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Multidisciplinary approaches towards compartmentalization in development: Dorsoventral boundary formation of the *Drosophila* wing disc as a case study

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Summary. Lineage restriction boundaries set stable barriers during tissue growth that compartmentalize the primordia and promote their patterning. This discovery was a major breakthrough in modern biology because of its powerful conceptual implications regarding the developmental plan in both vertebrates and invertebrates, the subject of this short review. As a leitmotif, we focus on our own recent contributions to the problem of dorsoventral boundary formation in the wing disc of *Drosophila*, paying special attention to recent multidisciplinary approaches that have shed light on the gene regulatory interactions and biomechanics underlying the compartmentalization process.

Keywords: compartmentalization · developmental biology · systems biology · biomechanics · gene regulatory networks

Resum. Els límits de restricció dels llinatges estableixen barreres durant el creixement tissular que compartimentalitzen els primordis i promouen el seu patró. Aquest descobriment va suposar un gran avenç en la biologia moderna, gràcies a les seves poderoses implicacions conceptuals sobre el pla de desenvolupament dels vertebrats i dels invertebrats, que és el tema d'aquesta breu revisió. Com a leitmotiv, utilitzem les nostres contribucions més recents al problema de la formació del límit dorsiventral del disc imaginal de l'ala de *Drosophila*, tot posant especial atenció en enfocaments multidisciplinaris recents que han aclarit la biomecànica i les interaccions gèniques reguladores subjacents al procés de compartimentalització.

Paraules clau: compartimentació · biologia del desenvolupament · biologia de sistemes · biomecànica · xarxes de regulació gèniques

Compartments, boundaries, and the developmental plan

In the late 1960s and early 1970s, induced recombination techniques enabled the marking of single cells and their progeny (clones, mosaics). This, in turn, made it possible to follow the locations of cell populations in the primordium, mapping their positions in the adult organism and thus dynamically tracing the developmental plan. *Drosophila* is a perfect model organism in these kinds of experiments because of the peculiarities in the development of its imaginal discs [27,48]. All cuticular structures, e.g., wings, antennas, and legs, of the adult organism (imago) distinctly derive from these groups of cells. Thus, for example, all cells of the wing

of an adult fly (~10^4) originate from the cells of its corresponding wing imaginal disc (~10^1). Using clonal analysis, García Bellido and colleagues showed that the wing imaginal disc is compartmentalized [20]. Clones of cells in the tissue grow and extend up to certain cellular boundaries that do not overlap, thereby preventing the intermingling of different cellular populations (Fig. 1A). The first of those boundaries to be recognized was the dorsoventral (DV) one, which segregates the prospective dorsal and ventral cellular domains of the wing (Fig. 1B). In that case, the boundary population itself becomes the wing margin. However, as demonstrated by García bellido and colleagues, boundaries are not necessarily associated with morphological hallmarks: the wing primordium is further subdivided into anterior and posterior (AP) domains that do not correlate with particular structures of the adult wing [21] (Fig. 1B). These seminal studies fostered further work on the compartmentalization process in other imaginal discs (e.g., legs, head), revealing similar features [27,48]. Moreover, Nature has widely followed this developmental strategy, as this mechanism is evolutionarily conserved in a number of tissues and organisms. Thus, now we know that lineage restriction boundaries also exist in vertebrates at the hindbrain, in the limb buds, and in the gut [15,30].

Compartments are established by the expression and activity of "selector genes" that encode homeodomain tran-

