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Organ size control: lessons from *Drosophila*

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Abstract

Of fundamental interest to biologists is how organs achieve a reproducible size during development. Studies of the developing *Drosophila* wing have provided many key insights that will help give a conceptual understanding of the process beyond the fly. In the wing, there is evidence for both “top-down” mechanisms, where signals emanating from small subsets of cells direct global proliferation, and “”bottom-up” mechanisms, where the final size is an emergent property of local cell-cell interactions. Mechanical forces also appear to have an important role along with the Hippo pathway, which may integrate multiple types of inputs to regulate the extent of growth.

Introduction

While we have witnessed tremendous progress in our understanding of the genetic regulation of pattern formation in recent years, our current understanding of the mechanisms that regulate organ or organism size is rudimentary, at best. It has been known for a long time that nutritional deprivation and hormone deficiencies are known to compromise growth and that tumors that secrete growth hormone can cause excessive growth. However, in the absence of such systemic perturbations, very little is known about how individual organs stop growing when they reach the appropriate size. Experiments involving organ transplantation in mice suggest that some organs such as the thymus rely on controls that largely function within the organ (Metcalf, 1963), whereas others such as the spleen rely on humoral factors (Metcalf, 1964). In reciprocal transplants of limb buds between salamanders of different sizes, it was concluded that the growth properties of the graft cells together with circulating host-derived factors determined the growth properties of the limb (Harrison, 1924). Ninety years after those experiments were done, we still have little understanding their underlying mechanisms!

The transformation of embryology from a set of detailed observations of cellular behavior to a series of events involving key molecular regulators happened, in significant part, because genetic studies in *Drosophila* led to the identification of important regulators of pattern formation (Lewis, 1978; Nusslein-Volhard and Wieschaus, 1980). Once these genes were

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identified and molecularly characterized, their function could be manipulated during embryonic development in a variety of ways, thus linking the function of individual genes to specific biological processes. In a similar vein, studies of the developing *Drosophila* wing, initially using approaches derived from experimental embryology, then with the application of genetic techniques of increasing sophistication, and most recently incorporating approaches used by physicists and engineers, are providing our first glimpse of the regulatory logic that underlies the mechanisms that regulate organ size. This Review article is written with the explicit intent of explaining, especially to non-Drosophilists, some of the key insights into our understanding of organ size regulation that have been obtained from the study of growth and development of the *Drosophila* wing. To simplify matters, I have focused mostly on the issue of size regulation and have therefore not covered mechanisms that regulate the shape of the wing and genetic pathways that specify patterns of gene expression in the developing wing.

Growth and development of the *Drosophila* wing-imaginal disc

The adult wing of *Drosophila* derives from a primordium, the wing imaginal disc (hereafter “wing disc”), composed of approximately 30 cells (Garcia-Bellido and Merriam, 1971; Madhavan and Schneiderman, 1977; Worley et al., 2013), whose fates have been determined at an early stage of embryogenesis. These cells invaginate from the surface and begin to resemble a flattened sac with the apical surfaces of the epithelial cells pointing towards the lumen of the sac. During the larval stages, while the cells that give rise to the larval body increase in size and become highly polyploid, the cells of the imaginal discs, including the wing disc remain diploid. The cells of the wing disc undergo, on average, approximately 9-11 rounds of cell division (Martin et al., 2009; Worley et al., 2013) and accumulate in the G2 stage of the cell cycle at the end of the larval stage.

By this stage this disc has a characteristic size and shape. The cells of the two layers of what was once a “flattened sac” are now very different from each other (Figure 1) One layer, the disc proper, accounts for the vast majority of cells in the disc and is composed mostly of cells of columnar morphology. It has a buckled appearance with several characteristic folds and ridges and represents the primordium for the wing blade, the hinge (which attaches the wing to the body wall), and portions of the dorsal and ventral parts of the thorax. In the dorsal portion of the disc, beneath the epithelial cells is a tracheal branch and numerous myoblasts that generate the flight muscles. The other epithelial layer of the disc, the peripodial epithelium, is composed of squamous cells and appears to be stretched tightly over the convoluted epithelium of the disc proper (Figure 1A-D).

During the pupal stage of development, most cells complete two additional divisions and arrest permanently in the G1 phase of the cell cycle (Milan et al., 1996a, b). This stage is also characterized by morphogenetic events that transform an epithelial sheet with characteristic ridges and folds into the adult wing, which is a relatively flat bilayered structure. This structure is derived by folding the disc epithelium along its dorsoventral boundary and by promoting adhesion between the now apposed basement membranes. The adult wing has veins at characteristic positions that separate intervein regions (Figure 1E).

Identification of the major pathway that regulate disc growth

All growth has to occur at the level of individual cells. Therefore, any mechanism that regulates the overall growth of the wing disc has to, at some level, impact the biosynthesis and degradation of cellular macromolecules. Mutations that perturb growth have been identified using a variety of approaches in *Drosophila* (reviewed in Hariharan and Bilder, 2006; St Johnston, 2002). Genetic studies indicate that there are six or seven main pathways that regulate the growth of imaginal disc cells (Figure 2). These are the insulin/PI3 kinase pathway (Leevers et al., 1996), the Rheb/Tor pathway (Saucedo et al., 2003; Stocker et al., 2003), the receptor tyrosine kinase (RTK)/Ras pathway (Prober and Edgar, 2000), Myc (Johnston et al., 1999), the JAK/STAT pathway (Bach et al., 2003) and the Hippo pathway (Justice et al., 1995; Xu et al., 1995). Each of these pathways is conserved among diverse metazoan species, thus emphasizing that the mechanisms that regulate growth at the cellular level are evolutionarily ancient. In addition, cyclin D, which in mammals primarily promotes progression through the G1 phase of the cell cycle, also promotes growth in *Drosophila* imaginal discs (Datar et al., 2000).

Why are so many growth-promoting pathways necessary? One extreme explanation would be that each pathway promotes the biosynthesis of a specific and non-overlapping subset of cellular macromolecules (a “qualitative” difference). Thus, activation of each pathway would be necessary to provide the complete set. At the other extreme is the possibility that all pathways promote the biosynthesis of all macromolecules but that, under physiological conditions, they each provide an insufficient stimulus for cellular growth (a “quantitative” requirement), thus necessitating the simultaneous activation of multiple pathways. We know that this latter explanation, at least in its purest form, is incorrect for several reasons. First, the changes in cell physiology elicited by activation of each pathway differ. For example, increasing Myc activity promotes ribosome biogenesis while increasing activity of PI3 kinase does not (Grewal et al., 2005). Second, at least in the few cases examined, the inactivation of one pathway cannot be compensated for by activation of another (for example, Tseng et al., 2007). A challenge for the future is to obtain a more precise definition of the dynamics of cell growth at the molecular level and to be able to link the activity of each of these pathways to those molecular changes.

