Boundary formation and maintenance in tissue development

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Abstract | The formation and maintenance of boundaries between neighbouring groups of embryonic cells is vital for development because groups of cells with distinct functions must often be kept physically separated. Furthermore, because cells at the boundary often take on important signalling functions by acting as organizing centres, boundary shape and integrity can also control the outcome of many downstream patterning events. Recent experimental findings and theoretical descriptions have shed new light on classic questions about boundaries. In particular, in the past couple of years the role of forces acting in epithelial tissues to maintain boundaries has emerged as a new principle in understanding how early pattern is made into permanent anatomy.

French flag model
A tissue-patterning scenario in which a gradient of secreted signal causes a concentration-dependent activation of three target genes in non-overlapping and abutting domains across a field of initially undifferentiated cells. The idea comes from Lewis Wolpert, and the name refers to the three fields of colour on the French flag.

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doi:10.1038/nrg2902

The concept of boundaries between gene expression domains is central to our current understanding of development. In the early and influential French flag model for the patterning of a field of cells, a signal gradient across the field is converted into gene expression domains by the concentration-specific response of target genes[1]. The boundaries between these domains are places where cells of different types are juxtaposed. Interactions between these abutting cell types were predicted to form secondary signalling centres that pattern the field locally[2]. These developmental boundaries are important because, if they are defective, the downstream patterning events are correspondingly perturbed.

Two classic questions about boundaries concern how the positions of boundaries are established and how sharp boundaries are maintained during growth and morphogenesis. Although the first question has received the most attention in the developmental biology community, the second question is relatively underexplored. Recent studies have highlighted the concept that physical forces must be exerted to maintain boundaries, and control of these forces is a crucial regulatory point.

Classical techniques that have been used to analyse boundary formation include cell labelling through genetic and microinjection techniques to follow the behaviour of clones of cells[3,4]. Mutant and overexpression studies have contributed to a list of the genetic components and their roles in boundary formation and maintenance. More recently, sophisticated live imaging has enabled researchers to follow changes in cell shape and position before, during and after boundary formation[5–8]. Precise temporal and spatial perturbations, including laser ablation of cell borders and inactivation of proteins, in conjunction with theoretical models of cell mixing and of the forces exerted by cells, have driven a more dynamic and fine-grained description of developmental boundaries[7,8].

The integrity of boundaries can be challenged by cell intercalation from division, or by physical disruption and dispersal during morphogenesis (FIG. 1a). In an undifferentiated tissue, two basic types of boundaries can be defined that differ in the behaviour of the cells at the boundary in response to perturbation. At non-lineage boundaries, cells can move across gene expression boundaries and adapt their fate to that of their local neighbours (FIG. 1b, left pathway). Fate determination usually requires an upstream signalling input that continuously instructs these cells, thus maintaining a sharp gene expression boundary — despite the intermingling of populations owing to cell division and larger-scale cellular rearrangements such as convergent extension. Subsequent differentiation and morphogenesis creates a physical boundary that restricts cell intermingling. At non-lineage boundaries, the restriction of cell movement between the domains is therefore a consequence of differentiation.

A different situation arises when the fate of cell populations on either side of a boundary is inherited and does not require constant input from a higher-order signalling centre (FIG. 1b, right pathway). In this case, mechanisms must already exist in the undifferentiated tissue to restrict intermingling and maintain a straight boundary between the growing populations. These lineage-based boundaries

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Paraxial mesoderm
The bilaterally symmetrical tissue extending from the tail to the head of the vertebrate embryo that forms somites and their derivatives, such as bone, muscle, tendons and skin.

(termed compartment boundaries) were first identified by lineage-tracing experiments in insects[4,9]. Single cells were genetically marked so that their progeny could be detected in the adult animal. When marked during early development, these single cells grew into large patches of cells (clones) that often ran along, but never strayed across, an invisible borderline in the adult structure. The borderline was termed a compartment boundary, and the two cell populations on either side of the compartment boundary were termed compartments. Later work has shown that the compartment boundary corresponds precisely to a gene expression domain boundary[10].

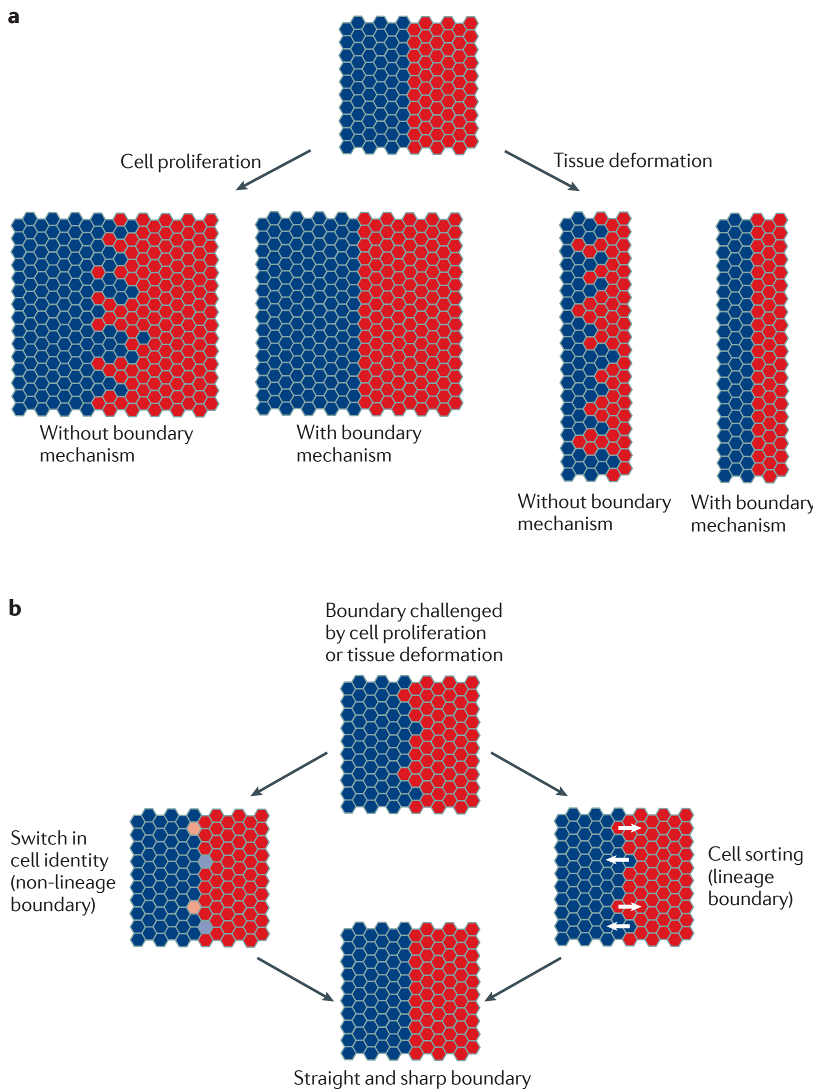

Figure 1 | **Boundary concepts: challenges and mechanisms. a** | Straight and sharp boundaries between two groups of cells (blue and red) are challenged by cell proliferation (left) or tissue deformation (right). Boundary mechanisms maintain straight and sharp interfaces. In the absence of a boundary mechanism, the initial straight and sharp boundary will become irregular and poorly defined. **b** | Two basic mechanisms maintain straight and sharp boundaries. At non-lineage boundaries, cells can move across gene expression boundaries and switch their identity to adapt to the identity of their local neighbours (left). At lineage boundaries, the identities of cells are inherited, and displaced cells are sorted back into the territory of cells with the same identity (arrows, right).