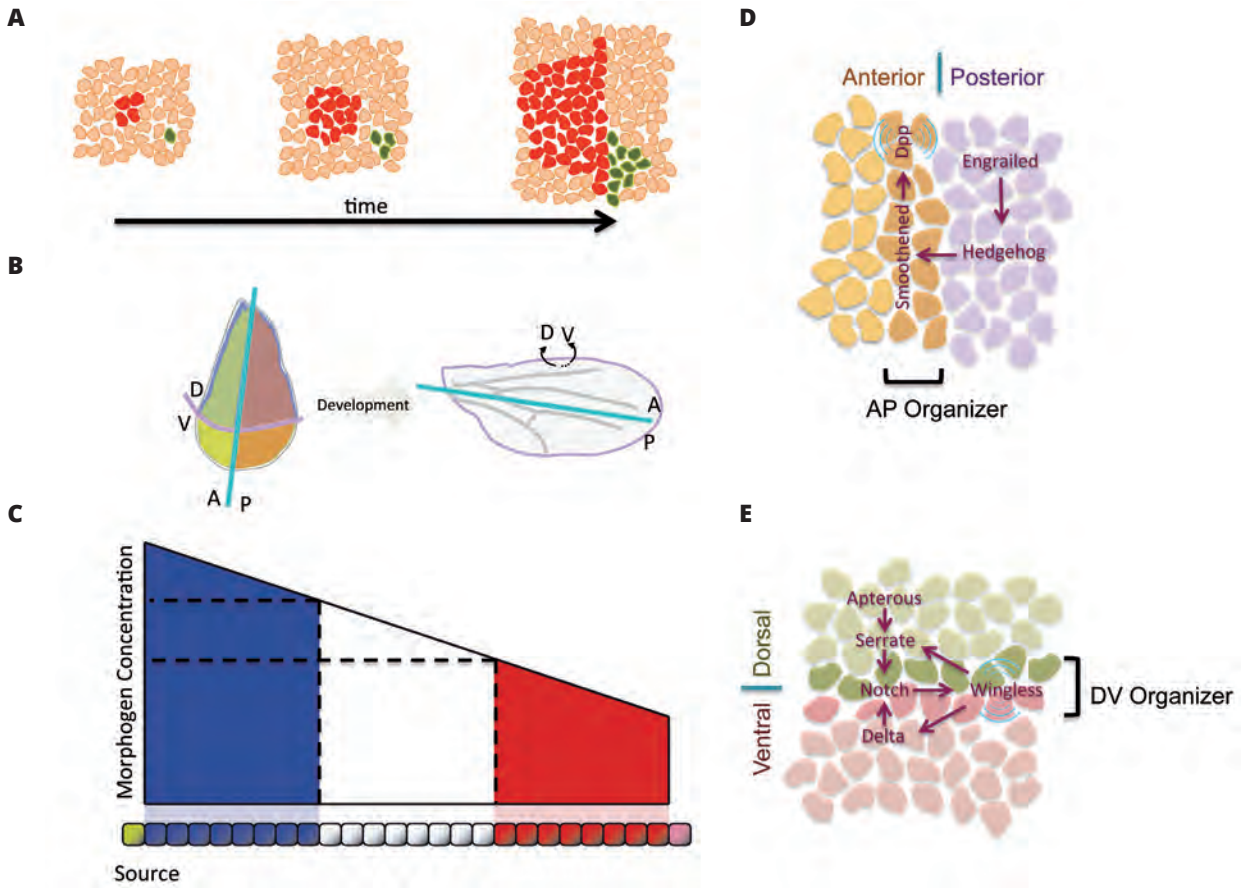

Fig. 1. (**A**) A growing tissue with two identified cellular clones. Lineage restriction boundaries are evident when the positions of cells and their progeny are followed over time: the established boundaries set a barrier that cannot be crossed. (**B**) Schematic representation of wing imaginal disc compartmentalization in *Drosophila* and the prospective positions of cellular populations within the adult wing. The dorsoventral (DV) boundary population becomes the wing margin whereas the anteroposterior (AP) one is not associated with any specific morphological hallmark. The expression and activity of selector genes establish a binary combinatorial mechanism that provides cells with coarse-grained positional information: dorso-anterior (DA), dorsoposterior (DP), ventral anterior (VA), and ventral posterior (VP). (**C**) The French flag model. Morphogens are synthesized at, and diffuse from, localized sources determined by the boundaries, the so-called organizers. Cells at the primordium obtain positional information by "reading" the morphogen concentration, resulting in further patterning. (**D**, **E**) Basic regulatory interactions shaping AP (**D**) and DV (**E**) compartmentalization. In the former, directional signaling because of the activity of Engrailed establishes the AP organizer at the posterior side of the anterior compartment, from which the morphogen Dpp diffuses. The action of Apterous modifies Notch ligands such that Notch becomes activated symmetrically in the cells at the DV interface (see text). Notch activity identifies the DV organizer and induces expression of the morphogen Wg.

scription factors and confer location identities at the single cell level. In the case of the wing imaginal disc of *Drosophila*, the transcription factors Engrailed and Apterous endow cells with posterior and dorsal characters, respectively [15,27,30,48]. These properties are heritable and prevent cells from mixing with those of "anteriorness" (lack of Engrailed activity) and "ventralness" (lack of Apterous activity) characters as proliferation progresses. This differential regulation of cell "affinities" due to the activity of selector genes has remained the fundamental hypothesis explaining the correct sorting of cells at the boundaries. It derives from the theoretical work of Steinberg, who proposed that tissues differing in their adhesiveness could rearrange and segregate just as immiscible fluids do, because of polar/non-polar interactions [53]. Accordingly, Capricious and Tartan are transmembrane proteins expressed in the dorsal compartment of the wing disc that promote cell adhesiveness [9,44]. Experiments have shown that in Apterous mutant backgrounds, in which the functionality of the DV boundary is compromised, the driven expression of these proteins can restore a functional boundary [9,44]. Likewise, cadherin expression downstream of Engrailed occurs along the anteroposterior (AP) boundary [51]. Other studies have shown that cadherins play a similar role during vertebrate development [29]. Still, as discussed below, the underlying biomechanics of cell sorting and segregation are complex and do not simply rely on adhesion properties.

The consequences of compartmentalization go far beyond keeping cellular populations separated; they also reveal an organized developmental plan for shaping organisms [15,30]. First, the gene patterning of the primordium, reflecting the activity of selector genes (or the lack of them), determines a coarse-grained cell fate by means of combinatorial overlap (Fig. 1B). Second, cellular interactions at compartment boundaries induce further patterning that provides detailed positional information to cells. Specifically, short-range signaling between cells at the compartment interface triggers new gene expression/regulation and establishes a cellular population at the boundary, the organizer, from which diffusive molecules, called morphogens, are secreted. This localized source of morphogen production sets up a morphogen concentration gradient that is "read" by cells at the compartments and elicits a long-range signaling mechanism. Examples of morphogen molecules are Decapentaplegic (Dpp), produced/secreted by the AP organizer, and Wingless (Wg), the DV organizer counterpart [15,30]. The positional information of cells within the bulk compartment is then achieved in a concentration-dependent manner following the so-called French flag model [11,57] (Fig. 1C), in which the organizers provide the axes of a coordinate reference system. Nonetheless, an organizer must display several key features to guarantee its reliability as a source of positional information: thus, the width of the cell population is constricted to a few (two, three) cells that must develop while the correct shape is maintained. Taken together, these findings were a major breakthrough in modern developmental biology because of their powerful conceptual implications in terms of the modular design of multicellular organisms, which is conserved in both vertebrates and invertebrates, and its genetic foundation.