The Hippo pathway (Halder and Johnson, 2011; Irvine, 2012) merits some additional discussion because it features prominently in many of the mechanisms that are thought to regulate the overall size of the wing disc that will be discussed in this Review. In *Drosophila*, the pathway consists of two protein kinases, Hippo and Warts, which function in series to restrict the nuclear localization of the growth-promoting transcriptional co-activator, Yorkie (Huang et al., 2005) (whose mammalian orthologs are YAP and TAZ). Importantly, the activity of this pathway is regulated by at least three different cell-surface proteins, Fat (Bennett and Harvey, 2006; Cho et al., 2006; Silva et al., 2006; Tyler and Baker, 2007; Willecke et al., 2006), Crumbs (Chen et al., 2010; Grzeschik et al., 2010; Ling et al., 2010; Robinson et al., 2010), and Echinoid (Yue et al., 2012). Fat (Ft) is a protocadherin that binds to another protocadherin, Dachsous (Ds) on adjacent cells. Crumbs and Echinoid can each engage in homophilic binding to Crumbs and Echinoid on adjacent

cells. Thus this pathway provides a mechanism by which cell proliferation can be regulated by a cell's immediate neighbors.

Framing the organ size problem – linking cell growth to organ size

Even in a complex multicellular organism, all tissue growth occurs at the level of individual cells. Importantly, cell growth and survival is determined by local cues; cells assess the levels of nutrients and growth factors in their immediate microenvironment. In contrast to growth that occurs at the level of individual cells, the overall size and shape of an organ is a collective property of large numbers of cells (typically thousands of cells). The challenge is to understand how the growth and proliferation of individual cells, scattered throughout the organ, is regulated so as to collectively generate, with considerable precision, an organ of the right size and shape.

From first principles, there seem to be two main ways to ensure that an organ can achieve a precise final size. One way involves a “top down” mechanism of size control that operates at the level of the entire organ from some kind of signaling center or organizer. An example of this type of mechanism is one that invokes a key role for morphogens that are secreted from specific locations within the developing organ. Individual cells at different locations in the growing organ assess some parameter associated with the morphogen (such as its absolute level or the slope of the morphogen gradient) and regulate their proliferation accordingly. The other type of mechanism involves a “bottom-up” mode of organization, where local cellular interactions govern cell proliferation. Thus, the overall size of the organ is an emergent property of the cellular interactions that occur throughout the organ. An important mechanistic distinction between the two types of mechanisms is that the “bottom-up” variety does not require any sensing of the overall size of the organ. As discussed in this Review, there are aspects of size determination in the wing disc that can be ascribed to both “top-down” and “bottom-up” mechanisms.

The wing disc has a robust disc-autonomous size control mechanism

When immature imaginal discs were transplanted into the abdomen of an adult female, they grew until they reached the approximate size and shape of a disc at the end of normal larval development (Bryant and Levinson, 1985), thus demonstrating a disc-autonomous size-control mechanism that could function even in a heterologous environment. Moreover, wild-type discs stopped growing at the appropriate size even when the larval phase was extended to allow for additional growth (Martin and Morata, 2006; Simpson et al., 1980). The classic experiments of Hadorn (Hadorn, 1963), Bryant (Bryant, 1971), and Schubiger (Schubiger, 1971) demonstrated that when fragments of discs were implanted in adult abdomens, in some cases, regenerative growth also generated a complete disc of approximately the appropriate final size. Thus the size-determination mechanism is operational not just during developmental growth but also during regenerative growth.

The final disc size is also determined independently of precursor cell number. Irradiating *Drosophila* at early stages of development, at doses that drastically reduce the number of cells in the disc, does not prevent a disc from developing to its normal final size, which would require additional cell divisions by the surviving cells (Haynie and Bryant, 1977).

Indeed, although a wing disc typically derives from around 30 cells, discs of normal appearance can be generated from fewer than 5 founders (Worley et al., 2013). Moreover, having patches of cells within the disc that grow at different rates does not obviously affect its overall size (Simpson and Morata, 1981). Thus, the number of divisions that individual founder cells have to complete appears irrelevant, thereby precluding models that rely on a mechanism where the number of cell divisions in precursor cells is counted.

Experiments have been conducted both in the larval and pupal disc that alter cell size in parts of the disc (Neufeld et al., 1998; Weigmann et al., 1997). In either case, the overall physical dimensions of the disc remain appropriate – the tissue can be composed of either fewer larger cells or a larger number of smaller cells. Moreover, when individual cells and all of their progeny (clones) are marked in specific ways, it is clear that even though the overall size and shape of the disc are predictable, the size and shape of the individual clones is not. Hence, the size control mechanism must specify the physical dimensions of the structure without much regard to its cellular composition.

The cessation of growth is contingent upon normal disc architecture

It is often incorrectly assumed that the phenomenon of organs or organisms stopping their growth at a fixed final size, as is observed in *Drosophila* imaginal discs, is universal in the animal kingdom. In diverse taxa (e.g. lobsters, most fishes), there is a pattern of growth (referred to as indeterminate growth [Sebens, 1987]) that never ceases completely, although it usually slows as the organism ages. In multiple taxa, growth is indeterminate in more basal branches and is determinate in more derived branches, suggesting that indeterminate growth represents the ancestral condition (Hariharan et al., (in press)).

The shift, during evolution, from a pattern of indeterminate growth to one of determinate growth could have occurred by changes that manifest at the level of individual cells, which limit their capacity of proliferate. Transplantation experiments have shown that this is not the case with the cells of the imaginal disc. As discussed before, a fragmented disc implanted in the adult abdomen is capable of regeneration. Indeed, Hadorn and colleagues were able to put disc fragments through 300 rounds of serial fragmentation and regeneration over a period of 12 years, thus demonstrating that these cells were capable of indefinite proliferation (Hadorn, 1978). During normal development, proliferation ceases when disc cells collectively generate a structure of a pre-determined final size. This implies that the cessation of proliferation is not limited by the proliferative capacity of individual cells, but rather is a collective property of disc cells that must result from the way that they interact with each other within the context of the disc epithelium.

Genetic studies in *Drosophila* have also uncovered mutations that prevent a definitive arrest of growth. Mutations in any of the so-called “neoplastic tumor-suppressor genes” (nTSGs) result in continued proliferation of disc cells either in the larva or following transplantation into adult abdomens (reviewed in Hariharan and Bilder, 2006). What is commons to these mutations is that they all disrupt the normal architecture of the disc epithelium to a varying extent, further supporting the notion that cell-cell interactions are necessary for timely growth arrest.