Non-lineage boundaries are common, and examples include the boundary between the wing and the notum in *Drosophila melanogaster*[11], and in vertebrate embryos the boundaries between the foregut and hindgut[12,13] and between the somites[14]. In this Review, to illustrate this boundary type we discuss somites, which are perhaps the most obvious series of boundaries in the early vertebrate embryo. Somites are transient structures — balls or blocks of paraxial mesoderm cells with an epithelial outer layer and a mesenchymal cell core — that emerge sequentially through a mesenchymal-to-epithelial transition (MET) from the morphologically unpatterned presomitic mesoderm (PSM)[15]. They give rise to the metameric anatomy of the vertebral column itself, and the associated skin, muscle and tendons. They merit attention here for two reasons: first, their formation is reiterated many times in development, thus requiring a resetting mechanism to allow each new boundary to form[16]; and second, although their lifetime is short, their boundaries must in some cases withstand large morphogenetic changes[5].

In *D. melanogaster*, compartment boundaries were identified in the embryonic ectoderm and in the wings, legs, halteres, head and abdomen[4,17–20]. Subsequently, compartment boundaries were discovered also in vertebrates, including in the hindbrain of developing chick and mouse embryos[3,21], at the mid–hindbrain boundary of zebrafish embryos[22] and mouse embryos[23], in developing chicken and mouse limb buds[24–27], in the developing chicken gut[28], in the corticostriatal boundary in the embryonic mouse telencephalon[29] and in the zona limitans intrathalamica of the mouse[30]. The formation of compartment boundaries along gene expression boundaries is therefore a mechanism that is common to both insects and vertebrates.

In this Review we discuss the compartment boundaries of the *D. melanogaster* embryonic ectoderm and larval wing imaginal discs (FIG. 2a–d), the vertebrate embryonic brain (FIG. 2e–g), and the non-lineage boundaries of the vertebrate somites (FIG. 2 h,i). We outline the similar overall logic behind the generation of all these boundaries, and contrast the different mechanisms used to withstand the distinct challenges faced by a particular boundary during development (TABLE 1). We have organized the Review to follow the key steps in the developmental lifetime of a boundary, first discussing the role of selector genes in positioning boundaries, then turning to the role of signalling systems in the maintenance of boundaries, and last covering the recent progress in understanding the regulation of physical processes that maintain the boundaries. For example, the physical mechanisms that counteract cell intermingling and thus maintain straight compartment boundaries have long been thought to depend on differential cell adhesion. New findings, however, indicate that differential mechanical tension might also have an important role.

Selector genes and positioning of boundaries

From the study of *D. melanogaster* compartment boundaries comes the concept that genes that define a territory and its identity are also important for the

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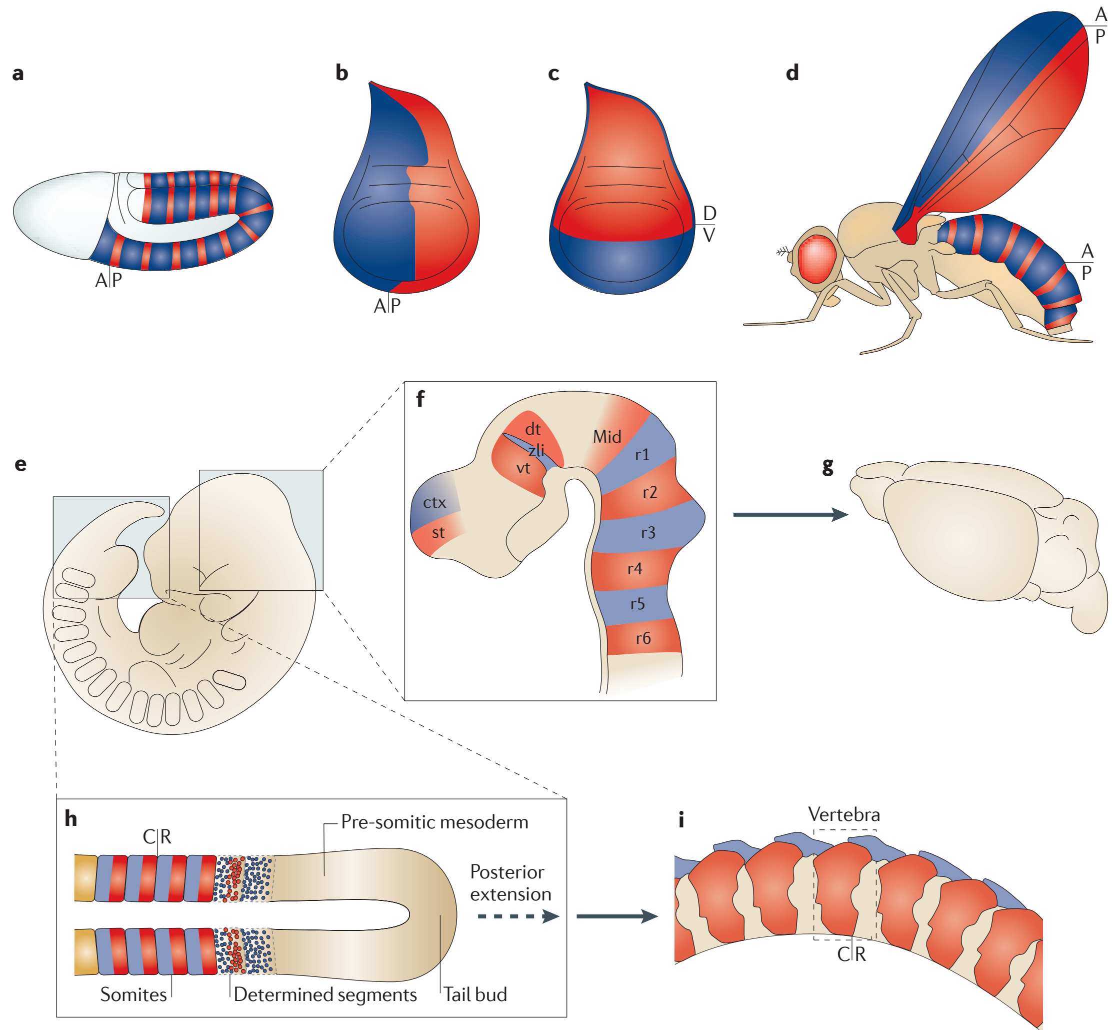

Figure 2 | **Developmental boundaries in the developing fruitfly and vertebrate embryo. a–d** | To illustrate compartment boundaries, cartoons are shown of a *Drosophila melanogaster* embryo during developmental stages 8–11 (**a**), larval wing imaginal discs (**b**,**c**), and an adult fly (**d**). Anterior compartments (**a**,**b**,**d**) or ventral compartments (**c**) are coloured blue; posterior (**a**,**b**,**d**) or dorsal (**c**) compartments are coloured red. A|P, anteroposterior boundary; D|V, dorsoventral boundary. **e** | Cartoon of a mouse embryo at 12.5 days post coitum (dpc) showing the developing brain and somites. **f** | Lineage-restricted boundaries of the neuroepithelium. Red and blue colours are used to illustrate the compartments and their boundaries discussed in the text. ctx, cortex; dt, dorsal thalamus; mid, midbrain; r1–r6, hindbrain segment rhombomere 1–6; st, striatum; vt, ventral thalamus; zli, zona limitans intrathalamica. **g** | The adult mouse brain. The anterior (left) two lobes are derived from the forebrain — for example, the cortex — and the next lobe is the cerebellum, which is derived from the mid-hindbrain junction. The protruding structure at the base (right) is the hindbrain, which connects to the spinal cord (not shown). At the scale shown, the segmented organization of the hindbrain that is derived from the embryonic rhombomeres is not evident. **h** | The posterior mesoderm of the extending vertebrate embryo during segmentation stages, highlighting the rostral (R)–caudal (C) polarity of the developing somites in blue and red, respectively. Solid colours are the completed, polarized somites, and the dotted patterns represent cells with emerging polarized identities. The location of the morphological boundary between the caudal domain of a given somite and the rostral domain of the somite immediately posterior to it is marked with C|R. The resulting segmented architecture of the axial skeleton of the mouse is shown with red marking the ossified bone of the vertebral body and pedicle, most of which is derived from the caudal somite, and blue marking the spinous process. Note that the segmental unit of the vertebral column is shifted by half a segment with respect to the initial segmental unit of the somites, a process termed resegmentation that allows the muscles from one somite to pull on the two neighbouring vertebrae. The position of the prior somite boundary is marked with C|R in the middle of a vertebra.

formation of the boundaries that delimit that territory. These so-called ‘selector’ genes have the following properties: their expression domain defines a unique territory, their loss eliminates identity in the territory, and their ectopic expression induces this identity in the ectopic territory. As we describe here, this concept is not strictly true for the lineage boundaries of the hindbrain, but recent findings suggest that, surprisingly, it seems to hold true for the non-lineage boundaries of the somites.

