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Forces Controlling Organ Growth and Size

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Abstract

One of the fundamental questions in developmental biology is what determines the final size and shape of an organ. Recent research strongly emphasizes that besides cell-cell communication, biophysical principals govern organ development. The architecture and mechanics of a tissue guide cellular processes such as movement, growth or differentiation. Furthermore, mechanical cues do not only regulate processes at a cellular level but also provide constant feedback about size and shape on a tissue scale. Here we review several models and experimental systems which are contributing to our understanding of the roles mechanical forces play during organ development. One of the best understood processes is how the remodeling of bones is driven by mechanical load. Culture systems of single cells and of cellular monolayers provide further insights into the growth promoting capacity of mechanical cues. We focus on the *Drosophila* wing imaginal disc, a well-established model system for growth regulation. We discuss theoretical models that invoke mechanical feedback loops for growth regulation and experimental studies providing empirical support. Future progress in this exciting field will require the development of new tools to precisely measure and modify forces in living tissue systems.

1. Introduction

Precise regulation of organ growth is fundamental for life. For example, it would be hard for us to walk if our legs were not roughly of the same length. Similarly, an insect would have problems flying if its wings were not scaled to body size. What are the mechanisms that ensure that organs and extremities acquire the right size and shape during development? Biologists in the early 20th century tackled this question with grafting experiments in salamanders. The zoologists Twitty and Schwind removed prospective leg anlagen from a big salamander species, and grafted them to embryos of a smaller salamander species. Interestingly, the grafted limbs grew to the large size they would have reached in their bodies of origin, while the host's other limbs remained small (Twitty and Schwind, 1931). Similar experiments were performed in mice with fetal thymus glands yielding similar results – the organs grew to their original size, even in a different environment (Metcalf, 1963). These experiments reveal that organs contain intrinsic information about their destined size. But what role do extrinsic factors, such as nutrition and hormones, play? In the above mentioned studies the feeding plan during the experiments also influenced the speed and extent of growth of the grafted organ. Thus, the interplay of intrinsic and extrinsic factors defines the final size of an organ.

Cell growth, death, proliferation and cell polarity constitute an organ's toolkit to grow and sculpt its shape. Growth is generally defined as the increase in mass. In an organ, the most common cause for this is cell proliferation, which results in an increased cell number. In addition to proliferation, tissue growth can also occur without cell division and an increase in cell size alone can significantly contribute to overall growth (Conlon and Raff, 1999). Although proliferation and cell growth are often used interchangeably, they are two separate processes as shown in the *Drosophila* wing

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imaginal disc (Neufeld et al., 1998; Weigmann et al., 1997). If proliferation is experimentally enhanced or blocked in the disc, the tissue still reaches its appropriate size. Hence, the tissue compensates for the reduced or increased cell number with increased or decreased cell growth, respectively. This indicates that the mechanism controlling organ size regulates the *overall* size rather than cell growth or proliferation alone. Further, it is not only the rate of growth but also its orientation that shapes an organ. Studies on the *Drosophila* wing have revealed a clear causal relationship between the orientation of cell divisions in the larval wing epithelium and the morphology of the adult wing (Baena-Lopez et al., 2005; Aigouy et al., 2010). In addition to growth, apoptosis also plays a role in shaping the final size of an organ. Apoptosis helps shaping organs e.g. by separating digits in the mammalian limb (Raff, 1998) or by reducing the amount of neurons in the developing brain (Roth and D'Sa, 2001).

In recent years, a vast amount of molecular factors has been described which drive and control cell growth, death, proliferation and cell polarity. However, in order to understand the regulation of the final organ size we need to understand how growth is stopped at the appropriate time-point. As mentioned above, grafting experiments revealed that final organ size seems to be an inherent property. This requires an organ to continuously monitor its dimensions to determine when the final size is reached.

In the prevailing hypotheses the mechanisms for size regulation and pattern formation are interconnected. Signaling proteins are secreted, form a gradient and act in a concentration dependent manner to provide positional information. Such morphogens are responsible for patterning organs and are able to restrict or promote growth (Day and Lawrence, 2000). This has been best studied in the *Drosophila* wing disc. Two prominent morphogens - Wingless (Wg) and Decapentaplegic (Dpp) - are important patterning factors in this system (Zecca et al., 1995; Zecca et al., 1996). Overexpression of either morphogen also leads to overgrowth, whereas discs lacking Dpp or Wg are significantly smaller (Day and Lawrence, 2000; Wartlick et al., 2011). These observations indicated the interplay between patterning and growth regulation. Nonetheless, despite being an attractive system for intrinsic size regulation, morphogenetic growth models alone fail to explain numerous experimental observations in the wing disc (Schwank and Basler, 2010; Restrepo et al., 2014).

It has become widely accepted that in addition to biochemical signals, mechanical cues have an impact on growth regulation (reviewed by LeGoff and Lecuit, 2015). Therefore, tissue mechanics was integrated into various growth models to explain observations which the instructive role of biochemical growth factors alone could not account for (Aegerter-Wilmsen et al., 2007; Hufnagel et al., 2007; Egginton, 2011; Uyttewaal et al., 2012).