Size regulation does not operate at the level of the entire disc

Before discussing the specific mechanisms that might allow the cells of the growing disc to generate a structure of a precise size, it is important to review evidence that size control may not operate at the level of the entire disc but rather at the level of major subdivisions. The wing disc is composed of lineage-restricted groups of cells – referred to as compartments – whose members do not intermingle during development (Garcia-Bellido et al., 1973). The first and major subdivision in the disc separates the cells into a larger anterior (A) compartment and a smaller posterior (P) compartment. The A cells and the P cells, which derive from separate populations of founder cells in the embryo, do not intermingle throughout disc development. In contrast to the wiggly boundaries of marked clones generated within one of the two compartments, clonal boundaries that abut a compartment boundary are relatively smooth. These cells may initially remain separate because of differences in their adhesive properties although, so far, the search for compartment-specific homophilic adhesion molecules has been unsuccessful. At later stages, there is evidence for an actomyosin cable that runs along the edge of cells that abut the compartment boundary, which is at greater tension than other cell boundaries (Landsberg et al., 2009). In the wing disc, a second lineage-restricted boundary develops at the end of the first larval instar that separates dorsal (D) cells from ventral (V) cells. This boundary coincides with the future margin of the adult wing. Still later, there are further subdivisions that are usually but not always restricted by lineage (Garcia-Bellido, 2009). For example, clones in the wing pouch are less likely to cross over into the hinge region and vice versa (Zirin and Mann, 2007). Clonal boundaries are also less likely to cross the rows of cells that are fated to become wing veins. It is possible that as compartment boundaries mature, they become more absolute with the A-P boundary being the earliest and hence most rigid compartment boundary.

Especially important to growth control is that the A and P compartment can tolerate growth rates that are considerably different (Martin and Morata, 2006) (Figure 2). For example, slowing the growth of the A compartment by restricting the effect of a *Minute* mutation to that compartment still allows the development of a wing of considerably normal shape and size. As the P compartment approaches its final size, it appears to slow its growth and even stop growing. The A compartment eventually catches up. This means that, at least in some ways, the wing disc can be thought of as two separate organs (albeit attached to one other), each with its own size control mechanism. It is possible, even likely, that size-regulation may also occur in a relatively autonomous way in further subdivisions that are generated when the A and P compartments are each divided into dorsal and ventral compartments. The subdivision of a developing organ into smaller and manageable sub-domains may allow for more precise control of its overall size.

A top-down view: regulation of disc size by morphogens

As discussed previously, size control mechanisms in the wing disc appear to operate at the level of its overall physical dimensions. How can individual cells compute organ size and adjust their proliferation accordingly? Morphogens are molecules that diffuse away from a source and specify cellular outputs, typically gene expression, in a concentration-dependent manner. If the gradient of morphogen concentration is predictable, then cells should be able to utilize some property of the morphogen gradient such as the local morphogen

concentration or the local slope of the gradient to assess their distance from the source. Most studies have focused on Dpp (reviewed by Restrepo et al., 2014), which is secreted by cells just anterior to the A-P compartment boundary, and Wingless (Wg) (reviewed by Swarup and Verheyen, 2012), which is made by cells near the D-V boundary (although the role of Wg as a secreted morphogen has recently come into question, Alexandre et al., 2014). There is now considerable evidence that the morphogen Dpp indeed has an important role in regulating the growth of the wing imaginal disc. Still to be clarified is whether Dpp has a clear instructive role in regulating final disc size or whether its role is more permissive, e.g. to sustain growth at levels that are more precisely specified by other mechanisms.

In the wing-imaginal disc, Dpp is expressed in a stripe of cells immediately anterior to the compartment boundary (Figure 3A). From here, Dpp spreads laterally in both directions and generates a gradient of Dpp signaling. Due to the absence of high-quality antibodies to Dpp itself, most studies of Dpp expression have visualized the spread of engineered Dpp proteins with GFP tags (Entchev et al., 2000; Teleman and Cohen, 2000). Under these conditions, Dpp is visualized as a gradient where levels appear to decrease in close to an exponential manner from the source to the lateral edges of the disc. The observed distribution could be the result of spread by free diffusion facilitated by extracellular heparan sulfate proteoglycans (Belenkaya et al., 2004; Zhou et al., 2012), receptor-mediated transcytosis (Kicheva et al., 2007), or even transport by thin cellular processes known as cytonemes (Hsiung et al., 2005; Ramirez-Weber and Kornberg, 1999). The relative importance of each of these mechanisms is still open to debate.

The evidence that Dpp promotes disc growth is unequivocal. Increasing Dpp signaling can cause wings of increased size with pattern duplications while mutations that reduce Dpp levels compromise wing disc growth (Burke and Basler, 1996; Capdevila and Guerrero, 1994; Martin-Castellanos and Edgar, 2002; Spencer et al., 1982; Zecca et al., 1995). Moreover, when the levels of Dpp and its spread are compared between the haltere disc (which generates a flight-stabilizing appendage that is smaller than the wing) and the wing disc, Dpp is made at higher levels and spreads further in the wing disc (Crickmore and Mann, 2006). This is consistent with the hypothesis that Dpp functions as a morphogen that directs tissue growth.

The simplest mechanism by which a gradient of Dpp could regulate organ size would be that cells require a critical concentration of Dpp to proliferate (Figure 3B). This concentration would be exceeded in medial portions of the disc and would be barely exceeded in lateral parts of the disc. According to this model, the disc would continue to grow until its most lateral cells receive a sub-threshold concentration of Dpp. One objection to this kind of model has been the observation that, at least in the third-instar disc there seems to be no obvious reduction in proliferation at the edges of the disc. Rather, proliferation across the disc appears uniform. However, a recent study has shown that in less-mature discs, the extent of cell proliferation in the central portion of the disc is indeed greater than in the lateral portions (Mao et al., 2013).

A second type of morphogen-based model is one where cells compare their current level of Dpp signaling with the level that was present during the previous cell cycle (Wartlick et al.,

2011) (Figure 3C). This model is based on the observation that the concentration of Dpp at the source increases over time and that the Dpp gradient scales with disc size. Based on measurements of GFP-Dpp levels, it has been suggested that cells divide each time their level of Dpp signaling increases by 50% or more when compared to the level at the preceding cell division. The appeal of this model is that it explains how a relatively even pattern of cell division can be generated across the disc. Cells are merely computing the relative rate of change in Dpp signaling and are indifferent to the absolute levels of Dpp. A molecular mechanism to mediate this kind of temporal comparison has not yet been discovered.