Mesenchymal-to-epithelial transition
The process whereby a mesenchymal population of cells rearrange their local positions and cell polarity to build an epithelium.

Telencephalon
The most anterior segment of the vertebrate brain. It gives rise to the forebrain and, in mammals, the neocortex.

Zona limitans intrathalamica
A zone that divides the dorsal and ventral thalamus of the forebrain.

Wing imaginal disc
An epithelial tissue that gives rise to the wings and parts of the body wall of adult flies. It is subdivided by the anteroposterior and dorsoventral compartment boundaries.

Tension
A force relating to the stretching of an object; the opposite of compression.

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Table 1 | **Boundary scenarios discussed in this Review**

Boundary	Type of tissue	Selector genes	Local signalling network	Challenge to boundary	Mechanical maintenance
Embryonic parasegment	Columnar epithelium	*engrailed*	Wingless	Intercalation from division	Differential mechanical tension
Wing disc A–P	Columnar epithelium	*engrailed*	Hedgehog, DPP	Intercalation from division	Differential mechanical tension
Wing disc D–V	Columnar epithelium	*apterous*	Notch	Intercalation from division	Differential mechanical tension
Rhombomere	Neuroepithelium	Combinatorial code?	Eph–ephrin	Intercalation from division, morphogenesis during neural tube/keel formation	Cell adhesion — repulsion
Somites	Mesenchyme then epithelium	Mesp genes	Eph–ephrin	Intercalation from convergent extension	Extracellular matrix

A–P, anteroposterior; D–V, dorsoventral; DPP, Decapentaplegic (a member of the bone morphogenetic protein (BMP) protein family); Eph–ephrin, Ephrin receptor–ephrin; Mesp, mesoderm posterior.

Selector genes at compartment boundaries. Compartment boundaries are established by the activity of selector genes[31], which control the identity and fate of cells within compartments. In *D. melanogaster*, a gene that encodes a homeodomain-containing transcription factor, *engrailed* (and its sister gene, *invected*[32]), acts as a selector gene for all posterior compartments[33] (FIG. 2a,b,d). *engrailed* is specifically expressed in all cells of the posterior compartments, where it specifies a posterior sorting property that prevents these cells from intermingling with anterior cells across the anteroposterior compartment boundary (A–P boundary). Similarly, the LIM domain-containing transcription factor Apterous is expressed in all cells of the dorsal compartment (FIG. 2c), where it acts as a selector gene to specify dorsal cell identity and to prevent mixing with ventral cells across the dorsoventral compartment boundary (D–V boundary)[34].

Compartment boundaries using a combinatorial selector code. The concept that compartments are units of gene expression holds true also for vertebrate neuromeres (FIG. 2e,f). Briefly, neuromeres were initially discovered on the basis of morphology[35–38], and in 1990 they were found to be lineage-restricted compartments, similar to their *D. melanogaster* counterparts[3]. They arise in the forming neural plate and neural tube. Neuromeres correspond to segmental, reiterated units of gene expression of members of the Hox gene family, the Ephrin receptor–ephrin (Eph–ephrin) gene family and other gene families. This is particularly obvious in the hindbrain primordium, where the expression of several gene families mark out alternating odd- and even-numbered rhombomeres. These segmental expression patterns are crucial for the formation and maintenance of compartment boundaries; for example, Eph–ephrin signalling at the boundary between adjacent expression domains restricts cell intermixing[39,40]. Boundaries between neuromeres are often associated with signalling centres, similar to the boundary between the compartments in the fly wing imaginal disc[41].

Despite these similarities, the transcription factors that are expressed earliest in segmental territories in the hindbrain do not meet the selector gene criteria: in no case has a gene been described for which expression is limited to a single neuromere, loss of function of the gene deletes this rhombomere's identity and ectopic expression of the gene drives this identity in an alternative rhombomere or brain region. Rather, in the hindbrain a restricted set of selector-like genes confers identity in a combinatorial manner. For example, the zinc finger transcription factor known as early growth response protein 2 (EGR2; also known as KROX-20), which is expressed in both rhombomeres 3 and 5, has a role similar to that of a selector gene, whereby its effects on hindbrain identity are altered in rhombomere 5 by the presence of the transcription factors HNF homeobox B, MAFB (also known as Kreisler) and HOX3 paralogues (reviewed in REF. 42).

Evidence for selector genes at a non-lineage boundary. The concept of selector gene function is accepted for the regional axial identity of the somites, in which Hox gene expression boundaries define regional territories, and gains and losses of Hox genes cause homeotic transformations between axial identities (reviewed in REF. 42). In this case, Hox selector genes are not involved in boundary formation *per se*. Recent work has revealed a case of apparent selector gene function in somite boundary formation, in which the identity of territories is repeatedly specified within each segment as it forms. Each somite is internally polarized in the rostrocaudal (R–C) axis, providing a segmental scaffold for migrating neurons and neural crest cells from the central nervous system (CNS) into the periphery. Understanding how segment polarity and morphological segmentation is coordinated is an ongoing challenge, but there has been substantial progress in recent years.

In the anterior PSM, classical grafting experiments[43] and segmentally striped patterns of gene expression showed that a segmental pre-pattern is laid down before the morphological boundaries of somites can be observed (FIGS 2h,3a). The central players in the pre-patterning process seem to be the mesoderm posterior (Mesp) basic helix–loop–helix (bHLH) transcription factors (MESP2 in mice and chicks (in which it is also known as cMeso-1) and Mespb in zebrafish). First, the *Mesp2* expression domain defines the size of the segment, then, as it is spatially refined into the rostral half of the presumptive somite, it defines the rostrocaudal polarity within each segment[44]. Loss of *Mesp2* in the mouse embryo causes a loss of somites and caudalization

Hox gene family
A family of homeobox DNA-binding domain-containing transcription factors that were initially identified by their function in homeotic transformations.

Rhombomere
The basic unit of segmental organization in the hindbrain. The rhombomere has lineage or compartment boundaries.

Basic helix–loop–helix
A family of transcription factors that are characterized by their basic helix–loop–helix DNA binding and dimerization domain structure.