In this review we will discuss mechanical forces that regulate growth and review experimental approaches to investigate them. Although there is a vast amount of literature about mechanics in plant systems, we will focus exclusively on animal model systems here. In the first part we will present evidence for the growth regulating roles of mechanical forces in different experimental systems. In the second part we will focus on the *Drosophila* wing disc, starting with mechanical feedback growth models and then describe technical means to measure and alter mechanical forces.

2. Mechanical Forces in Growth

The idea that the mechanical environment influences size and shape of a tissue is not new. Biologists recognized the importance of physical forces for establishing a functional organ a long time ago.

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D'Arcy Thompson's "On Growth and Form" is a popular book from the early twentieth century discussing biophysical principles during animal development (Thompson, 1917). Later on, the rapid technical progress in molecular biology directed the focus of developmental biologists onto genetically encoded information rather than physical one. Additionally, the lack of tools to measure and manipulate mechanical properties in a living tissue rendered research on the physical principles in development challenging. In the 1970s the discovery of focal adhesion provided a mechanism by which a cell can sense its mechanical microenvironment (Izzard and Lochner, 1976). This, together with findings about the actomyosin machinery attached to these adhesion sites, stimulated work on mechanotransduction between a cell and its surrounding (Heath and Dunn, 1978).

Subsequently the implementation of new techniques allowed the investigation of the growth promoting effects of mechanical forces. Early evidence came from cancer cells that were grown in increasing agarose concentrations that increased the compressive stress onto the spheroid tumors (Helmlinger et al., 1997; Cheng et al., 2009). These experiments revealed that increased mechanical stress inhibited growth: compression of the spheroid suppressed proliferation and induced apoptosis. Another elegant study with endothelial cells, seeded on varying substrate rigidities, indicated that individual cell growth was influenced by the geometry of the cell, rather than the direct contact with the substrate (Chen et al., 1997).

These studies illustrate that cellular growth is dependent on its mechanical environment. In the following chapter we will briefly describe the mechanical architecture of an epithelial cell and its contact points to the physical surrounding.

2.1. Mechanics of an epithelial cell

2.1.1. Epithelial architecture

The majority of tissues in metazoans are organized as epithelia. Epithelial cells are defined by their polarity along an apical-basal direction (Fig. 1A): the apical side is exposed to the outside or luminal space whereas the basal side is attached to a substrate (Tepass, 2012). The adhesion between neighboring cells happens mostly at the apical side, with the adherens junctions playing a major role. E-cadherin is the most prominent adhesion protein and governs the connection between a cell and its neighbors as well as to its cytoskeleton. Nectins (Takai et al., 2008) and desmosomes (Green and Simpson, 2007) are additional complexes that allow adjacent cells to adhere to each other. Together, these adhesion complexes ensure tissue integrity (Tepass, 2012). Moreover, due to its association with the cytoskeleton, E-cadherin is believed to transduce mechanical signals between cells. At the basal side the focal adhesion complex (FA) has an analogous function: integrins - at the core of the FA - form strong attachments to the ECM and anchor to the cytoskeleton at the other end. Thus, integrins connect the cytoskeleton to the mechanical environment at cell-ECM adhesion points in a similar fashion as E-cadherin does at the cell-cell junctions (Geiger et al., 2009).

The shape of an epithelial cell is governed by internally and externally generated forces. In order to minimize mechanical damage to a cell and to ensure tissue integrity, internal and external forces must be balanced. Therefore, the actomyosin cytoskeleton constantly adjusts to the internal hydrostatic pressure and to mechanical stresses from outside the cell. Two cytoskeletal structures share this responsibility: actin cortex and stress fibers (SF). Lying under the apical cell membrane and more rarely at the basal side, the actin cortex comprises a mesh of actin filaments, myosin motors and actin binding proteins (Salbreux et al., 2012). The actin cortex is considered to be the main determinant of

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cell surface stiffness and to associate with the adherens junctions; cortical actin senses external forces and responds by remodeling. Accordingly, SF form a highly dynamic actin network when forces are required. SF can sense and respond to the external environment via attachment points at the FA. In contrast to cortical actin, SF span most of the cell and therefore can transmit forces over a longer distance range (Smith et al., 2014). Due to the high activity of actin crosslinkers and myosin motors, the turnover of the actin cortex and SF happens within seconds, enabling a fast response to an applied stress (Salbreux et al., 2012).

The activity of the actomyosin network, however, does not only passively resist against internal or external stress, but also actively shapes the cell. In concert with other cytoskeletal components, such as microfilaments and intermediate filaments, actin filaments stimulate cellular shape changes by altering apical, basal or lateral domains of the cell (Huber et al., 2015; Mao and Baum, 2015). The simultaneous activation of such transformations in a group of cells alters tissue architecture and contributes to morphogenetic development. A good example for this is apical constriction in which shortening of the apical cortex results in a wedge like shape of the cell. If coordinated tissue-wide, this can trigger the folding and invagination of a tissue such as in ventral furrow formation during *Drosophila* gastrulation or in *Caenorhabditis elegans* (Mao and Baum, 2015). Alternatively, actomyosin and/or microtubule activities modify the lateral dimension of a cell which causes flattening. If happening tissue-wide, epithelial flattening elongates a tissue as in the amnioserosa of *Drosophila* (Pope and Harris, 2008).