Since the discovery of compartments, much progress has been made regarding the processes that lead to their formation and function. Yet, many aspects remain puzzling, including the gene regulatory networks responsible for the robust establishment and maintenance of gene patterning, and the biomechanical features of tissues that ensure precise and regular boundaries/organizers as cells proliferate and/or the tissue deforms. Systems-like approaches, including mathematical modeling, computational biology, and biophysics, have contributed to shedding light on these topics. Within this framework, in the following we review recent advances in the field, using the DV boundary of the wing imaginal disc as a case study.

Patterning the primordium: the establishment of boundaries and organizers

How do a given cell and its progeny "know" which genes should and should not be expressed in order to perform a particular task? As mentioned above, patterning of the primordium imparts positional information to cells and establishes a "map," by means of which the fate of a cell is determined depending on its position relative to other cells that form the tissue. Consequently, the question underlying the positional information problem is: how does genetic regulation establish such a map?

In the wing imaginal disc, Engrailed induces the expression of Hedgehog, a short-range signaling molecule, in posterior cells. Anterior cells at the compartment interface transduce the Hedgehog signal via the transmembrane protein Smoothened (and the Patched receptor), which in turn induces the expression of the morphogen Dpp. As a result of this directional signaling, from posterior to anterior, the AP organizer is established at the posterior side of the anterior compartment [15,30] (Fig. 1D).

While the DV case shares the basics with the AP one, there are some relevant differences. Given the morphological implications of DV compartmentalization in the adult organism, formation of the DV organizer implies more complex and bidirectional signaling between adjacent compartments. The latter might reflect the need for symmetrical positioning of the organizer since the dorsal and ventral compartments are to give rise to the apposed, specular-like surfaces of the adult wing [15,27,30,48]. Thus, Apterous in

the D cells of the early wing primordium activates the expression of the transmembrane ligand Serrate and the glycosyltransferase Fringe and restricts the expression of Delta, another transmembrane ligand, to V cells. Fringe modifies the Notch receptor and makes D cells more sensitive to Delta and less sensitive to Serrate. Conversely, unmodified Notch in V cells responds better to Serrate than to Delta. The preferential response of the Notch receptor to the ligands expressed in the opposite compartments ensures the activation of the Notch pathway only at the DV interface, i.e., symmetrically and bidirectionally (see [10] and references therein) (Fig. 1E). Other compartmentalization problems, such as separation of the animal cap cells in zebrafish, also involve bidirectional signaling [43]. Notch receptor activity causes expression of the signaling molecule Wg at the DV boundary population, which in turn drives the expression of Notch ligands, thus sustaining receptor activity and leading to the establishment of the DV organizer [10]. Yet, experimental studies aimed at elucidating the details of the genetic interactions that sustain both Notch and Wg activities revealed a few inconsistencies. Specifically, Dishevelled, a cytoplasmic mediator of the Wg signaling pathway, binds the intracellular domain of Notch, which blocks Notch signaling and reduces receptor activity [5]. This finding necessitated that a new property be ascribed to boundary cells that allowed them to stably maintain the organizing center: they must be refractory to the Wg signal. Indeed, refractoriness to a morphogenetic signal also develops in the AP case: Engrailed not only induces Hedgehog expression but also causes posterior cells to become refractory to it [54,59]. These observations pointed out the need to redefine the aforementioned regulatory interactions patterning DV compartmentalization—a need that was met using a multidisciplinary approach.