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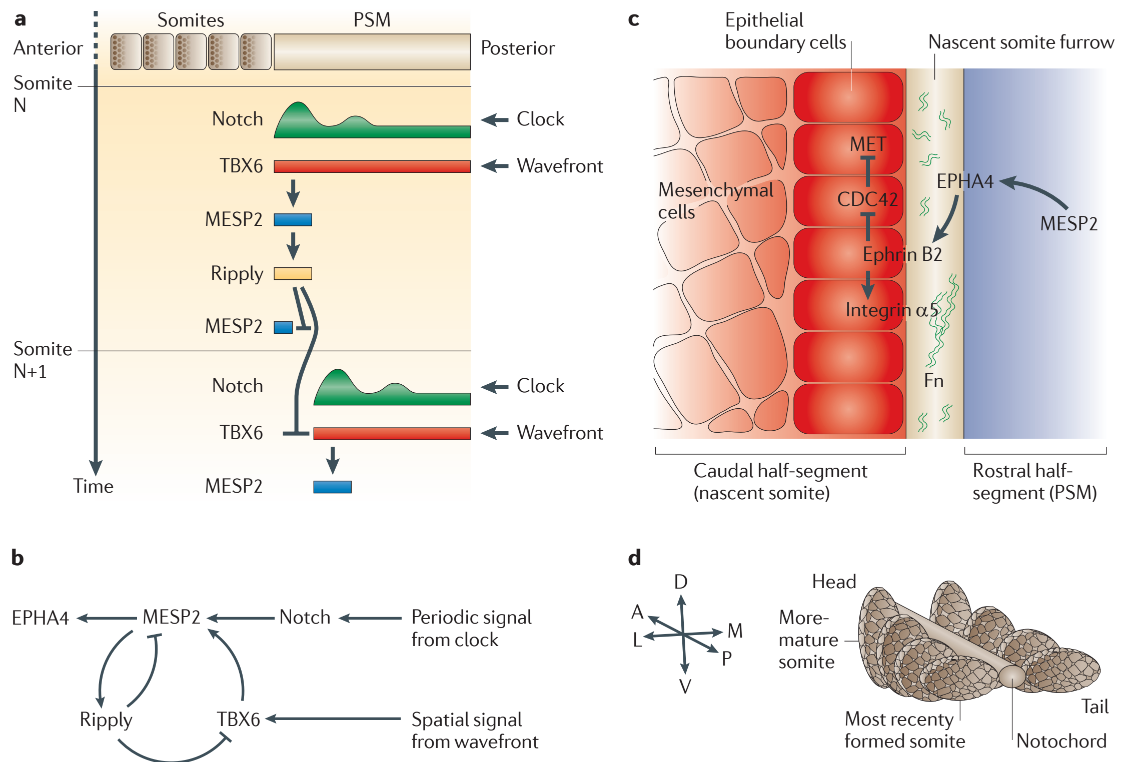

Figure 3 | **Formation and maintenance of the somite boundary. a** | Positioning of the future somite boundary in the presomitic mesoderm (PSM) by the mesoderm posterior 2 (*Mesp2*) gene network during segmentation stages. Shown here for the mouse embryo, this network is thought to integrate an oscillating signal from a segmentation clock ('clock') in the posterior tissue via Notch and a spatial signal from a wavefront of cell maturation via the T-box transcription factor TBX6. The output is a sharp stable boundary of *Mesp2* expression (coloured bars show the region of expression), which is restricted to the rostral half of the segment, and a posterior-ward shift of the *Tbx6*-expressing domain, allowing a repeat of the network's function for the next segment (somite *N* + 1). The restriction of *Mesp2* expression to the rostral half-somite is brought about through repression by Ripply (indicated by a T-bar). **b** | A network diagram of the genetic interactions that occur in the anterior PSM. **c** | Epithelialization of the somite through ephrin–ephrin signalling and Rho GTPase activity, shown for the mouse or chicken embryo during segmentation stages. MESP2 drives EPHA4 expression in the rostral territory of the pre-patterned PSM. EPHA4 binding to ephrin B2 across the boundary activates ephrin B2 in the border cells of the anterior somite, causing the local inhibition of the Rho GTPase CDC42 at the boundary. This leads to epithelial cell shape, and the maintenance of the morphological somite furrow. Below, the events associated with integrin clustering and fibronectin deposition, as they occur in the zebrafish somite, are indicated. The location of fibronectin extracellular matrix is shown in green. **d** | The deformation of early zebrafish somites by convergent extension. Immediately after formation, zebrafish somites of the trunk are extended along the mediolateral axis (M–L), but the rapid cell and tissue movements of convergence extension deform the somites into dorsoventrally extended structures without changing the anterior–posterior dimensions.

of the paraxial mesoderm and axial skeleton[45]. *Mesp2* overexpression, on the other hand, drives a rostralization of the paraxial mesoderm[46]. Thus, in the terminology of compartment boundaries, *Mesp2* behaves as a selector gene that controls the rostral domain. Although traditionally the term selector gene has been used only in association with lineage boundaries, the functional parallel between *Mesp2* in the somite boundary and selectors such as *engrailed* in the posterior compartment of the *D. melanogaster* wing imaginal disc points to deeper similarities in the processes, and it may justify the use of the term 'selector gene' in this case.

Segmentation regulatory network. The formation of the segmental pre-pattern is best understood in the mouse embryo, and various studies are building up a picture of a regulatory network that controls boundary specification (FIG. 3a,b). Segmentation in vertebrates is regulated by a clock and wavefront mechanism[15,47,48]. The T-box transcription factor TBX6 is expressed in the entire PSM downstream of the wavefront; its anterior limit is positioned by the previously defined segment. During each cycle of somite formation, expression of *Mesp2* is induced in cells that express *Tbx6* and simultaneously experience a temporal pulse of Notch signalling downstream of the clock in the anterior PSM. MESP2 is then required for repression of *Tbx6* expression and degradation of the TBX6 protein[16,49–53]. The mechanism of repression of *Tbx6* involves the Ripply family of bHLH repressors, which are activated by MESP2 (REFS 54–58). Shutting off *Tbx6* expression in this manner throughout the newly generated *Mesp2* expression domain completes

Clock and wavefront
A mechanism for segmentally patterning the vertebrate paraxial mesoderm, involving a cellular oscillator, the clock, in the cells of the presomitic mesoderm, and a wavefront of differentiation that arrests the clock as it moves across the presomitic mesoderm.

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a feedback loop that effectively shifts the anterior end of the *Tbx6* expression domain by one segment length to the posterior, ready to start the next cycle of somite formation[16]. Thus, the *Mesp2* regulatory circuit seems to couple inputs from the clock and the wavefront to produce stable segment boundaries.

Feedback loops can be slippery concepts, but recent description of the repetitive switching processes discussed above using mathematical models of the gene regulatory network (GRN) has enabled the proposed interactions to be explicitly stated[58,59]. Although the parameters and some of the connections in these models are still poorly constrained by data, the models mark an important step because they foster unambiguous experimental testing, which will enable further model refinement[60]. As in other areas of developmental biology, in which the temporal interactions of multiple elements must be considered, the dynamics of boundary GRNs will be important for understanding boundary formation.

As in the mouse, gene networks involving proteins in the Notch, T-box, Mesp and Ripply families seem to be involved in establishing the segment boundary in zebrafish and *Xenopus* spp.[16,49–53,55,56,61–63]. In each species the key output signal is the activation of a boundary cascade downstream of Notch and headed by a Mesp protein, but the details of how the genes interact are different. Further work is needed to determine whether these differences derive from different switching mechanisms or whether gene duplications and divergence have simply produced variations on a theme.

Segment polarity. What is the role of R–C patterning in generating the morphological somite boundary? By removing the epidermis from PSM explants[64] or by inactivating transcription factor 15 (*Tcf15*; also known as Paraxis) gene in mice, which encodes a bHLH protein[65], the emergence of the epithelial somite boundary can be blocked while leaving the molecular R–C polarized prepattern intact. These and other studies have led to the idea that R–C polarity lies upstream of somite boundary formation. However, this idea was challenged by the surprising observation that, in a mouse carrying a hypomorphic *Mesp2* allele, most segment polarity markers are absent, but at least two epithelial somites can form transiently[66]. This suggests that morphological segmentation may proceed independently from segment polarity. Thus, segment polarity may be a parallel process that is important only after the somite is completed, in order to maintain the epithelium and to allow migrating cells to find their way. Additional mutations or other experimental conditions that dissociate these events will be necessary to allow us to fully understand the contribution of segment polarity to somite boundary formation.