2.1.2. Types of mechanical stress and mechanotransduction

Here, we will briefly outline the types of external forces to which an epithelial cell is exposed and how the mechanical cues are transduced to generate a cellular response (Fig. 1B, C). An epithelial cell is constantly exposed to tensile stresses from adjacent cells. By definition, tensile stress leads to expansion or compression in the direction of the force. This can be caused by active shape and volume changes of the neighbors or through proliferation or apoptosis. According to current knowledge, mechanical cues from adjacent cells are transduced via E-cadherin to the actin filaments. The cytoskeleton directly senses the stress and causes a relevant response. It is further hypothesized that α-catenin, which links E-cadherin to actin, acts as mechanosensor and triggers a biochemical signaling cascade (Nowotarski and Peifer, 2014; Rooij, 2014). An epithelial cell is also sensitive to the rigidity of the ECM. Mechanical cues from the ECM are transferred via integrins to SF, which sense and respond directly to the stress by remodeling. Alternatively, the actin adapter protein Talin acts as a mechanosensor – when stretched it exposes buried binding sites for effector proteins (Austen et al., 2015). Finally, mechanical stresses can be exerted from outside the epithelium or the lumen. For example in endothelial tubes, cells are exerted to a shear stress which is caused by the frictional force of a flowing fluid. In contrast to tensile stress, shear stress is oriented longitudinally to the surface. Mechanotransduction of a shear stress is thought to be mediated via cilia bending, stretching sensitive ion channels or junctional proteins (Roman and Pekkan, 2012).

Figure 1. Schematic representation of an epithelial cell and its mechanical environment. (A) A polarized epithelial cell is connected via adherens junctions to its neighboring cells and via focal adhesion to the substrate. Desmosomal, septate and tight junctions are not shown because they do not appear in all epithelial cell types. (B, C) Epithelial cells are exposed to various types of mechanical stresses from intrinsic forces, neighboring cells and the extracellular space. (C) Cells respond to increasing substrate stiffness by reinforcing adhesion and active spreading on the substrate in an actomyosin- dependent process.

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2.2. Models for mechanical growth control

2.2.1. Bone remodeling

The function of bones is to give a framework for the body, to provide attachment sites for skeletal muscles and to protect inner organs. Bones are therefore exposed to varying mechanical loads.

Bones are constantly remodeled by the balanced activity of their constituent cell types, resulting in gain or loss of bone mass. The bone-forming osteoblasts and the bone-resorbing osteoclasts reside on top of the mineralized bone matrix beneath the periosteum (Fig. 2A). Osteoblasts produce the organic bone matrix and support mineralization, whereas osteoclasts dissolve bone matrix. The osteoblast-derived osteocytes are dispersed throughout the bone matrix and form dendritic networks between each other, osteoblasts and osteoclasts. Osteocytes stimulate osteoblast and osteoclasts to form or resorb the bone matrix (Bonewald and Johnson, 2008; Crockett et al., 2011).

Several observations indicate that bone remodeling strongly depends on the mechanical load: the playing arm of professional tennis players is enriched in bone mass whereas persons with long-term bed rest have reduced bone mass, as do astronauts (Armbrecht et al. 2011; Vico et al., 2000). *In vivo* studies in mice show an increased bone mass of the caudal vertebrae following increased loading (Christen et al., 2014). These studies reveal the strong response to mechanical loads on the level of the tissue. On the other side, *in vitro* studies on single osteocytes indicate a change in cellular behavior if exposed to mechanical stress (Crockett et al., 2011). It has been reported that osteocytes activate Nitric Oxide (NO) signaling and Wnt signaling upon the application of mechanical stress (Jacobs et al., 2010; Crockett et al., 2011). In the absence of osteocytes bone remodeling does not respond to mechanical loading (Tatsumi et al., 2007). Osteocytes are therefore the mechanosensors which mediate instructive signals for bone remodeling.

The current model of bone adaption to mechanical load is a multiscale process (Fig. 2A). Initially, a mechanical load onto the organ causes a tissue–level strain. But the transmitted tissue-level strains are too small to activate a cellular response (You et al., 2001). Thus, it has been proposed that mechanical loading induces a fluid flow in the extracellular fluid surrounding the osteocytes (Klein-Nulend et al., 2012). The fluid flow enhances the mechanical strain and thus serves as the mechanical stress which is sensed by the osteocytes. *In vitro* models indicate that shear stress of the fluid interacts with membrane-associated proteins which stimulate signaling pathways such as BMP, Wnts and NO (Jacobs et al., 2010; Klein-Nulend et al., 2012). The signaling factors from the osteocytes modulate the activity of osteoblasts and osteoclasts and promote their bone forming and resorbing potential, respectively (Crockett et al., 2011).

Apart from correlational studies, this multiscale process is very difficult to analyze *in vivo*, hence most evidence was derived from either *in vitro* studies or theoretical modelling. As a consequence the relationship between bone remodeling and its mechanical environment remains under debate. Concerns were raised whether osteocytes *in vivo* are stimulated by mechanical stress or whether regulation could be explained by an altered biochemical environment due to different flow dynamics in the bone (Jacobs et al., 2010). With the current methods it is difficult to separate cellular responses that are triggered by mechanical versus biochemical cues. Recent studies combined advanced high resolution computer tomography with computational methods to calculate local strain distributions and microstructural changes: a strong correlation was observed between bone remodeling and local strain patterns in murine vertebrae and human tibiae *in vivo* (Schulte et al., 2013; Christen et al.,

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2014). This supports the hypothesis of a multi-scale process: Organ-scale stresses provoke a change in the local microenvironment which in turn drives the adaption of specific micro-structures. These mechanisms allow bones to resist to external forces and to adjust to these forces by remodeling of the organ.

