simplest way to create a sharp tissue interface (Fig. 6B), and should be revisited. Molecules such as Ed and PAPC could become paradigms for a fundamentally different mechanism: tension and/or adhesion may be regulated indirectly by molecules that are specifically absent from heterotypic contacts (Fig. 6C).

intrinsic nature of each tissue (e.g. the need for an epithelial layer to resist stretching or for mesoderm cells to be highly motile). The mechanism of separation must be compatible with these conditions, yet robust enough to maintain a boundary despite potential variations in these conditions. Reciprocally, it is fair to assume that tissue properties can accommodate some flexibility, which might be important to achieve separation. These considerations probably account for the fact that several components seem to influence both separation and the morphogenetic properties of tissues (e.g. PAPC; see Medina et al., 2004).

Perspectives

Recent years have taught us that there is an intimate cross-talk between adhesion and tension that goes beyond the simple antagonistic contribution of the two forces in inanimate physical systems. Modulation of one or both of these parameters can produce a discontinuity at tissue/compartment interfaces. Retrospectively, separation mechanisms based on classical DAH or DITH suffer from the need for large differences in adhesion and/or contractility, which would impose strong constraints on tissues. These might be tolerated in some rare situations in which one of the tissues could be either very loose or exceptionally compact. Most morphogenetic processes, however, may not be compatible with such extreme differences. The establishment of specific regulatory mechanisms restricted to the tissue interfaces relieves these constrains. The system becomes highly versatile, as each tissue may adjust its biophysical parameters to the level appropriate for its other functions. In principle, the only strict requirement is that local reactions can still produce an acute difference at the interface. In this view of tissue morphogenesis, global tissue tension and adhesion are still obviously important, but I would argue that they should not be considered as determinants of separation, but rather as basic parameters that must be entered in the final equation.

We have reached an exciting point at which we can finally catch sight of the general principles that control tissue separation in animal development. Several key questions remain to be addressed (Box 3), but the model proposed here provides a framework within which these issues can be explored.

Competing interests

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