A third type of models posits that the extent of cell proliferation is determined by the local slope of the Dpp gradient (Day and Lawrence, 2000; Rogulja and Irvine, 2005). If the edges of the disc function as a morphogen sink, then mathematical models of gradient formation would predict that the slope would decrease as the distance between the source and sink increases. Cells might be able to detect a decrease in slope of the gradient by sensing the drop in Dpp concentration between the medial and lateral edges of the cell. A second possibility is that specific properties of each cell (e.g. expression of a cell-surface protein) are determined by the local Dpp concentration and thus the information contained in the Dpp gradient has been translated into a gradient of positional values. By interacting with their neighbors, cells could assess their differences (Figure 3D). When these differences in positional values between neighboring cells exceeds a threshold, additional cells are generated to intercalate between these neighbors and “smooth out” the differences. Growth would stop when these differences fall below a critical threshold. In this view, the growth that occurs during normal development is mechanistically similar to the growth elicited by juxtaposing tissue fragments with disparate positional identity in studies of regeneration conducted with *Drosophila* imaginal discs (Haynie and Bryant, 1976) or with cockroach limbs (Bohn, 1970). An important aspect of this type of model is that a transient Dpp gradient might suffice to set up a gradient of positional identities.

Experimental manipulations that generate large local differences in Dpp signaling promoted cell proliferation at the boundaries (Rogulja and Irvine, 2005). If local differences in Dpp signaling are generated, how can cells compare their signaling levels with those of their neighbors, and furthermore, how can these differences be translated into a pro-growth signal? The discovery of the Hippo pathway (reviewed by Halder and Johnson, 2011; Irvine, 2012) and the demonstration that its activity can be modulated by several cell-surface proteins that are capable of binding to ligands on adjacent cells provides the molecular machinery necessary for a growth response that is based on a comparison between neighbors. If morphogens such as Dpp regulate the levels of these ligands or their ability to bind to each other, then differences in signaling levels between adjacent cells would alter the relative occupancy of these interactions. Molecules such as these could therefore serve, at least in principle, as a way that cells compare themselves with their neighbors. Indeed, both for Ft/Ds signaling (Rogulja et al., 2008; Willecke et al., 2008) and for Crumbs (Chen et al., 2010; Hafezi et al., 2012), differences in expression levels between adjacent cells can generate “boundary effects”. Moreover, changes in Dpp signaling can influence the extent of Ft/Ds signaling (Rogulja et al., 2008). The three models discussed thus far in the “top-down”

category are not mutually exclusive. Indeed, one study found evidence that proliferation in medial portions of the disc are regulated by the slope of the Dpp gradient, whereas proliferation in the lateral portions of the disc depends more on the absolute levels of Dpp (Rogulja and Irvine, 2005).

A key experiment that has questioned the importance of graded Dpp expression in driving growth was one where graded Dpp signling was abolished by generating discs that lack the function of the Dpp receptor Tkv as well as the repressor Brinker (Schwank et al., 2008). In the disc, Dpp regulates target gene expression, in significant part, by alleviating Brinker-mediated repression. In the absence of the Dpp/Brinker system, discs growth is remarkably normal with the exception that overgrowth is observed in lateral regions of the disc. This experiment argues that the Dpp gradient per se is not necessary for disc growth. It does not, however, exclude the possibility that the Dpp gradient functions redundantly with other mechanisms. Building from this observation, the authors subsequently advocated a model where the protocadherin Fat, which activates the Hippo pathway, functions in medial regions of the disc to repress growth while Brinker represses growth in the lateral regions and that these two systems function in parallel (Schwank et al., 2011) to generate a disc of an appropriate size. In this scenario, the Hippo pathway does not function as part of the mechanism by which cells proliferate in response to local differences in Dpp signaling, but rather, functions in parallel.

Bottom-up mechanisms: the Entelechia model and a “feed-forward” model

Of the “bottom-up” models, the most elaborate is the Entelechia model advocated by Antonio Garcia-Bellido based on evidence from detailed studies of the patterns of cell proliferation in wild-type as well as genetically manipulated wing discs (Garcia-Bellido, 2009; Garcia-Bellido and Garcia-Bellido, 1998). A recurrent theme in his experiments was that clones of mutant cells have different proliferative properties when generated in different parts of the wing disc implying that their proliferative properties are determined by their interactions with neighboring cells. The essence of the Entelechia model is that local cellular interactions determine the extent of proliferation and that the final size of the disc is an emergent property of these interactions.

According to this model (Figure 4A), cells adjacent to boundaries (the A-P boundary being the first to be set up) express a high level of a “Martial (M) gene”, which encodes a nuclear protein, as a result of interactions with cells across the boundary. The level of M is proposed to keep increasing during development until it reaches a value that is specific for that species. Cells further away from the boundary express lower levels of M. The level of M determines the level of specific ligands on the cell surface. These ligands bind to receptors on adjacent cells and promote their proliferation by reducing M gene expression in those cells. Thus, if there is an initial disparity in M gene expression between two adjacent cells that exceeds a threshold (the “increment value”), this would result in the division of the cell with the lower value. The daughter cells generated by the division then upregulate their M values to approximate the average value of their neighbors (intercalation). This cascade of proliferation that is driven by increasing levels of M near the boundary continues until (1) M gene expression at the boundary has reached its maximal value and (2) the differences

between neighbors in terms of M gene expression have dropped below the increment value or threshold of detection. It is also proposed that this process occurs concurrently (using different signals) on the A-P and proximo-distal (P-D) axes. Additionally, as new boundaries are set up defining subdomains (e.g. veins and interveins), a similar process is repeated to regulate growth at higher resolution within those sub-domains. When these processes are complete, the disc has reached the Entelechia condition (perfection or completeness) and proliferation ceases.

One reason why the Entelechia model has received less attention than it deserves is that when it was proposed, many of its components were hypothetical entities whose properties could not be easily correlated with specific molecules. We now know of many molecules whose level and/or activity is graded throughout the disc, most notably the activity of the Fat protocadherin (Ma et al., 2003). It is possible that molecules that bind homophilically between adjacent cells including E-cadherin, Echinoid, and Crumbs could also be expressed in subtle gradients. The expression levels of these cell-surface proteins could, either individually or in combination, be a read out of a cell's positional identity. Homophilic binding (or heterophilic binding in the case of Ft and Ds) between such molecules on adjacent cells also provides a mechanism by which disparities in positional identity between adjacent cells can be computed. Moreover, signaling downstream of these molecules can regulate cell proliferation via the Hippo pathway. Given that actual molecules have been discovered that have the properties of some of the hypothetical ones originally postulated in the Entelechia model, we might yet witness a resurrection of a view of size control that is primarily driven by local cell-cell interactions.