Figure 2. Model systems for mechanical growth control. (A) Mechanical load is driving bone remodeling in a multi-scale process: Mechanical load on the bone leads to fluid flow surrounding the osteocytes. Subsequently, shear-stress induced activation of osteocytes stimulates osteoblasts or osteoclasts to form and resorb the bone matrix, respectively. (B) Endothelial cells switch from proliferation to apoptosis when cell size is decreased by using micropatterned islands of adhesive substrate (Chen, 1997). (C) Proliferation patterns of MDCK cells analyzed with Fucci cell cycle marker. Cells constrained with a PDMS barrier do not proliferate, but progress in cell cycle when the barrier is removed. Similarly, when cell area is increased by stretching the substrate, cells continue in the cell cycle (Streichan et al., 2014).

2.2.2. Epithelial culture systems

Systems such as the culture of epithelial monolayers allow controlled mechanical manipulation to be performed to investigate the behavior of multicellular tissues in response to force patterns. It has been shown for endothelial cells that the mechanical properties of the substrate strongly govern cell shape which in turn influences growth and viability (Fig. 2B; Chen, 1997). Endothelial cells were seeded on micropatterned substrates coated with extra-cellular matrix (ECM) components. By changing the spacing between the substrate islands it was possible to alter either the cell spreading or the cell-ECM contact zone (Fig.2B). The results indicated that individual cell growth was governed by the cell geometry rather than the area providing contact with the substrate. Epithelial cells also respond to substrate rigidity by changing their geometry: they spread more on stiff than on soft substrates (Pelham et al., 1997). But how do cells behave if they are not separated from each other but in contact with neighboring cells? In an interesting study, Nelson et al. grew cell sheets on micropatterned substrates to control their spatial organization (Nelson et al., 2005). These authors explored how growth generates a global mechanical stress pattern in the tissue and how this feeds back to form asymmetric patterns of proliferation. When seeding cell sheets on microfabricated islands of different forms, they observed that the proliferative patterns changed according to the size and shape of the islands. Furthermore, computational modelling of the mechanical stress patterns within the tissue revealed a high correlation between mechanical stress and proliferation, suggesting that it is mechanical stress that is driving proliferation. Experimental depletion of junctional components showed that intercellular junctions are required for, and hence likely mediate, force-dependent proliferation.

Similar feedback loops between tissue mechanics and proliferation patterns were observed in studies with Madin-Darby canine kidney (MDCK) cells. The term “contact inhibition” describes the drastic decrease of motility and proliferation rate in a confluent epithelial monolayer (Fig. 2C) (Martz and Steinberg, 1972). Contact inhibition depends on cell-cell contact. Puliafito et al. performed a quantitative characterization of contact inhibition in MDCK cell culture by long-term tracking of single cells and monitoring tissue behavior (Puliafito et al., 2012). An outward growing colony reaches the point where cells at the periphery cannot expand fast enough to accommodate for the proliferation in the bulk. Hence, cell density in the bulk increases as a consequence of mechanical constraints. This marks the onset of contact inhibition and mitotic activity sharply decreases. In this

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transition phase, cell area is reduced by cell division and converges to the critical point where proliferation is arrested. Puliafito et al. concluded that cell contacts are necessary, but not sufficient for mitotic inhibition. Rather, proliferation is arrested as a consequence of the reduced cell area, which is imposed by mechanical constraints on tissue expansion. In another study, the correlation between mechanical constraints, cell area and proliferation rate was examined by experimental perturbation of the mechanical constraints (Fig.2C; Streichan et al., 2014). Restricting the overall area of the MDCK epithelial layer or actively stretching and compressing the tissue confirmed the conclusions of Puliafito et al. Furthermore, Streichan and colleagues proposed that mechanical constraint inhibits proliferation by regulating cell cycle entry at the checkpoint from G1 to S Phase.

In sum, *in vitro* proliferation is regulated by the mechanical constraints stemming from tissue dynamics. Similar mechanisms could also control tissue growth during organ development.

2.3. Drosophila wing imaginal disc

2.3.1. Introduction

The *Drosophila* wing imaginal disc is currently one of the best studied model systems for which mechanical signaling is integrated into growth models; we will therefore focus on this system. The wing disc is the larval progenitor organ that develops into the wing of an adult fly (Fig. 3A). Development starts out with an anlage of around 30 cells and reaches roughly 50,000 cells at the onset of metamorphosis (Milan et al., 1996). The wing disc is an epithelial monolayer which forms a sac-like structure. The columnar cells on one side of the sac are the focus in most studies. At the center of the disc is the wing pouch which gives rise to the adult wing blade. Due to its relatively flat geometry and the well characterized set of morphogens and growth factors involved in its patterning, most of the growth studies have concentrated on the wing pouch.