Cell signalling and maintenance of boundaries

After a boundary is formed, it needs to be maintained to enable tissue development. In addition to the activity of selector genes, the two main influences on maintenance are signalling among cells and their physical interactions. In this section we discuss examples that show the importance of cell signalling.

Part of the identity specified by selector genes is which signals cells send and respond to, and it has been found that local signalling between cells from adjacent compartments is important for maintaining sharp and straight compartment boundaries. In the developing *D. melanogaster* wing, anterior cells need to receive signals from Hedgehog and Decapentaplegic (a member of the bone morphogenetic protein (BMP) protein family) to maintain the A–P boundary[67–70]. Bidirectional signalling of Notch between dorsal and ventral cells is required for maintaining the D–V boundary[71,72].

Eph–ephrin signalling at compartment boundaries. In the vertebrate embryonic hindbrain, bidirectional Eph–ephrin signalling is important for maintaining the boundaries between adjacent odd- and even-numbered rhombomeres[39,40]. Embryo explant experiments suggested that complementary populations of cells expressing an Eph and its cognate ephrin are sufficient to drive segregation into two non-mixing populations with relatively smooth boundaries[39]. In the zebrafish hindbrain, examination of the sorting behaviour of cells with reduced ephrin receptor A4 (EphA4) and ephrin B2a (EfnB2a) function has provided elegant *in vivo* evidence that these proteins mediate both a repulsive function between hindbrain rhombomeres and an independent cohesive function within a segment[6,73]. Interestingly, the neural progenitors that express EphA4 receptor and EfnB2a seemed to be most sensitive to reduction of these proteins' functions during and immediately after cell division. Using time-lapse analysis of cell sorting in mosaic zebrafish embryos, Kemp *et al.* found that EphA4 and EfnB2a are specifically and individually required to facilitate normal integration of newborn progenitor cells back into the neuroepithelium[6]. This finding indicates that the demands on maintaining mechanical integrity within hindbrain segments is highest during and immediately after cell division, similar to the wing compartment boundary in *D. melanogaster* (see below).

Eph–ephrin signalling at non-lineage boundaries. After the patterning events that define the position of the future segmental boundary are completed, as described above, a new somite must be separated from the anterior end of the PSM tissue. The somite's morphological boundary is generated by a rapid MET that maintains the previous Mesp gene expression boundary. In various species, the expression patterns of the Eph–ephrin genes suggested that maintaining somite boundaries might involve cell–cell repulsion through localized expression of Eph and ephrin in complementary segmental domains[74–77] (FIG. 3c). Uniform overexpression of Eph and ephrins in early embryos disrupts somitogenesis[74,78]. In zebrafish that are mutant for the gene *fused somites* (also known as *tbx24*), which encodes a T-box transcription factor, overexpression of activated EphA4 in transplanted cells drives somite formation cell-autonomously; otherwise, these mutants lack both EphA4 expression and morphological somites[79].

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Grafting experiments in chick embryos have been instrumental in revealing the key steps that connect the patterning of the segmental boundary to the emergence of epithelial somite boundaries and their mesenchymal cores[80]. These boundary-forming assays showed that the cells of the PSM just posterior to a presumptive boundary instruct cells that lie anteriorly to become separated and epithelialized. Overexpression in the transplanted cells of Lunatic fringe, a modulator of Notch signalling, or of a constitutively activated Notch intracellular domain, showed that the signal depends on Notch[80].

As described above, in the mouse TBX6 and high Notch activity induce *Mesp2* expression[52]. MESP2 drives ephrin type-A receptor 4 (EPHA4) expression in the cells at the somite's presumptive anterior boundary[46,66]. In the chick, the TBX18 transcription factor seems induce boundaries, similar to Notch[81], suggesting that it may be involved along with MESP2. Downstream of Eph–ephrin signalling, regulation of Rho family GTPase signalling allows the cells to assume and stabilize the mesenchymal versus epithelial state[82]. A role for specific small GTPases in MET was revealed by electroporation of chick embryos with activated or dominant-negative versions of the Rho family members CDC42 or RAC1, generating a mosaic tissue. By measuring the expressing cell's location in the somite, it was shown that CDC42 activity must be suppressed in the posterior cells to allow MET[82]. By contrast, either elevated or reduced RAC1 activity disrupted epithelialization, and reduced RAC1 activity blocks the ability of the TCF15 bHLH transcription factor to drive cells into the epithelial fate[82]. Clearly, a precise regulation of GTPase activity is essential for epithelialization at the somite boundary.

Recent technical advances using an inducible expression system have added precise temporal control of gene activity to the chick PSM grafting assays[83]. This has revealed a role for ephrin B2, the EphA4 ligand, in coordinating morphological boundary formation and cell epithelialization[84]. This study shows that signalling through ephrin B2 phosphorylation is responsible for the suppression of CDC42 activity, which enables cells in the posterior border of the forming somite to epithelialize.

Thus, the role of Eph–ephrin signalling is a shared theme of signalling to maintain compartment boundaries in the hindbrain and to maintain the non-lineage boundaries of the somites. Whether Eph–ephrin signalling is important to maintain compartment boundaries in *D. melanogaster* is not known; this may prove a fruitful avenue of investigation.

Physical mechanisms of boundary maintenance

After the positioning and formation of boundaries by selector genes and cell signalling, the long-term maintenance of straight and sharp boundaries is crucial for subsequent patterning events. The shape of boundaries is challenged by cell rearrangements that take place during cell proliferation and morphogenetic tissue movements. The maintenance of straight boundaries depends crucially on the regulation of cell proliferation and the physical interactions among cells. In this section, we focus on four maintenance mechanisms: regulation of cell proliferation, deposition of extracellular matrix, regulation of cell adhesion, and regulation of mechanical tension. We discuss how these mechanisms match the particular challenges to boundary integrity that are thrown up by the developmental context in which the boundaries are located.

Reduced rate of cell proliferation. Cell proliferation is a hallmark of most developing tissues. Cell divisions, however, can lead to cell rearrangements[85,86] that can challenge straight and sharp compartment boundaries. The wing imaginal discs of *D. melanogaster* undergo rapid cell proliferation during larval stages. Interestingly, during late larval development, the rate of proliferation of cells in the vicinity of the D–V boundary is strongly decreased in this tissue[87]. It has been proposed that this zone of non-proliferating cells is important for the maintenance of the D–V boundary[8,87,88] (however, see also REF. 89). Cell proliferation is also reduced at rhombomere boundaries in the chick embryo hindbrain[90], so a reduced rate of cell proliferation might be a common signature of compartment boundaries. It will be interesting to test what precise role, if any, a reduced rate of cell proliferation has in maintaining compartment boundaries.

Deposition of extracellular matrix. It is well established that extracellular matrix (ECM) accumulates between rhombomeres[91,92]. Although a function for this material in the formation or maintenance of the rhombomere boundary is not clear, recent advances indicate that ECM has an important role in the maintenance of the somite boundary. During somitogenesis, the vertebrate embryo converges and extends — a process that is driven largely by cellular rearrangement. This can cause the somite to change shape, becoming shortened by many fold in its mediolateral axis and elongated in its dorsoventral axis; at the same time, its length along the anteroposterior axis is barely altered (FIG. 3d). The tissue and cellular rearrangement involved in the convergence of somites is particularly striking in the anterior trunk of the zebrafish embryo, in which convergence is rapid. Time-lapse analyses of the cells in the somite during convergence show that, despite the rearrangement, convergence occurs without the somite losing its boundaries or exchanging cells with its neighbours[93–96]. This challenge to the somite boundary by convergence-driven deformation is an interesting contrast with the situation in the wing imaginal discs of *D. melanogaster*, where the main challenge to boundary integrity is thought to come from cell division.