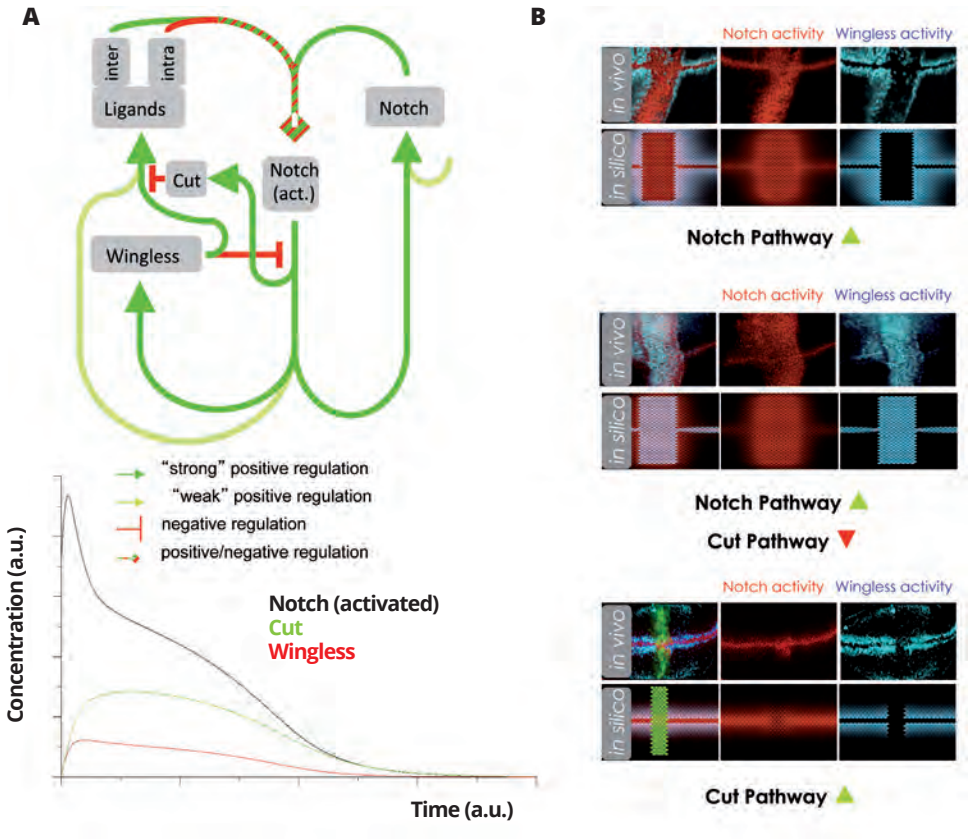

Fig. 2. (**A**) As shown by in silico experiments, putative regulatory interactions between Notch and Wg pathways (top) cannot sustain Notch activity at the DV boundary population; as time progresses Notch and Wg expression fades in those cells (bottom). (**B**) When the correct regulatory interactions are included (see Fig. 3A) in silico experiments are able to reproduce their in vivo counterparts with respect to boundary formation patterning. **Top:** Induced expression of Notch in a strip perpendicular to the DV boundary generates a pattern of Notch and Wg activities resembling that of the boundary, indicating that refractoriness to the morphogen signal is downstream of Notch (red: Wg expression; blue: Senseless expression). **Middle:** The same experiment in a Cut mutant background cannot induce refractoriness to the Wg signal. **Bottom:** Ectopic expression of Cut in a strip perpendicular to the DV boundary (green) drives refractoriness to the Wg signal in that region.

Modeling approaches to the genetic regulation of compartmentalization

In parallel with the experimental efforts aimed at identifying gene regulatory interactions, mathematical modeling approaches have become an increasingly powerful tool based on their predictive capabilities [18]. At the root of the concept of boundaries and their functions lies the theoretical work of Meinhardt, who proposed that boundaries serve as reference points for positional information as sites of morphogen synthesis [41,42]. As reviewed here, his predictions have been experimentally confirmed to a large extent. AP patterning and boundary formation have been addressed through modeling, which has unveiled the regulatory interactions that confer robustness in terms of parameter variations [16]. In the context of DV boundary formation in the *Drosophila* wing, continuous [10,23] and Boolean [35] regulatory networks have been proposed. Both types of studies have contributed to a better understanding of the temporal modularity of the developmental plan and they have shed light on the respective genetic interactions. Thus, in silico studies helped to clarify the dynamics of patterning along developmental instars [23] and provided evidence that the putative regulatory interactions in the DV boundary could not provide a plausible explanation for its establishment [10] (Fig. 2A). The combination of experiments, mathematical modeling, and computer simulations further showed that the expression and activity of Cut are both necessary and sufficient to inhibit Wg target gene expression in boundary cells [10] (Fig. 2B) and helped to reverse-engineer a consistent DV axis regulatory network (Fig. 3A). Thus, refractoriness to Wg via Cut in the DV boundary population blocks Wg signaling pathway in these cells: the Notch ligands Serrate and Delta as well as other genes transcriptionally regulated by Wg activity