Evidence for an integrative role of mechanical interactions in growth regulation first came from computational modelling (see 2.3.2.); these were then complemented by experimental data (see 2.3.3. and 2.3.4.). Below we first briefly describe the computational growth models that invoke mechanical forces and then discuss the options available to experimentally measure and modify mechanical forces within the wing disc.

Figure 3. *Drosophila* wing imaginal disc. (A) Top and lateral view of the wing imaginal disc at third instar. The wing pouch will develop into the wing blade of the adult fly. (B) The concentration of morphogens is highest in the center of the wing pouch. Growth models suggest that the morphogen distribution promotes growth in the center, resulting in a growth gradient at early stages (Aegerter-Wilmsen et al., 2007; Hufnagel et al., 2007). This gradient changes the global tensions in the wing pouch which in turn stimulates proliferation at the periphery and suppresses proliferation at the center. This feedback loop leads to homogenous growth throughout the wing pouch at later stages as well as controlling size.

2.3.2. Modelling forces

It has been suggested that patterning and growth are coupled. In the wing disc the morphogens Dpp and Wg are supposed to play key roles in patterning and also in promoting growth (e.g. Day and Lawrence, 2000). But the role of Dpp and Wg in growth regulation remains controversial. Recent work pointed out that Dpp and Wg are only partially essential for growth and are not directly required

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to set the final size of the wing disc (Akiyama and Gibson, 2015, Alexandre et al., 2014, Harmansa et al., 2015). In the wing pouch Dpp and Wg form gradients perpendicular to each other with the highest concentrations in the center. The observation that proliferation is homogenous throughout the wing pouch lead to a conundrum: How can the graded morphogen distribution result in a uniform proliferation pattern (Milan et al., 1996)? This paradox inspired scientists in the field to think about alternative models for growth control - considering mechanical signals as an additional carrier of instructive information for cells.

B. Shraiman initially postulated that mechanical interactions allow cells to compare their growth rate and trigger an appropriate cellular response (Shraiman, 2005). In a tissue with non-uniform growth certain patches of cells grow faster than the surrounding cells. Due to tissue rigidity and spatial restrictions, the faster growing cells get compressed whereas the surrounding tissue is stretched. Under the assumption that mechanical compression negatively regulates growth, the growth rate of the faster growing cells would eventually slow down until it is similar to that of the surrounding tissue. A prediction of this mechanical feedback mechanism is that in a healthy tissue different growth rates will converge and result in uniform growth. This mechanism would also prevent the local accumulation of mechanical stress and ensure tissue integrity.

This initial assumption was further integrated into two similar growth models which proposed a feedback loop between mechanical forces and morphogen induced proliferation in the wing disc (Fig. 3B; Aegerter-Wilmsen et al., 2007; Hufnagel et al., 2007). These models both offered a solution to the paradox of uniform growth driven by graded morphogens as well as providing a mechanism for final size determination. Hufnagel et al. suggested that cells proliferate above a certain threshold of Dpp concentration (Hufnagel et al., 2007). Although contradicted by more recent work (Hamaratoglu et al., 2011, Wartlick et al., 2011), they experimentally showed that the Dpp gradient is fixed over time and does not scale to disc size. According to the model, proliferation is arrested when Dpp levels fall below the threshold in the marginal cells resulting in increased compression in the center of the disc. This compressional stress feeds back on the proliferation rate and reduces growth. The model of Aegerter-Wilmsen et al., suggested that the high abundance of Dpp and Wg in the center of the disc initially promotes growth (Aegerter-Wilmsen et al., 2007). As the center grows, the surrounding cells are stretched, stimulating growth. Simultaneously, compression builds up in the center which leads to a competition between the growth promoting effects of morphogens and the inhibitory effects of compression. Growth stops when the stimulating effects of morphogens can no longer overcome the inhibitory effects of compression; the disc has then reached its final size. According to both models, mechanical feedback can explain the homogenous proliferation pattern as well as acting as a determinant of the final size of the wing disc. Aegerter- Wilmsen and colleagues extended their model to integrate molecular signaling pathways into the mechanical growth regulation (Aegerter-Wilmsen et al., 2012). The extended model includes tested, and also hypothetical, interactions between the factors which are known to be involved in wing disc growth. The network incorporates the morphogens Dpp and Wg, growth factors like Yorkie and Vestigial, and polarity factors such as Four-jointed and Dachsous. The model was able to make predictions of cell size and shape which were subsequently confirmed by experimental data.

Initially, the mechanical feedback model was hypothetical as it was not based on empirical evidence in the wing disc, but rather extrapolated from different studies in other tissues (see 2.2.2). Consequently, parameters were not derived from underlying experimental data but were fitted manually. In the next two chapters we will highlight experimental approaches that are being used to directly assess the role of mechanical forces in wing disc growth.

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2.3.3. Measuring forces

The underlying premise of the mechanical feedback models is that mechanical tensions are distributed heterogeneously over the wing disc. Initially, cell area was used as a read-out for mechanical stress. As predicted, a gradient of cell area can indeed be observed in the wing disc, with smaller cells in the center and larger, tangentially elongated cells at the periphery (Aegerter-Wilmsen et al., 2012). However, cell area can also be affected by other mechanisms; therefore it is a somewhat unspecific measure for mechanical stress.