A tissue-level mechanism underlying boundary integrity in converging somites that involves the ECM has been revealed through zebrafish mutations that cause the somite boundaries to form correctly but not be maintained[97,98]. In such mutants, the somitic furrows are lost within hours, and cells are exchanged across the previously existing somite boundaries. Analysis of these mutants showed that the integrin–fibronectin signalling and adhesion system is required for the localization of fibronectin-based ECM to the forming boundary[97,98]. This intersomitic furrow ECM is likely to be a key

Integrin
A cell surface transmembrane protein that binds fibronectin; integrins are usually associated with focal adhesion complexes.

Fibronectin
An extracellular matrix glycoprotein that is capable of forming fibrils, a ligand for integrins.

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mechanical constraint in holding the boundary together under the deformation of embryonic convergence. Although the cellular movements of somitogenesis vary between different vertebrate species, a conserved role for integrin–fibronectin interactions in regulating these movements is supported by studies on *Xenopus laevis*[99], chicks[100,101] and mice[102].

Recent studies using transgenic reporters of integrin localization in zebrafish have provided insight into the molecular steps that are involved in regulating the deposition of the ECM around the newly forming somite[5]. Live imaging of GFP-labelled integrin α5 showed that it clustered at the newly formed somite boundary before matrix assembly, independently of fibronectin. This clustering is initiated by Eph–ephrin signalling across the nascent somite boundary, and ephrin reverse signalling seems to be sufficient, evidence that links integrin activity to the previous stage in somitogenesis (FIG. 3c). In the absence of activation by ephrin reverse signalling in a cell, integrin α5 in the same cell prevents integrin α5 clustering on adjacent cells. Therefore, this is a form of reciprocal integrin inhibition that suppresses ECM assembly by non-boundary cells. Together, these results lead to a model for somite integrity in the zebrafish in which interplay between Eph–ephrin signalling, ligand-independent integrin clustering and reciprocal integrin inhibition restrict the formation of ECM production to somite boundaries. It will be important to investigate whether this scenario is a general mechanism for integrin–fibronectin interaction at the somite boundaries of other species.

Differential cell adhesion. In addition to interactions of cells with their ECM, physical interactions between neighbouring cells are important to maintain boundaries. There is a long-standing hypothesis to explain the separation of cells along compartment boundaries that is based on differences in the adhesion (or affinity) between cells from neighbouring compartments[31]. The concept of differential cell adhesion is derived from earlier theoretical work by Malcom Steinberg[103], who compared the rearrangements and sorting of cells to the behaviour of immiscible liquids. Work on cadherins in tissue culture and genetic studies in *D. melanogaster* ovaries, for example, provided evidence that differential cell adhesion guides cell sorting[104–106]. Circumstantial evidence also suggests that cadherins have an important role in maintaining compartment boundaries. For example, during mouse embryonic development, cadherin-4 (also known as R-cadherin) and cadherin-6 are differentially expressed on either side of the corticostriatal compartment boundary[29], and misexpression of either protein results in the mixing of cells across this boundary[29]. Moreover, in *D. melanogaster* wing imaginal discs, Capricious and Tartan — two leucine-rich repeat transmembrane proteins that confer cell adhesion *in vitro*[107] — are transiently expressed in the dorsal but not the ventral compartment[108]. Forced expression of these proteins in *apterous* mutants, in which this boundary is irregular, restores a normal straight D–V boundary[108]. However, clones of cells that are double mutant for these two genes do not affect the straightness of the D–V boundary[108,109]. At vertebrate rhombomere boundaries, Eph–ephrin signalling is important for repulsion of cells from adjacent compartments[39,40]. In addition, more recent results from zebrafish indicate that EphA4 has a role in cell–cell adhesion in the rhombomere, thus indirectly contributing to the formation of the rhombomere boundary[73]. In future, it will be an important challenge to measure cell-adhesion strengths at boundaries in the developing tissue.

Differential mechanical tension. More recent hypotheses to explain cell sorting during animal development are based on differential surface contraction[110] or differential interfacial tension[111]. Unlike Steinberg's hypothesis, these hypotheses also take into account the fact that cells can generate mechanical tension that allows them to contract their surfaces that are in contact with neighbouring cells, and thereby become sorted from them.

Mechanical tension can be generated by contractile elements at the cell cortex, including actomyosin filaments (FIG. 4a) (reviewed in REF. 112). A local enrichment in the levels of Filamentous (F)-actin and non-muscle Myosin II (referred to here as Myosin II) has been reported at several compartment boundaries in *D. melanogaster*, including the parasegment boundaries of the embryonic epidermis[8] (FIG. 4b) and the A–P and D–V boundaries of the larval wing imaginal disc[7,88,113] (FIG. 4c,d). The local increase of F-actin and Myosin II at the parasegment boundaries and the D–V boundary is controlled by Wingless and Notch signalling, respectively[8,88,113]. As Wingless signalling is required to maintain parasegment boundaries[8] and Notch signalling is required to maintain the D–V boundary[71,72], it seems that the enrichment of F-actin and Myosin II at compartment boundaries is an important regulatory step in the maintenance of compartment boundaries in *D. melanogaster*. Future work will be required to reveal the molecular mechanism by which these signalling pathways control the enrichment of F-actin and Myosin II.

Further experiments showed that perturbation of myosin activity, either by mutations in *zipper*, the gene encoding Myosin II heavy chain, or by expressing a dominant-negative form of *zipper*, resulted in irregular compartment boundaries[7,8,113]. In an elegant experiment, Monier *et al.* locally reduced Myosin II activity using chromophore-assisted laser inactivation (CALI)[8]. Cell divisions along the parasegment boundary normally do not disturb the boundary, but when Myosin II activity was reduced along the parasegment boundary by CALI, cell division resulted in an irregular boundary. Taken together, these experiments reveal an important role for Myosin II in maintaining sharp and straight compartment boundaries in the *D. melanogaster* epithelia.

A further signature of differences in mechanical tension along cell borders, in addition to increased levels of F-actin and Myosin II, is a distinct shape of cells and angles between adjacent cell borders[86,114]. Quantitative image analysis of the network of adherens junctions in *D. melanogaster* wing imaginal discs showed that the two rows of cells on either side of the A–P boundary have

Ephrin reverse signalling
The activity of ephrin cell surface proteins, initially thought to be ligands only, to transduce a signal from the Eph-type receptor tyrosine kinase.

Cadherin
A cell surface transmembrane calcium-dependent cell-adhesion protein that is capable of homophilic binding. Cadherins are usually associated with adherens junctions in epithelial tissue.

Chromophore-assisted laser inactivation
The use of high-intensity laser light delivered to subcellular locations with fluorescently tagged proteins of interest to inactivate them through the local release of free radicals from the stimulated chromophore.

Adherens junctions
Multiprotein membrane complexes that mediate adhesion between epithelial cells. Adherens junctions contain cadherins, α- and β-catenins, and p120, the cytoplasmic faces of which connect to the actin cytoskeleton.