The studies of Zecca and Struhl provide evidence for another size-regulation mechanism that is based on local cell-cell interactions (Figure 4B). Here, expansion of the wing pouch is not driven by intercalation, but rather by a feed-forward mechanism where cells are progressively recruited to a wing-pouch fate from a nucleating event at the dorsoventral boundary (Zecca and Struhl, 2007a, b, 2010). Cells at the D-V boundary express *vestigial* (*vg*). These cells then induce *vg* expression in adjacent cells resulting in waves of recruitment proceeding bidirectionally from a dorsoventral boundary. The inductive mechanism can be explained by Fat in the committed cells binding to Ds in the adjacent uncommitted cells and requires Wingless which is made by cells near the D-V boundary.

This mode of growth by accretion via inductive events at the periphery is highly reminiscent of the process by which cells are committed to a retinal fate in the eye-imaginal disc (reviewed by (Baker, 2007)). In that tissue, the wave of recruitment proceeds over time from the posterior to the anterior end of the disc. Cells anterior to the wave front represent a proliferating, uncommitted pool of precursor cells. Posterior to it, cells are progressively recruited to specific fates including those of photoreceptors and other types of accessory cells. In the eye disc, the wave of recruitment eventually encounters cells that are refractory to recruitment and hence stops moving. Similarly, cells at the peripheral regions of the wing disc, which give rise to the hinge, might have been similarly rendered insensitive to the recruitment process by patterning mechanisms. Thus, when the wave front stops moving, it might cause a proliferation arrest over the entire pouch by a hitherto undescribed mechanism. Zecca and Struhl proposed that the feed-forward mechanism at the edge of the

growing pouch is fueled by continuously increasing levels of diffusible Wg from the cells near the D-V boundary (Zecca and Struhl, 2010). This aspect of the model needs to be reconciled with the recent observation that a membrane-tethered (and hence non-diffusible) form of Wg can replace Wg function to a remarkable degree in allowing growth of the wing pouch (Alexandre et al., 2014).

A role for mechanical forces in size regulation

A recent and important development in the field has been the appreciation that mechanical forces generated by growing tissues can impact cell proliferation. As a result, there has been an attempt to incorporate the role of forces into prevailing models of disc growth regulation. Models that take mechanical forces into account have been especially useful for reconciling the graded expression of morphogens with the observation that the pattern of cell proliferation is relatively uniform throughout the disc (Aegerter-Wilmsen et al., 2007; Aegerter-Wilmsen et al., 2012; Hufnagel et al., 2007; Shraiman, 2005). This could happen if the compressive effect of growth in the central portion of the disc neutralized the additional proliferation that could be caused by increased morphogen levels. Conversely, a morphogen deficit at the peripheral edges could be compensated for by a proliferative stimulus provided by cell stretching (Figure 5A). These models also provide a way of explaining how a signal that shuts off cell proliferation could potentially function over the entire disc. Once cells at the periphery arrest their proliferation after morphogen levels fall below a critical threshold, strong constricting forces would propagate throughout the disc and cause a global arrest in cell proliferation. A more sophisticated recent version of one of these models that includes inputs from multiple signaling pathways predicts that disc proliferation stops when compression exceeds a critical level at the center of the disc and when the slope of compression from the center to the edge falls below a critical level (Figure 5C) (Aegerter-Wilmsen et al., 2012).

Experimental evidence is accumulating that cells at the periphery of the disc are being stretched and those at the center are being compressed. Measurements of recoil velocity following the scission of actomyosin cables along cell edges (Legoff et al., 2013; Mao et al., 2013) and using photoelasticity (Nienhaus et al., 2009) have shown that cells in the periphery are indeed at higher tension than those in the central portions of the disc. Additionally, in later larval stages, as discs approach the end their growth phase, there is a reduction in mechanical tension (Rauskolb et al., 2014). This is consistent with compressive forces being generated from the peripheral parts of the disc.

Excitingly, many recent observations point to links between mechanical forces and the Hippo pathway that are mediated by the actin cytoskeleton. First, the activity of the mammalian orthologs of Yki, YAP and TAZ, are regulated by cell shape (Aragona et al., 2013; Dupont et al., 2011; Wada et al., 2011). Cells that are stretched out have increased YAP/TAZ activity while those that have a more globular shape have decreased YAP/TAZ activity. In imaginal discs, some, but not all, manipulations that increased actin polymerization, which would be predicted to cause cell stretching, increased Yki activity and promted overgrowth especially in the proximal wing (Fernandez et al., 2011; Sansores-Garcia et al., 2011). Conversely, disruption of the actin cytoskeleton promotes the activation

of Wts by Merlin thus reducing Yki activity (Yin et al., 2013). More recently, a second pathway linking mechanical tension to Yki activity has been assembled (Rauskolb et al., 2014). Increased mechanical tension results in increased apical localization of the Jub protein, which recruits and inhibits Wts function thereby promoting Yki activity. By analogy with studies in mammalian cells (Yonemura et al., 2010), the recruitment of Jub could be mediated by a mechanical-force-dependent conformational change in α-catenin.

Two recent studies have also demonstrated that reducing the levels of different spectrin subunits can promote tissue overgrowth, at least in part, by activating Yki-mediated gene expression. This implies that a normal function of the spectrin cytoskeleton is to reduce growth by promoting the retention of Yki in the cytoplasm. The exact mechanism by which spectrin regulates Wts activity remains to be elucidated. One group proposed a mechanism based on effects on clustering of the transmembrane protein Crumbs (Fletcher et al., 2015) while the other invoked the actomyosin network as an intermediary with spectrin constraining the activity of kinases that phosphorylate the regulatory subunit of myosin (Deng et al., 2015). The spectrin-mediated pathway seems to function in parallel to the Jub-Wts pathway. Additional pathways linking force transduction to the Hippo pathway and other growth-promoting pathways will undoubtedly be discovered in the near future. Taken together, the studies discussed so far indicate that the Hippo pathway is capable of integrating signals from cell-surface receptors with those generated by mechanical forces.