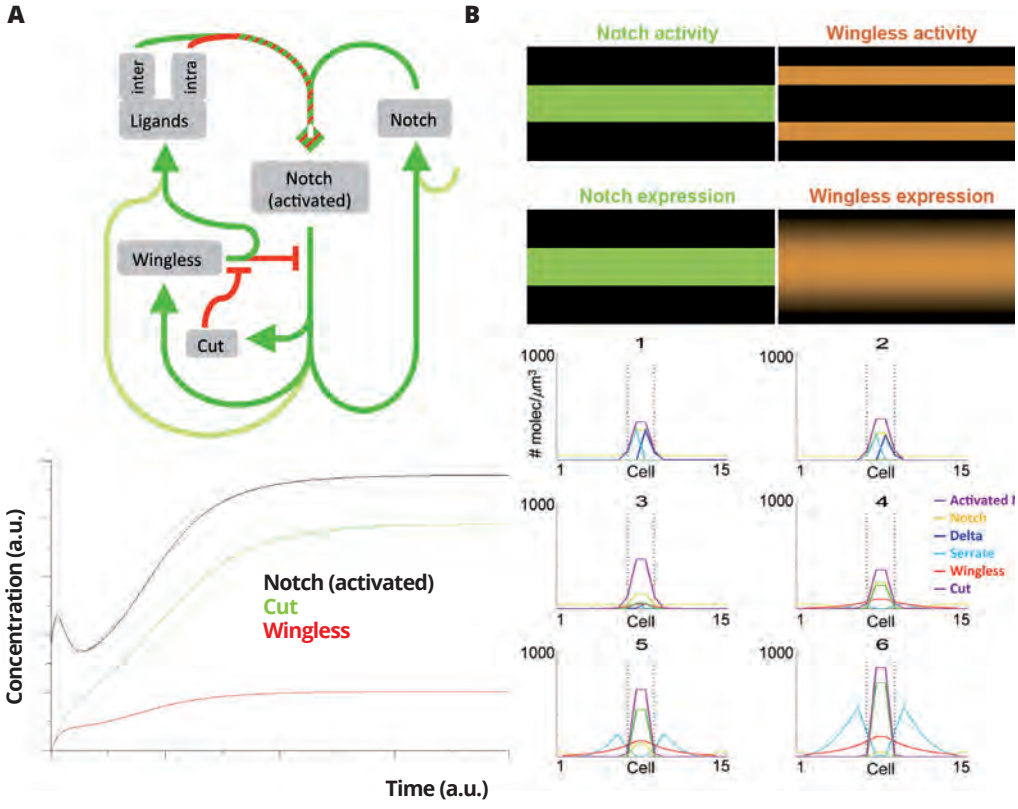

Fig. 3. (**A**) A reverse-engineering approach reveals the gene regulatory network underlying DV boundary formation (top). The role played by Cut is complex and includes the repression of Notch ligands. Cut also induces total refractoriness to the Wg signal (cf. Fig. 2A). The model takes into account cell-autonomous and intercellular processes, including Wg diffusion and intracellular (*cis*) and intercellular (*trans*) interactions between the Notch receptor and its ligands Delta and Serrate. This network "circuitry" gradually generates the robust and sustained expression of Notch and Wingless in boundary cells (bottom). (**B**) Depiction of Notch and Wg expression and activity profiles at the DV boundary and neighboring regions (top). Wg expression is maximal at the DV boundary but its activity is null at that domain. The interplay between Notch and Wg pathways generates mutually exclusive spatial domains in terms of their activities. In silico experiments have reproduced the evolution of the pattern that shapes the DV boundary and neighboring regions (bottom). Note the spatial refinement of Notch activity, the symmetrization of Delta and Serrate expression (Wg activity reporter), and the formation of a Wg gradient as the DV organizer becomes established.

(e.g., Senseless) are not expressed in boundary cells. Refractoriness mediates the regulatory interplay between Notch and Wg and promotes the formation of mutually exclusive spatial domains in terms of their activities in patterning the DV organizer (Fig. 3B). This systems biology approach also led to the recognition of: (a) the role of Cut in terms of the functionality of the DV boundary (Cut is not required for the formation but for the robust maintenance of patterning) and (b) so-called boundary refinement dynamics (Wg activity restricts the width of the organizer and forces polarized signaling by Notch receptor and its ligands).

As the complexity of the developmental model increases in terms of the details of the interactions in gene regulation (e.g., *cis* versus *trans* interactions between ligands and receptors) and/or in terms of the number of "players" involved in a signaling pathway, so does the dimensionality of the parameter space. Thus, depending on the amount and quality of the experimental data, fitting or estimating parameters can set a limitation for the predictive capabilities of modeling. Moreover, conceptual abstractions about the fundamental mechanisms driving a particular process become more difficult as the modeling process becomes less and less reductive. In this regard, a powerful approach for understanding the properties and functionalities of genetic regulation is the analysis of reduced functional blocks, namely, *network motifs* [4]. Crosstalk between motifs has been shown to be useful for characterizing the spatial and temporal patterning that arises in developmental processes [14,31]. Accordingly, a motif-like formalism also has been applied to the DV boundary formation problem [12], thus confirming the robustness of DV patterning as well as the basics of the mechanism underlying the formation of mutually exclusive domains of activities. In addition, this approach has allowed the output of different mutant backgrounds to be easily analyzed (Fig. 4).

All in all, the modeling of gene regulatory networks in combination with experiments has been a valuable tool for comprehending the genetic interactions that lead to a stable and robust pattern for boundary formation. Nonetheless, in spite of all the knowledge gained about the genetic regulation underlying boundary formation, the biomechanics of cell segregation long remained a conundrum. Note that during development the number of cells in the wing imaginal disc increases by approximately 1000-fold. This poses several intriguing questions: How do organizer cells deal with division events while maintaining straightness, width, and stability? How does patterning become scaled as proliferation progresses? What are the roles played by cytoskeletal remodeling, adhesiveness, and cortical tension in ensuring reliable compartmentalization?