A purely visual and non-invasive method is based on birefringence, which is an optical response of the tissue to stress anisotropies (Fig. 4A). Birefringence refers to the differences in refraction index of differently oriented material axes and can be measured by the retardance of polarized light transmitted through the tissue. Since forces can influence the material orientation, birefringence provides an indirect readout for mechanical stress (Nienhaus et al., 2009; Sugimura et al., 2016). Birefringence maps of the wing disc revealed that the retardance in the center is highest and decreases towards the edges, indicating a mechanical stress gradient with the maximal value in the center, which is predicted by the models (see 2.3.2., Nienhaus et al., 2009). However, the interpretation of these data is complicated by the fact that besides mechanical stress, birefringence also depends on the thickness and density of the sample or on tissue anisotropies not induced by mechanical forces. It is difficult to correct for these additional parameters in a biological tissue. Further, it has to be considered that the measurement takes the entire tissue into account, which comprises two cell layers and two ECMs in the case of the wing disc. Thus, birefringence measurements alone do not allow a differentiation between mechanical tensions in overlapping layers.

Force inference (FI), another non-invasive method, is a computational approach which infers mechanical tensions from the analysis of cell shape (Fig. 4C). Given that the tissue is at mechanical equilibrium, the cell shapes are determined by the balance of contact forces between cells and the internal pressure. Thus, deviations from regular cell geometry enable the estimation of cellular tension and pressure (Sugimura et al., 2016). From any image which represents an apical surface marker, FI infers a map for junctional tension as well as internal pressure. In the wing disc FI confirmed the presence of a global mechanical gradient with highest compression in the center (Chiou et al., 2012; Ishihara and Sugimura, 2012). The advantage of FI is that it gives an overview over global mechanical patterns resolved at the cellular edge level. However, the limiting factor of the technique is that it has to rely on several assumptions. First, a requirement for FI is that cellular forces are dominating at the apical side because cell geometries are obtained from the apical cell surface. This neglects the contribution of more basally located cell-ECM junctions to cell mechanics. Further, FI greatly depends on prior assumptions of mechanical equilibrium, force balance and homogenous mechanical properties. Video force microscopy (VFM) relaxes some of these assumptions by using temporal cell shape changes rather than static images (Brodland et al., 2014). Finally, FI only provides relative information about pressure and tension and does not give absolute values.

The findings of FI in the wing disc were supported by laser ablation (LA) experiments (Fig. 4B). In contrast to the above described methods, LA is an invasive measurement. For LA, a focused two-photon-laser ablates a cellular structure which is under tension and the reaction of the cell is recorded. In the wing disc LA has been used to disrupt the cortical actomyosin in order to measure the recoil velocity of the remaining cell edges (LeGoff et al., 2013; Mao et al., 2013). The recoil velocity provides a measure of the tension state of the cortical actomyosin. LA confirmed that cells in the center of the wing disc are compressed and cells at the periphery are stretched tangentially, in accordance with all previously observations.

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In addition to its invasiveness, LA has two limitations. First, when interpreting results it has to be considered that only the tension of the cortical actomyosin is measured; adhesion strength and hydrostatic pressure are neglected in this analysis. Second, the recoil velocity does not only depend on the cortical tension, but also on the material properties of the structure. More precisely, the measurement only provides the ratio of force/viscosity, which means that no absolute values of forces can be gained from LA (Campas, 2016).

FRET based sensors were used in different systems to measure mechanical forces between cells (Fig. 4D). In contrast to other methods, FRET sensors measure forces along specific proteins. The core of a FRET sensor is two fluorophores connected by an elastic linker. Analysis of the FRET efficiency reveals the distance between the fluorophores, which correlates with the tension on the sensor. Being genetically encoded, such sensors were integrated into several proteins which are known to be involved in mechanotransduction and used for *in vitro* studies: Vinculin, Talin and E-cadherin (Grashoff et al., 2010; Borghi et al., 2012; Austen et al., 2015). We tested a sensor for the wing disc to measure tensions across E-cadherin at adherens junctions, but could not reliably measure mechanical forces (manuscript in preparation). The general problems of this method are: (1) FRET efficiencies not only depend on the distance between the two fluorophores but also on their microenvironment and their conformation to each other. This complicates the interpretation of the results. (2) Technical limitations impede the ratiometric method - the most commonly used scheme for FRET analysis. Being an intensity-based method it works well in cell culture but includes measurement artifacts when applied in living tissues. We believe that currently FRET based sensors are not an optimal tool for force measurements in the wing disc.

Figure 4. Methods to measure forces in the wing disc. (A) Birefringence measurement: The polarization state of a linearly polarized beam of light is changed when passing through a birefringent material. This is described by a phase difference in the different states called retardance and can be due to stress anisotropies in the material. (B) Laser ablation: When the actomyosin cortex is cut with a focused laser beam, the remaining edges retract, indicating a positive tension at the cortex. The velocity of the displacement provides a measure for this tension. (C) Force inference: The cell geometries of the input image reflect the balance between internal pressure and apical tensions. Solving force balance equations returns maps of cell pressure and tensions at cell-cell junctions. (D) FRET tension sensor: Sensor module is composed of two fluorophores connected by an elastic linker. The module is genetically integrated to a protein of choice, here E-cadherin. E-cadherin mechanically connects adjacent cells, thus tension between neighboring cells is transduced via E-cadherin. This moves the two fluorophores apart and can be measured by FRET efficiency.