While invoking mechanical forces offers good explanations for some observations such as the relatively even distribution of proliferating cells in the disc, there are other observations that are less easily explained. First, as discussed previously, when one compartment has a *Minute* mutation and the other is wild type, the two compartments grow in a seemingly autonomous manner at different rates and generate wings of normal size and shape (Martin and Morata, 2006). One would imagine that the compressive forces generated by the compartment that has nearly approached its terminal size would be transmitted across the compartment boundary to the slow-growing compartment (where cells would be more compliant) and result in the fast growing compartment being larger than usual. Second, while most modeling approaches make the simplifying assumption that the disc is relatively circular, the disc, in reality has a reproducibly irregular shape. Thus, even if globally acting mechanical forces are important for shutting off proliferation, those signals are likely enhanced or overridden by local signals so that proliferation can be arrested at different distances from the morphogen source in different parts of the disc. Third, the model shown in Figures 5A and B assumes that the disc is a flat monolayer. In fact, by the end of the third larval instar, the epithelium of the disc proper becomes buckled in a characteristic way. In contrast, the peripodial epithelium, the layer of squamous cells, appears to be stretched tightly over the disc proper (Figure 1A and B, Figure 5D). In this regard, is surprising that the role of the peripodial epithelium in size regulation has received so little attention. The folds in the disc proper could be easily explained by differential growth between the disc proper and the peripodial epithelium. Indeed such differential growth mechanisms have been used to explain the formation of the gyri in the brain (Tallinen et al., 2014) and the villi in the gut lumen (Shyer et al., 2013). It is therefore possible that a force-sensing mechanism in the peripodial epithelium could arrest its growth before growth ceases in the disc proper.

This might initially cause buckling in disc proper but might eventually arrest its growth by some kind of signaling between the two layers. The development, both of methodologies that will allow us to visualize forces in live discs, and of techniques for manipulating forces within discs, should help clarify the role of mechanical forces in arresting growth.

Approaching a unified explanation of how the wing disc achieves its final size

The wide variety of genetic tools available to *Drosophila* geneticists has facilitated a series of sophisticated experimental manipulations that have enabled the discovery of several types of mechanisms that operate during size regulation in the imaginal disc. Each type of experimental perturbation offers insights into specific mechanisms, and at first glance, some of these findings may seem to contradict each other. For example, on one hand, generating discontinuities in the levels of Dpp signaling can promote cell proliferation, possibly by generating discontinuities in positional identity. On the other hand, eliminating the Dpp gradient seems compatible with near-normal growth of the disc. These findings could be reconciled if, in reality, Dpp functions redundantly with other mechanisms to generate the subtle differences in positional identity that might drive proliferation during the normal growth of the disc. In such a situation, the elimination of the Dpp gradient could be compensated for by other mechanisms. However, generating drastic differences in Dpp signaling between adjacent populations of cells could override compensatory mechanisms and activate intercalary proliferation. Similarly, the “top-down” and “bottom-up” models are not mutually exclusive. Some of the “top-down” models rely on local mechanisms for cells to interpret morphogen gradients. Conversely, the two “bottom-up” models discussed rely on starting conditions that are set up by patterning mechanisms that likely rely on long-range signals. Indeed it is likely that both types of mechanisms operate concurrently and together comprise a robust system for size regulation, where the “top-down” mechanisms might place broad limits on growth while the “bottom-up” mechanisms might provide precision.

Experimental perturbations are often necessary to uncover mechanisms that might otherwise operate in subtle ways. However, each type of manipulation might, because of its design, emphasize the importance of one type of mechanism over another. The real challenge is to understand the part that each of these mechanisms plays in an unmanipulated disc under physiological conditions. To that end, developments in the generation of biosensors for each growth-regulating pathway and developments in live imaging will provide important insights in the near future (Heemskerk et al., 2014; Rebollo et al., 2014). We live in exciting times!

How general are the lessons learned from the study of imaginal discs likely to be? Size regulation in vertebrate organs will likely be much more complex since those organs are three-dimensional structures of greater complexity. However, even in vertebrate embryos, many organs derive from primorida that are invaginations of epithelia, and the initial size of these primordia might be determined by mechanisms that are similar to those operating in imaginal discs. It is also likely that different kinds of mechanisms (e.g. local versus global) might predominate in individual organs or even in different parts of the same organ. After all, in contrast to “intelligent design”, evolution has a tendency to build systems that are often composed of an inelegant patchwork of solutions.

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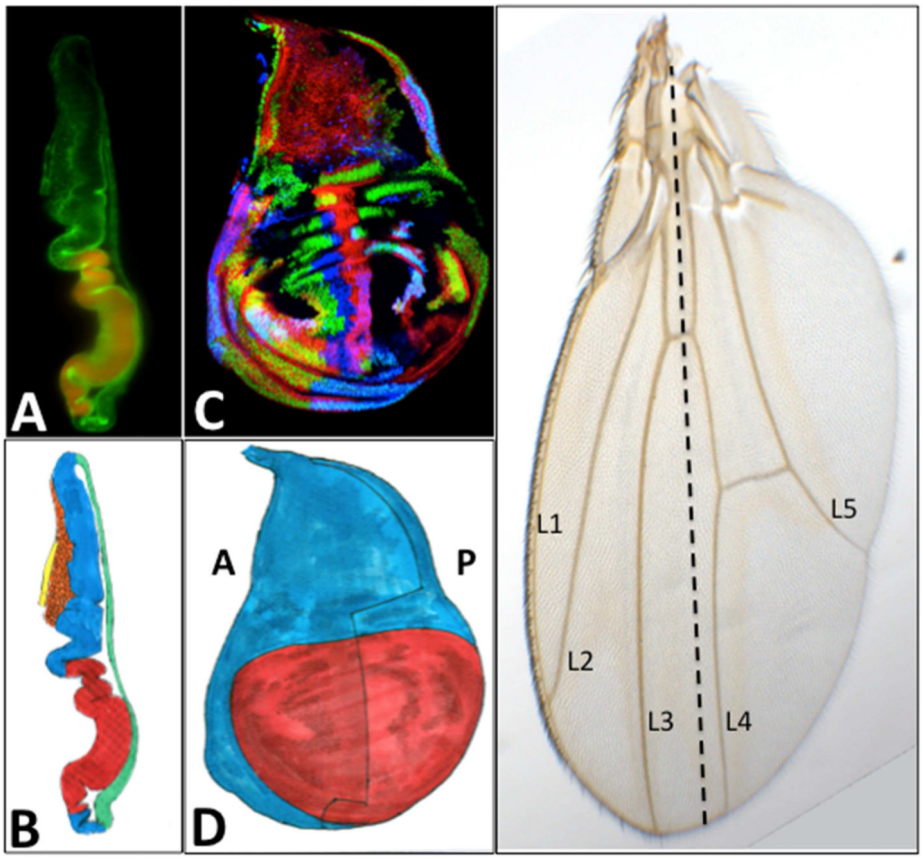

Figure 1. The wing-imaginal disc from late third instar larvae

(A) An image of a disc mounted in agarose and captured using a light sheet microscope. E-cadherin is shown in green. The wing-pouch, the primordium of the wing blade, is red (*nb>GAL4, UAS-GFP*). **(B)** An artistic representation of the same disc. The different parts of the disc are shown: wing pouch and adjacent folds (red), remainder of the disc proper (blue), peripodial epithelium (green), myoblasts (orange), tracheal branch (yellow). **(C)** Clonal populations in the disc are shown using the TIE-DYE system (Worley et al., 2013). **(D)** The same disc with the wing pouch shown in red and the remainder of the disc (notum and hinge) in blue. The black zig-zag line running through the disc is the A-P compartment boundary. A and P refer to the anterior and posterior compartments, respectively. **(E)** The adult wing. The longitudinal veins are indicated (L1-L5). The dashed line represents the approximate position of the A-P compartment boundary.