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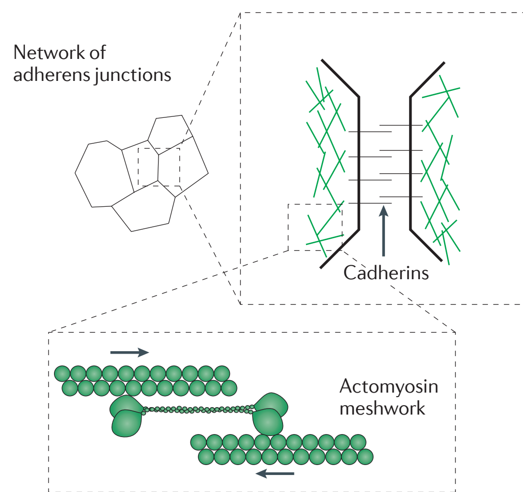

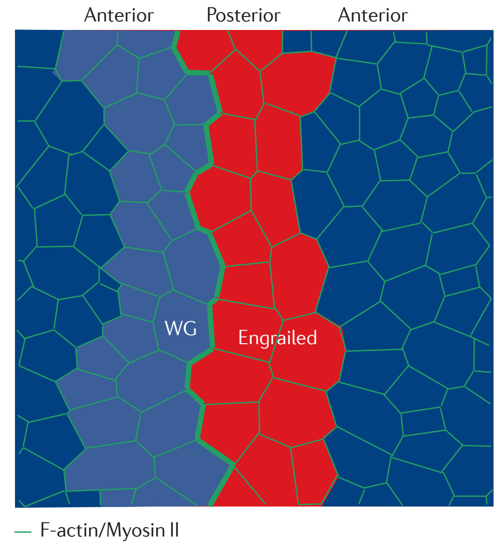

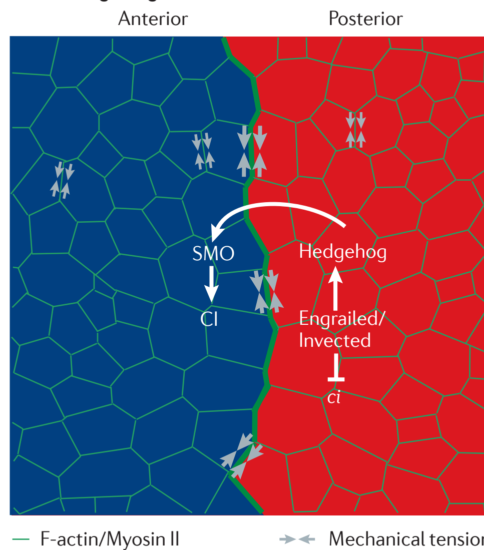

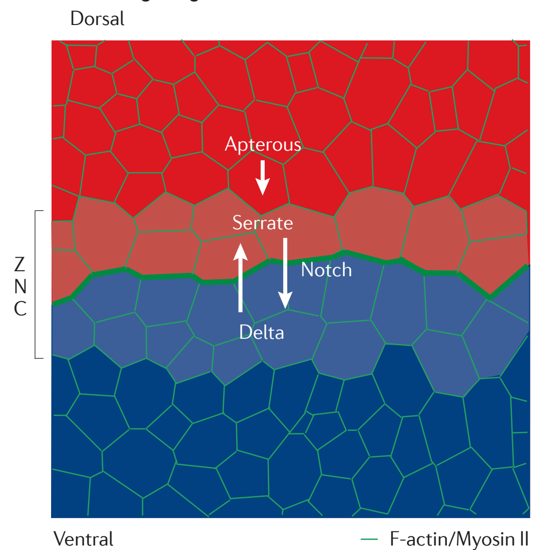

Figure 4 | **Mechanical tension in embryonic and larval *Drosophila melanogaster* epithelia. a** | Molecular basis of mechanical tension along cell borders. Tension is generated by contractile elements, including actomyosin, that are present at adherens junctions, where cell–cell adhesion is mediated by cadherins. **b** | Embryonic epidermis. Cells of the posterior compartments (red) express Engrailed. Filamentous (F)-actin and non-muscle Myosin II (Myosin II) are elevated at parasegment boundaries (green) in response to Wingless (WG), which is expressed from cells in the two to three rows immediately anterior to the parasegment boundary (light blue). **c**,**d** | Larval wing imaginal discs. In **c**, maintenance of the anterior–posterior (A–P) boundary requires the selector genes *engrailed* and *invected* in posterior cells and a response to the Hedgehog signal, which is mediated by the transmembrane protein Smoothened (SMO) and the transcription factor Cubitus interruptus (CI), in anterior cells. (Expression of the *ci* gene is repressed in the posterior cells.) F-actin and Myosin II (green), and mechanical tension (inferred from laser ablation experiments) (arrows), are elevated along the A–P boundary. In **d**, maintenance of the dorsal–ventral (D–V) boundary requires the selector gene *apterous* in dorsal cells and bidirectional Notch signalling across the D–V boundary (antiparallel arrows). Apterous induces Serrate and represses Delta, which are both Notch ligands. F-actin and Myosin II are elevated along the D–V boundary (green) in response to Notch signalling. Cells in the vicinity of the D–V boundary show a reduced proliferation rate (zone of non-proliferating cells (ZNC)), which is marked with a lighter colour.

a distinct shape and that angles between cell borders along this boundary are widened compared to the angles between cell borders in the remaining tissue[7]. Distinct cell shapes and widened angles between cell borders were also seen at ectopic interfaces between cells that do and do not transduce Hedgehog, or between cells that do and do not express *engrailed*[7]. These results indicated that the same regulatory mechanisms that set up the A–P boundary also determine the distinct shape of cells along this boundary.

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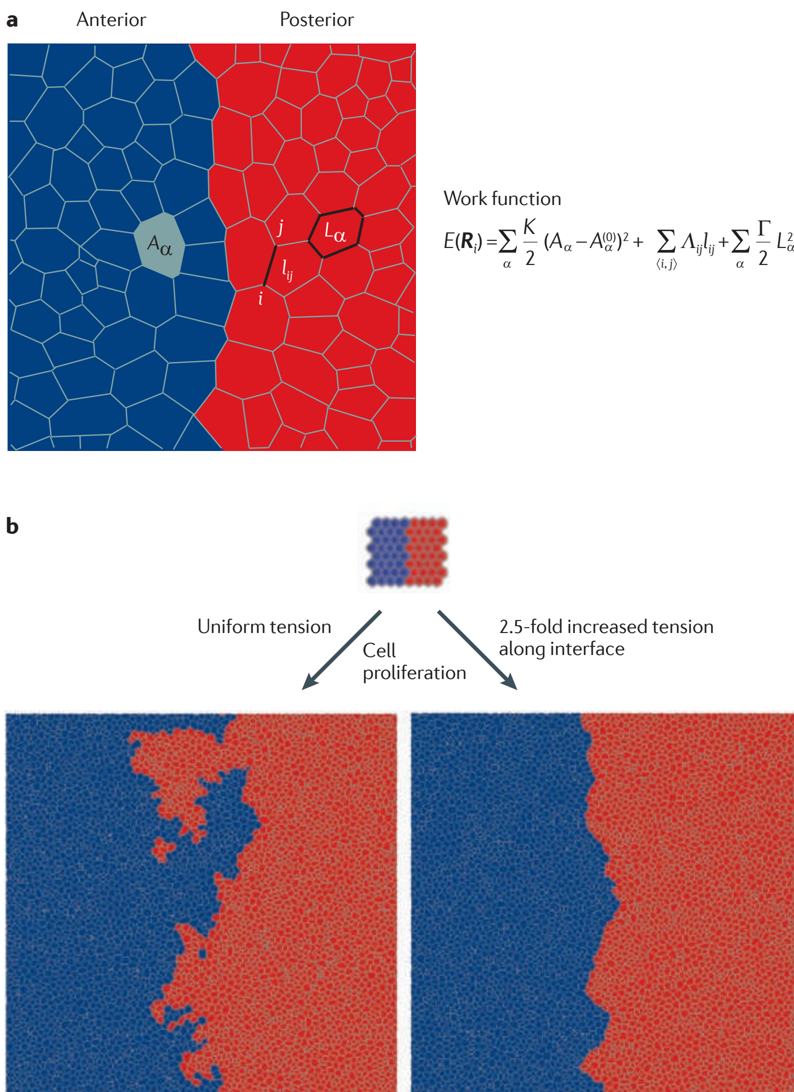
Box 1 | **Vertex model and simulation of tissue growth**