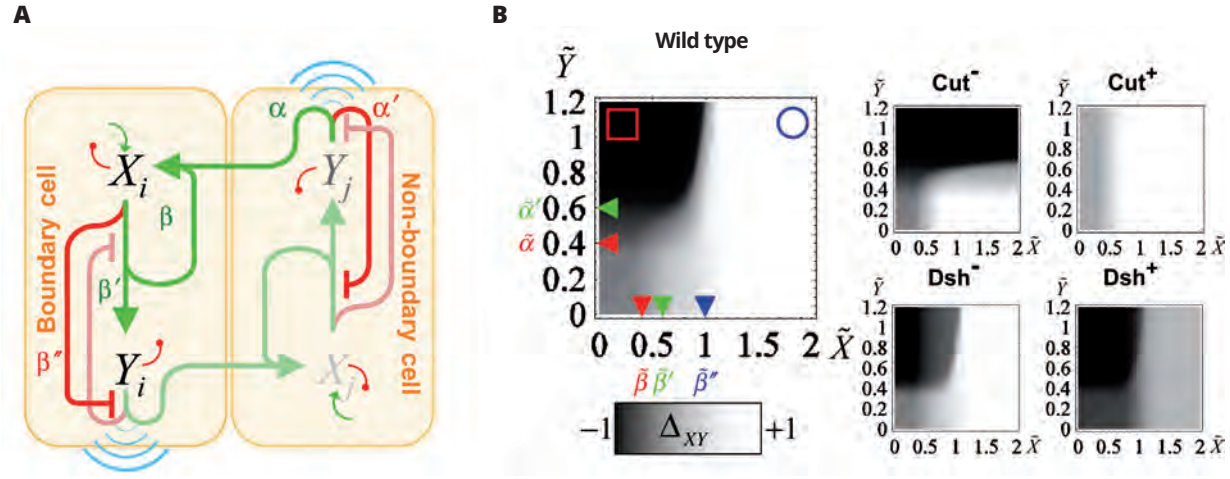

Fig. 4. (**A**) Network motifs capture the central functionality of interacting pathways, or species, using a minimal number of elements. Intertwined negative and positive feedback loops between two species, *X* (Notch) and *Y* (Wg), reproduce the formation of mutually exclusive activity domains and all the basic phenomenology of DV boundary formation. (**B**) The reduced dimensionality of the motifs enables representation of network potentiality in terms of expression and activity profiles. Here, the differences between Notch and Wg activities are shown by means of a density plot as a function of their expression levels. The triangles indicate the expression thresholds controlling network functioning. The sharp transition between full Notch (blue circle) and Wg pathway (red square) activation is marked by small variations in Notch levels (hypersensitivity). In addition, the signature of mutant backgrounds can be easily analyzed and compared with that of the wild-type (see [12] for details).

Biomechanics of compartmentalization

Recent research has pointed out that mechanical effects play a central role in the function of organizers/boundaries [15,30,36,47,48,56]. Thus, it has been shown that both F-actin and myosin II accumulate by the zonula adherens at the junctions of the DV border [39,40]. Running along the boundary, these components putatively promote cell adhesion and increase the cortical tension of cells. In agreement with these studies, it was reported that actomyosin-based barriers (cables) are effective inhibitors of cell mixing in other developmental stages of *Drosophila* (parasegmental boundaries of the embryonic epidermis) and that the reduction of myosin activity causes boundary disturbance [46]. These results provided support for the crucial and active role of the cytoskeleton, and consequently of its mechanical effects, in keeping the regularity and fence-like features of boundary/organizers. Note that it was recently shown that, in addition to differences in cell affinity, some of the contributions of the cytoskeleton, e.g., increased cell tension, underlie the functioning of AP/DV boundaries [3,37]. In laser ablation experiments, mechanical tension was greater (by approximately three-fold) on cell bonds along compartment boundaries than within the remaining tissue (Fig. 5A) [3,37]. These findings were in line with theoretical work challenging Steinberg's "differential adhesion hypothesis" and they led to the "differential surface contraction hypothesis," in which contractility plays a crucial role in cell sorting. More recently, the "differential interfacial tension hypothesis," combining elements of both theories, was proposed. Clearly, the biomechanics underlying cell sorting are more complex than first thought (see [34] and references therein).

At the same time, other studies have indicated that dynamic and morphologic factors related to the cell cycle must be taken into account to understand the stability and