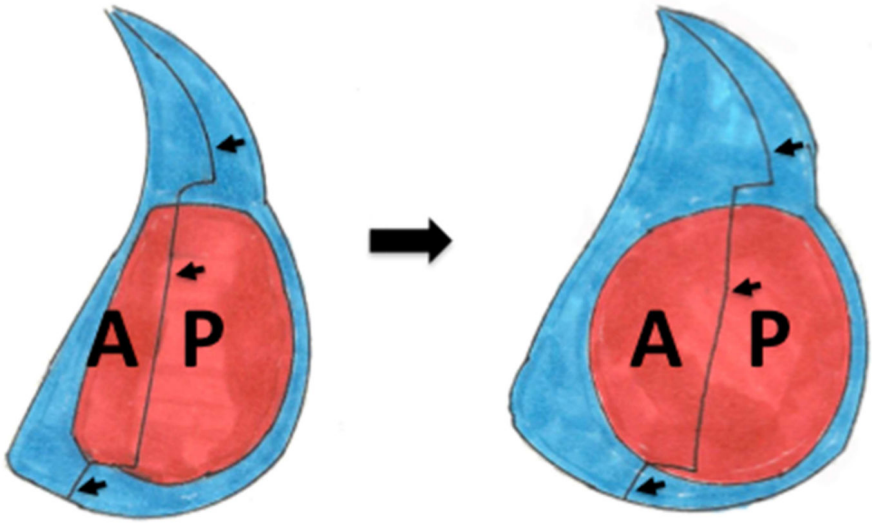

Figure 2. Different growth rates in the two compartments

The fast-growing posterior (P) compartment slows its growth when it approaches its final size and the slow-growing anterior compartment (A) eventually catches up. Arrows point to the compartment boundary.

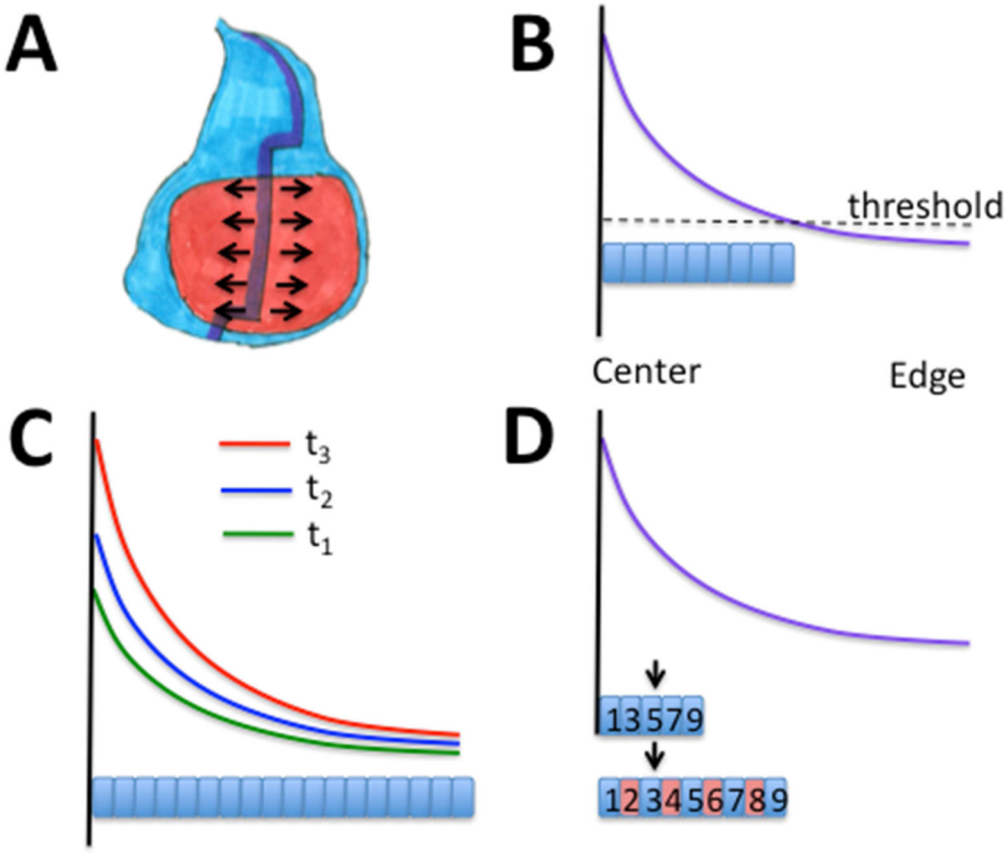

Figure 3. "Top-down" models of growth

(A) The morphogen Dpp is expressed just anterior to the A-P compartment boundary (violet) and diffuses from there in both directions. **(B-D)** Dpp concentration (Y-axis) is shown as a function of distance from the compartment boundary **(B)** A model where cell proliferation stops when the cells at the edge of the disc are exposed to levels of Dpp that are below a threshold. **(C)** A model where cells assess temporal changes in Dpp signaling. The amount of Dpp in the disc increases during development and hence, cells throughout the disc are exposed to increasing levels of Dpp. **(D)** A model where cells compare the levels of Dpp signaling with their neighbors and adopt "positional values" commensurate with the level of the signal, which are denoted by numbers. As long as the difference in positional values exceeds a threshold, cells are generated to adopt intermediate positional values. Once the difference drops below the threshold, proliferation stops.