2.3.4. Modifying forces

Measurements of force distributions show a clear circumferential pattern of mechanical stress with compressed cells in the center and stretched cells in the periphery of the wing disc, supporting the mechanical feedback models. In order to show a causal relationship between mechanical cues and growth, however, methods to experimentally modify tensions over the wing disc are required. For this, improvements of *in vitro* culturing techniques are essential, as they allow for long term *ex vivo* studies of wing discs (Zartman et al., 2013).

The most direct approach to evaluate the relationship between forces and growth has been the mechanical stretching of the wing disc *in vitro* (Fig. 5A). For this, the disc was attached with polylysine onto two movable coverslips which are pulled apart with a defined force. Imaging the

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dynamics of mitotic cells during stretching, an increase in proliferation upon mechanical stretching was observed in the wing pouch (Schluck et al., 2013).
While suggesting a link between mechanical tension and proliferation, the time-window for this experiment was one hour - the minimum length of one cell cycle is 8 hours (Milan et al., 1996). Thus currently, a major drawback of this method of the relatively short experimental window provided by the *in vitro* culturing technique. An additional concern is that *in vitro* cultivation interferes with cell cycle progression (Handke et al., 2014). This also limits the potential of wing disc culture to investigate growth and proliferation. Further efforts have to be made to either improve the culturing conditions of the wing disc or to establish manipulation techniques *in vivo*. *In vivo* imaging approaches have already been developed, but the handling and manipulation of the wing disc *in situ* is hampered by the accessibility in the larvae (Nienhaus et al., 2012; Heemskerk et al., 2014).

The induction of clones of overproliferating cells is one approach to increase mechanical tensions in the wing disc (Fig. 5B). Manipulation of the Hippo pathway in a patch of cells stimulates these cells to overgrow and surrounding tissue is stretched (LeGoff et al., 2013; Mao et al., 2013). This induced a tension pattern resembling that of the entire wing pouch. Consequently, adjacent cells that were stretched oriented their division plane according to the force field (Mao et al., 2013). Interestingly, in an analogous experiment in the *Drosophila* pupal notum, cell clones were stimulated to grow by overexpression of the oncogene RasV12. But instead of stretching the adjacent cells, tissue crowding was observed around the clone. Tissue crowding compressed the neighboring cells and drove apoptosis and cell delamination (Levayer et al., 2016). Thus, the effect of overproliferating clones on tissue mechanics is unclear, as it remains unresolved whether this discrepancy is an outcome of different biological or analytical tools.

Mechanical tension in the wing disc has also been modified indirectly by targeting cytoskeletal components (Fig. 5C). Genetic perturbations or pharmacological drugs were used to alter the actomyosin cytoskeleton. Inducing extra actin formation by the loss of actin capping proteins stimulated overgrowth in the wing disc. Analogous to experiments in mammalian cells (Aragona et al., 2013) this overproliferation was mediated by Yorkie (Fernandez et al., 2011; Sansores-Garcia et al., 2011). The downregulation of myosin by targeting the myosin regulator Rho-associated protein kinase (ROCK), either via RNAi or the drug Y-27632, also reduced wing disc growth. Similar to actin dependent overgrowth, the growth effect was mediated by increased Yorkie activity (Rauskolb et al., 2014). Thus, there is a clear link between the actomyosin cytoskeleton, Yorkie activity and growth. This suggests that mechanical stretching of a cell is enhancing cytoskeletal assembly which in turn promotes proliferation (Rauskolb et al., 2014). However, this mechanism remains hypothetical. Actin and myosin are essential for a plethora of cellular processes. Therefore it remains to be assessed whether the growth promoting effect of actin and myosin activation is mechanically driven or initiated by another cellular process.

Figure 5. Methods to manipulate forces in the wing disc. (A) Stretching device: The wing disc is attached *in vitro* onto two flexible coverslips with poly-lysine. The coverslips can be moved apart with a defined force to stretch the wing disc tissue. (B) Overproliferating clone: Clonal manipulation of the Hippo pathway stimulates a patch of cells to overproliferate. Cells within the clone are compressed and the surrounding tissue is stretched tangentially. (C) Cytoskeletal modification: Pharmacological drugs applied *in vitro* or RNAi against actin or myosin regulators reduces tension of the actomyosin network, especially at the apical cortex. Overexpression of myosin regulators increases the tensional state.

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3. Concluding remarks

There is increasing evidence that in addition to biochemical signaling events, mechanical forces also impact on cellular processes in developing organs. *In vitro* experiments have shown that mechanical cues and tissue architecture have the potential to modulate cellular behavior and therefore to actively drive developmental events. Mechanical signals can coordinate cell movement, stimulate proliferation (Chen, 1997; Helmlinger et al., 1997), orient cell division (Campinho et al., 2013; Mao et al., 2013) and trigger differentiation (Guilak et al., 2009). Do mechanical cues also similarly affect the development of organs *in vivo*?