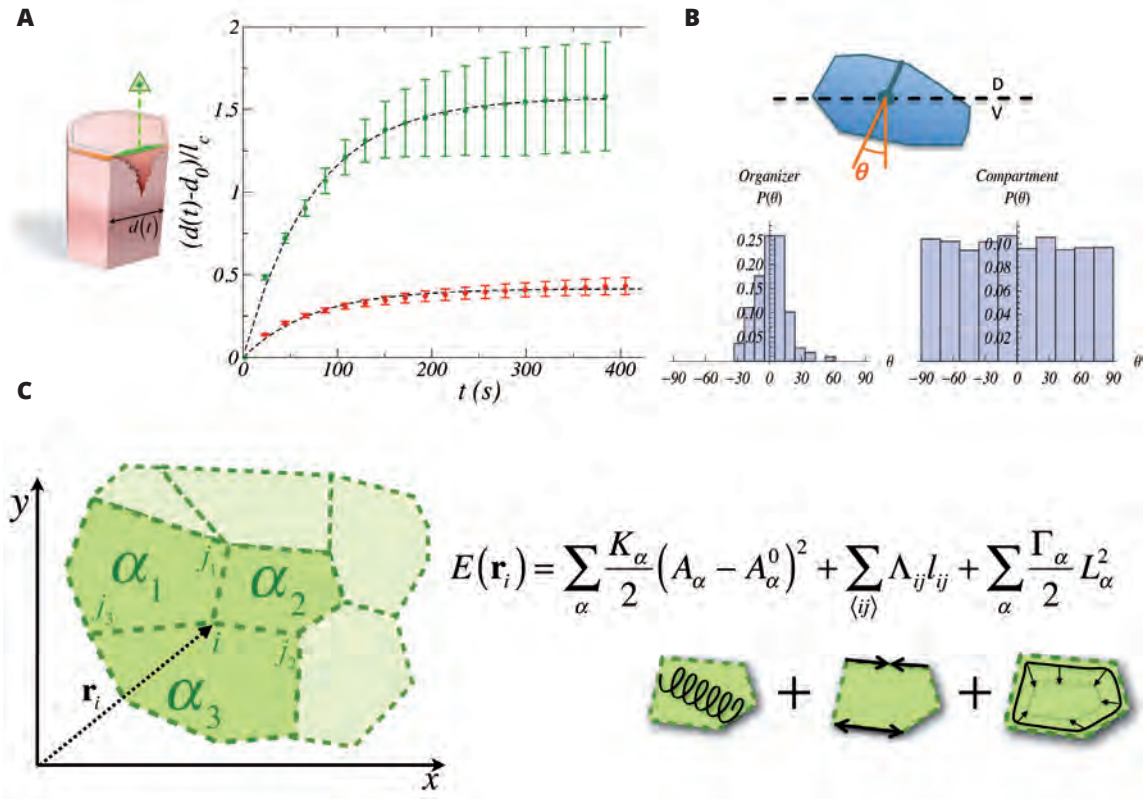

Fig. 5. (**A**) Laser ablation experiments shed light on, and allow quantification of, cellular tension in tissues. The distance between the vertices of ablated edges changes with time because of the energy dissipated in the tissue. The initial velocity of the expansion is related to the accumulated tension: the higher the velocity, the greater the tension. The curve indicates that the tension is greater at the edges of the DV boundary (green circles) than in the bulk compartment (red circles). (**B**) Cleavage orientation determines the shape of organs and challenges the stability of boundaries. At the DV boundary, cleavage orientation is clearly favored over the bulk compartment, with the cells preferentially dividing perpendicular to the DV axis. (**C**) In the vertex model, each cell is represented by a reduced number of points (the vertices). Associated with each vertex is an energetic contribution that takes into account the different elements, such as the elastic energy of cells, their adhesion, and the cortical tension.

robustness of organizers. Thus, it has been demonstrated in different contexts that the orientation of cell divisions determines the shape of developing tissues and organs [2,6]. In particular, it is now clear, from measurements of the orientation of the mitotic spindle and from post-mitotic cellular allocation, that cells of the DV organizer follow a division pattern that is different from that of cells within the bulk of the compartments: in the former the division plane is perpendicular to the DV interface (Fig. 5B) [6,39,40]. In addition, the rate of proliferation decreases in the vicinity of some boundaries. In the DV case, Notch activity eventually controls cell proliferation at the organizer by arresting the G1-S cell cycle progression; also, by late third instar the DV organizer and neighboring cells clearly define the "zone of non-proliferating cells" (ZNC) [28,32]. In vertebrates it has been also shown that during segmentation of the chick embryo hindbrain, the duration of the cell cycle is longer at the rhombomeres interface [25].

In silico experiments are also a powerful and effective tool for studying the biomechanics of tissues. The so-called vertex model exploits the polygonal-like morphology, monolayer character, and apicobasal mechanical polarization of epithelial cells to characterize them by a reduced set of points (the apical vertices) and to compute the dynamics of each cell vertex depending on the applied forces, which derive from mechanical considerations, e.g., cytoskeleton activity [49] (Fig. 5C). In the literature different examples show that the vertex model successfully describes the biomechanics of the wing imaginal disc. These examples include its packing [19], AP compartmentalization [37], the effects of mechanical feedback on tissue topology [1], the alignment of planar cell polarity domains with the proximal-distal axis of the wing [2] and, more recently, the DV compartmentalization biomechanics [3,13]. For example, it has been shown that cellular mechanical properties are coupled to cleavage orientation by means of the Hertwig rule [45,55] (cells divide perpendicular to their longest axis) and that this is key to the organizer stability (Fig. 6) [13]. However, some contradictions persist in terms of the influence of the cell cycle duration on the maintenance of the DV organizer. On the one