The vertex model[7,86] (see the figure, part **a**, and equation) describes the network of adherens junctions within epithelia by polygons identified by their vertex positions. Stable network configurations in the model are defined by local minima of a work function that describes the separate contributions from a cell's area elasticity, the line tension between two vertices and the elasticity of the cell's perimeter. Specifically, the work function $E(\mathbf{R}_i)$ describes an energy at vertex positions $\mathbf{R}_i$, where the index i numbers the vertices. The area elasticity of cells, indexed by α, with area A_α, preferred area $A_\alpha^{(0)}$ and elastic coefficient K, is described by the first term of the equation. Line tension is given by the second term, which describes the effects of tension Λ_{ij} along a cell border $\langle i,j \rangle$ of length l_{ij} that connects vertices i and j. The elasticity of the cell perimeter is given by the length of the cell perimeter L_α and by the coefficient Γ, as described by the last term. To represent the compartment boundary, an interface between anterior (blue) and posterior (red) cell populations is introduced. Simulation of cell proliferation proceeds by randomly selecting a cell, increasing its preferred area twofold and then introducing a new cell border to divide it. Energy minima are calculated for the whole 'tissue' and the procedure is repeated. In an epithelium, line tension depends on the contractility of the cortical actin–myosin meshwork and cell–cell adhesion. Part **b** of the figure shows simulations of tissue growth based on the vertex model. Cell proliferation results in irregular and unstable boundaries when the mechanical tension on cell borders is uniform. A local increase in mechanical tension on cell borders along the interface results in a sharper boundary during tissue growth. Figure is modified, with permission, from REF. 7 © (2009) Elsevier Science.



More direct evidence for a different mechanical tension along the A–P boundary in *D. melanogaster* wing imaginal discs was obtained by analysing the relaxation of the tissue after ablating single cell borders with an ultraviolet laser beam[7]. Ablating cell borders results in the displacement of cell corners (vertices). The relative initial velocity of cell vertex displacement after laser ablation is a relative measure of the mechanical tension on cell borders[115]. Landsberg *et al.* performed a systematic analysis of relative mechanical tension on cell borders located in the vicinity of the A–P boundary. They found that the initial velocity of cell vertex displacement was similar when cell borders either between two anterior cells or between two posterior cells were ablated[7]. However, when cell borders between anterior and posterior cells (that is, the A–P boundary) were ablated, initial velocity was increased approximately 2.5-fold. The increase in initial velocity was decreased by previous incubation of the tissue with Y-27632, a drug that inhibits Rho-kinase[116]. Rho-kinase is the main activator of Myosin II activity in fruitflies[117]. These results demonstrated that actomyosin-dependent mechanical tension along the A–P boundary is increased in wing imaginal discs. It will be interesting to test whether increased mechanical tension, as measured by tissue relaxation in response to laser ablation of cell borders, is a signature that is common to boundaries in insects and vertebrates.

Is a local increase in mechanical tension along compartment boundaries sufficient to maintain boundaries? Although this has been difficult to address experimentally, recent computer simulations provide evidence that this might be the case. Landsberg *et al.*[7] used a vertex model (BOX 1) to simulate the growth of a tissue. Using this model, simulation of cell proliferation renders initially straight interfaces between the two cell populations irregular[7]. When mechanical tension on cell borders along the interface was locally increased, however, the interface remained more smooth and straight as the tissue grew in size[7]. The higher the increase in relative tension at the interface, the 'straighter' the interface became. These results suggest that a local increase in mechanical tension on cell borders is sufficient to maintain straight interfaces.

Taken together, these data suggest a model in which the local increase in actomyosin-dependent mechanical tension maintains compartment boundaries in *D. melanogaster*. It will be interesting to explore whether a similar mechanism is also involved in maintaining compartment boundaries in vertebrates. A role of Myosin II in aligning cells along straight interfaces is not limited to compartment boundaries. In the ventral epidermis of *D. melanogaster* embryos, for example, cells are aligned into columns — a process dependent on Myosin II[118]. Thus, differential actomyosin-based mechanical tension might be a more general mechanism to maintain straight interfaces between any two kinds of populations of cells.

Is there a functional relationship between differential mechanical tension and differential cell adhesion? Mechanical tension depends on contractile actomyosin bundles as well as adhesive contacts between cells. Increased contractility is thought to elevate mechanical tension, whereas increasing adhesive

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contacts diminishes tension. Thus, in principle, a local de-adhesion between cells from neighbouring compartments could result in elevated mechanical tension along the intersecting boundary. In light of this, we speculate that the Eph–ephrin interactions that mediate cell repulsion between rhombomeres[39,40] might be a first step in modulating mechanical tension at these boundaries. A second mechanism by which differential cell adhesion could contribute to differential mechanical tension comes from the observation that the differential expression of some cell-adhesion molecules can result in the local enrichment of F-actin and Myosin II. In *D. melanogaster*, for example, cellular interfaces between wild-type cells and cells that are mutant for the gene encoding the cell-adhesion molecule Echinoid show elevated actomyosin levels[119]. Thus, local differences in mechanical tension along boundaries could be the result of the differential expression or activity of cell-adhesion molecules in the adjacent cell populations.

Conclusions and future directions

In the past couple of years, several common themes have become apparent with respect to how boundaries are established and maintained during animal development. First, the establishment of compartment boundaries in *D. melanogaster* was long known to depend on selector genes. The recent findings regarding the function of MESP2 in somite boundary formation suggest that non-lineage boundaries might also use selector genes for their establishment. Second, compartment and non-lineage boundaries both use cell signalling for their maintenance. In particular the importance of the Eph–ephrin signalling pathway in maintaining both compartment boundaries in the hindbrain and somite boundaries in vertebrate embryos has been revealed. A third emerging theme, at least in *D. melanogaster*, is that differential mechanical tension is an important physical mechanism for maintaining compartment boundaries. It seems equally clear, however, that there is not a single physical mechanism that maintains boundaries, and that even different mechanisms might be (sequentially) used to maintain one and the same boundary. Future work will need to explore whether these different physical mechanisms reflect the different challenges that boundaries are exposed to.

A further important question is how the patterning machinery that establishes and maintains boundaries is connected to the physical mechanisms. For somites, the recent work on Eph–ephrin signalling, integrin clustering and ECM deposition is starting to outline a molecular pathway. There is much to be learnt to understand how mechanical tension is controlled at compartment boundaries. Sophisticated live imaging and precise temporal and spatial perturbations of cells and proteins, in conjunction with mathematical modelling, have advanced our understanding of how boundaries are formed and maintained. Combining these experimental and theoretical approaches promises to continue to shed light on the mechanisms that establish and maintain boundaries in tissue development.

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Acknowledgements

The authors would like to thank Y. Saga, Y. Takahashi and S. Holley for comments on the manuscript. We thank D. Umetsu for help in preparing Figure 2. We apologize to all authors whose primary work we could not cite owing to space limitations. Work in the laboratory of C.D. is supported by the Max Planck Society, the Deutsche Forschungsgemeinschaft and the Human Frontiers Science Program. A.C.O. is supported by the Max Planck Society and the European Research Council under the European Communities Seventh Framework Programme (FP7/2007-2013) / ERC grant no. 207634. M.B. is supported by the TU Dresden, the Deutsche Forschungsgemeinschaft (SFB 655 and CRTD) and the European Union (ZF Health).

Competing interests statement

The authors declare no competing financial interests.

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