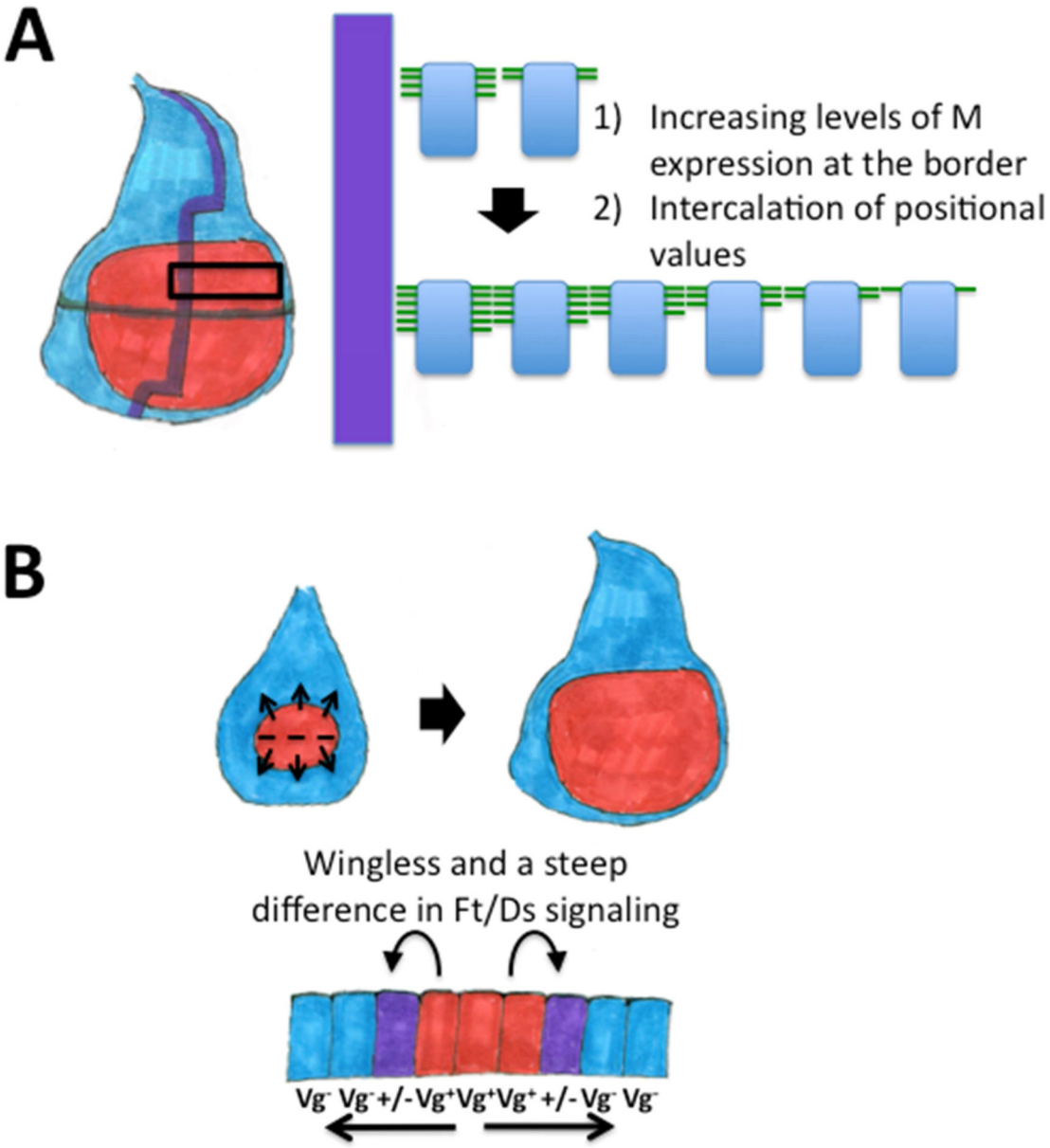

Figure 4. "Bottom-up" models of growth

(A) The Entelechia model. Cells adjacent to the boundary (purple) express higher levels of the Martial gene (M) and hence a higher level of a cell-surface protein, which reflects its positional value. A difference in the level of the cell-surface protein between adjacent cells promotes division of the cell with lower levels. The daughter cells adopt intermediate positional values. Proliferation stops when M reaches its maximal level and the differences in positional value fall below a threshold. **(B)** The feed-forward model. The pouch expands bidirectionally from the dorsoventral boundary (dashed line). Vestigial expressing cells (red) recruit adjacent cells (purple) to a fate where they also express Vestigial. This recruitment mechanism requires Wingless and is directed by Fat expression in the Vg-expressing cell and Ds expression in the cell awaiting recruitment.

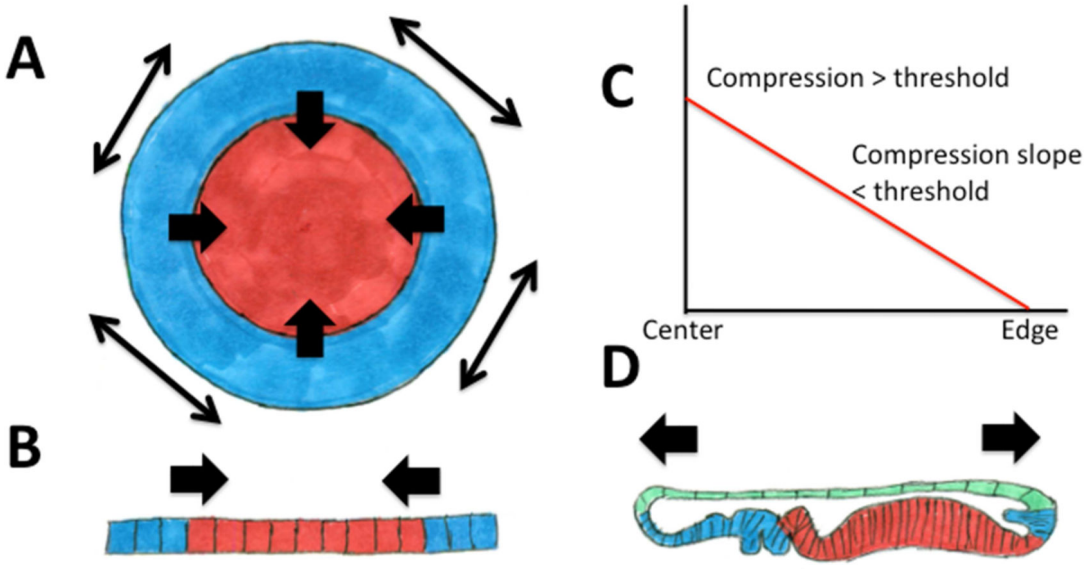

Figure 5. Mechanical forces in growth

(A-C) The model of growth regulation by mechanical forces described in Aegerter-Wilmsen et al. (2007) and Aegerter-Wilmsen et al (2012). **(A, B)** Stretching at the periphery of the disc promotes cell proliferation while compression inhibits proliferation. **(C)** Proliferation ceases when compression at the center exceeds a threshold and when the compression slope across the disc drops below a threshold. **(D)** A comparison of the morphology of the epithelium of the disc proper (buckled) and the peripodial epithelium (stretched) is suggestive of differential growth between the two epithelial sheets.