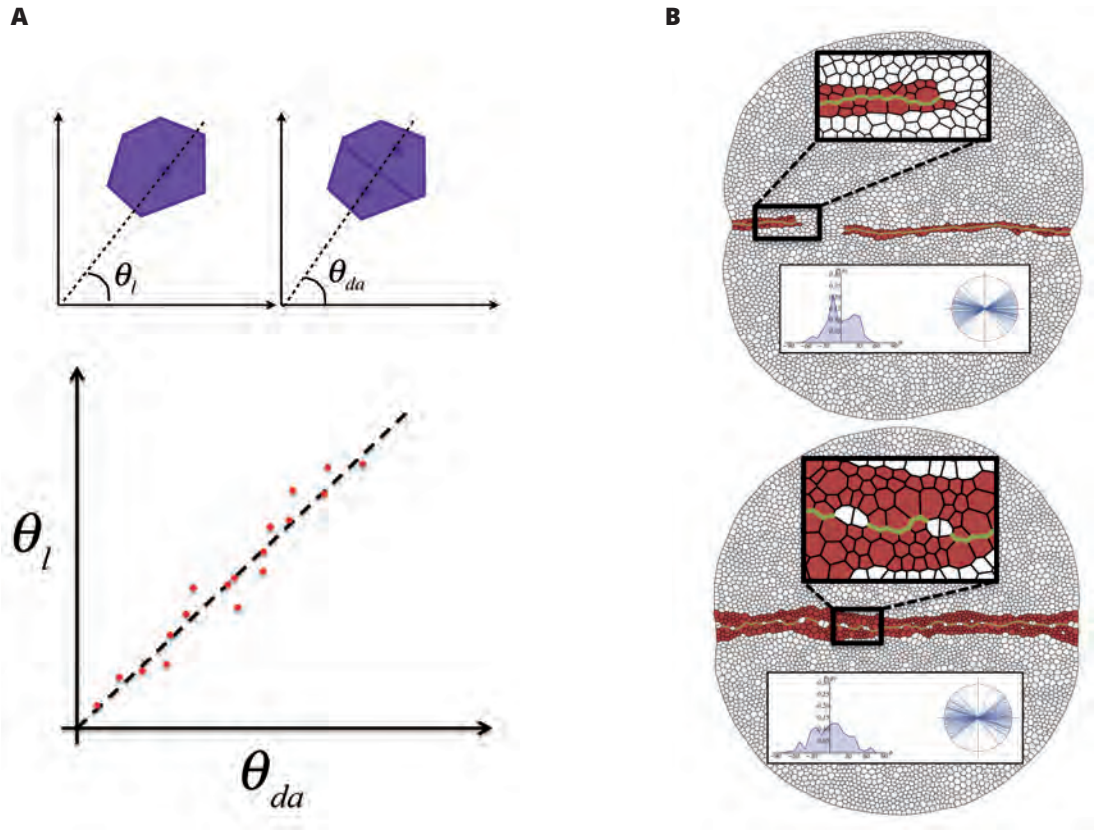

Fig. 6. (**A**) The Hertwig rule establishes a phenomenological relation between the angle that defines the longest axis of a cell (l) and the division angle (da). (**B**) In silico experiments, in agreement with experimental data, indicate that the stability of the boundary is challenged by the cleavage orientation and the duration of the cell cycle. A randomized cleavage orientation (top) breaks the DV boundary. In the absence of distinct regulation of the cell cycle duration at boundary vs. bulk compartment (bottom) cells, the DV organizer becomes wider such that some cells cannot maintain Notch activity and DV organizer stability is therefore compromised.

hand, the actomyosin cable at the DV interface is not present when the ZNC is evident [39,40]. A similar phenomenon is observed at the parasegmental boundaries of the *Drosophila* embryo [46]. This suggests that the challenge posed to the stability of boundaries by cell proliferation necessitates the involvement of additional, stabilizing mechanisms. Moreover, there is ample evidence (analytical, computational, and experimental) that a distinctive regulation of cell cycle duration is needed at the DV organizer in order to maintain its features [7,13] (Fig. 6B). On the other hand, a recent study showed that a decreased cell proliferation rate is not important to the maintenance of a straight and sharp DV boundary and that tissue elongation is a major factor [3]. In any case, these studies together have confirmed that an understanding of how the physical properties and cytokinetic processes of cells are regulated is crucial to understanding compartmentalization.

Conclusions and perspectives

Herein we have reviewed the dynamics of the formation and maintenance of boundaries and organizers, using DV compartmentalization as a leitmotif. In a discussion that ranged from its genetic foundations to its biomechanical properties, we showed how signaling events establish the onset of organizer formation, which further patterns the primordium and delivers positional information to cells. In this context, systems biology approaches have helped researchers to unravel some of the genetic interactions that underlie boundary formation. In addition, we pointed out that the identification and characterization of biomechanical elements are fundamental for understanding the robust maintenance of the compartmentalization process. In this regard, modeling techniques have provided both quantitative and predictive insights into the morphogenetic mechanisms driving boundary dynamics. However, interesting questions remain regarding the relative importance of signaling versus mechanical and dynamic effects in the maintenance of boundaries/organizers during development. At every developmental stage, signaling is indeed fundamental but, at the same time, once the onset of segregation has been established, mechanical and dynamic contributions become fundamental for the organizer to appropriately guide tissue growth.

Systemic approaches have also been useful in revealing many aspects of the compartmentalization process, a common theme in developmental biology. Still, the big picture requires further progress. For example, the link between biomechanics and signaling and the feedback between these processes remains unclear [58]. Recently there have been advances in force-sensing, the homeostasis of cell–cell junctions [22], and the interplay between mechanical signals and gene regulation [26,50]. However, additional work is required to understand how genetic regulation determines biomechanical features and vice versa [24]. Moreover, multiscale aspects are far from being understood. Also, while we have reviewed some of the techniques and studies that connect single cell behavior with that of the tissue, how molecular effectors determine collective cellular behavior and the interplay between tissues remain to be determined [17,33]. Quantitative approaches through imaging and modeling, in which the underlying biophysics are connected with the biological realm, will definitively help to answer this and other questions in the coming years [8,52].

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