For the mammalian bone it was confirmed by long-term studies that size and shape are determined by mechanical loads (Christen et al., 2014). Mechanical forces feedback onto bone remodeling in a multi-scale process to ensure that the bone adapts to mechanical loads (see 2.2.1). Similarly, during remodeling of vertebrate blood vessels, blood pressure and flow shape the developing vessels in order to preserve their integrity (Hoefer et al., 2013). In the bone and the vascular system, mechanical stress originates from outside the tissue and is clearly of biological relevance. In other developmental systems, mechanical stress that is internally generated by tissue growth is also supposed to play a role as developmental regulator (see 2.3.2.; Campinho et al., 2013; Uyttewaal et al., 2012). Computational growth models, like those developed for the *Drosophila* wing disc, integrate mechanical feedback loops to modulate growth according to size (see 2.3.2). However, despite intense efforts, causal empirical evidence for the contribution of mechanical signaling *in vivo* remains elusive. For a deeper understanding of the complexity of mechanics *in vivo*, new tools are needed to quantify and modify mechanical cues (see 2.3.3. and 2.3.4.)

An epithelial cell is exposed to mechanical forces from different origins, which in turn lead to stresses on different subcellular structures. Additionally, the material properties of the tissue, such as viscosity or elasticity, also contribute to the mechanics of a cell (see 2.1.; Campas, 2016). Because different mechanical stresses could affect the cellular behavior in a different manner, it is important to consider that most experiments affect or measure only one of these mechanical quantities (see 2.3.2. and 2.3.3.). For example it was shown in mammalian cell culture and in the *Drosophila* wing disc that the homologs Yorkie/YAP are activated by increasing cytoskeletal tension, which would suggest a similar mechanism operates in the two systems (Aragona et al., 2013; Rauskolb et al., 2014). However, experiments in mammalian cells were performed by changing substrate stiffness, whereas in the wing disc the cytoskeleton was manipulated at cell-cell junctions. Substrate stiffness is sensed at the basal side while alterations of cell-cell junctions act at the apical side of a cell – which suggests two potentially different mechanisms exist.

Further, the time-scales of developmental processes are relevant to understand the interplay of mechanics and cellular behavior. Mechanical perturbations can change cellular structures within a few seconds (Le Duc et al., 2010, Salbreux et al., 2012, Tabdili et al., 2012) while transcriptional events or cell divisions take several hours. Fast cytoskeletal turnover could therefore relax and dissipate mechanical stresses on a short time scale. Then it would be unclear how mechanical information can be stored in the long-term to trigger a response over a longer period (Salbreux et al., 2012). However, it was shown that mechanical stress can remain and influence cellular behavior over long time scales (Schluck et al., 2013, Wyatt et al., 2012), possibly with stresses being stored in less viscous structures such as the ECM (Wyatt et al., 2016).

In conclusion, it has been shown that mechanical forces have the potential to regulate growth and size of tissues. But we are just beginning to understand the underlying mechanisms. In order to deepen our

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knowledge of mechanobiology in organ growth, techniques to precisely quantify and modify forces need to be further developed.

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References

Aegerter-Wilmsen, T., Aegerter, C.M., Hafen, E., Basler, K., 2007. Model for the regulation of size in the wing imaginal disc of Drosophila. Mechanisms of development 124, 318–326.

Aegerter-Wilmsen, T., Heimlicher, M.B., Smith, A.C., Reuille, P.B. de, Smith, R.S., Aegerter, C.M., Basler, K., 2012. Integrating force-sensing and signaling pathways in a model for the regulation of wing imaginal disc size. Development (Cambridge, England) 139, 3221–3231.

Aigouy, B., Farhadifar, R., Staple, D.B., Sagner, A., Roper, J.-C., Julicher, F., Eaton, S., 2010. Cell flow reorients the axis of planar polarity in the wing epithelium of Drosophila. Cell 142, 773–786.

Akiyama, T., Gibson, M.C., 2015. Decapentaplegic and growth control in the developing Drosophila wing. Nature 527, 375–378.

Alexandre, C., Baena-Lopez, A., Vincent, J.-P., 2014. Patterning and growth control by membrane-tethered Wingless. Nature 505, 180–185.

Aragona, M., Panciera, T., Manfrin, A., Giulitti, S., Michielin, F., Elvassore, N., Dupont, S., Piccolo, S., 2013. A mechanical checkpoint controls multicellular growth through YAP/TAZ regulation by actin-processing factors. Cell 154, 1047–1059.

Armbrecht, G., Belavy, DL., Backström, M., Beller, G., Alexandre, C., Rizzoli, R., Felsenberg, D., 2011. Trabecular and cortical bone density and architecture inwomen after 60 days of bed rest using high-resolution pQCT: WISE 2005. J. Bone Miner. Res. 26, 2399–2410

Austen, K., Ringer, P., Mehlich, A., Chrostek-Grashoff, A., Kluger, C., Klingner, C., Sabass, B., Zent, R., Rief, M., Grashoff, C., 2015. Extracellular rigidity sensing by talin isoform-specific mechanical linkages. Nature cell biology 17, 1597–1606.

Baena-Lopez, L.A., Baonza, A., Garcia-Bellido, A., 2005. The orientation of cell divisions determines the shape of Drosophila organs. Current biology : CB 15, 1640–1644.

Bonewald, L.F., Johnson, M.L., 2008. Osteocytes, mechanosensing and Wnt signaling. Bone 42, 606–615.

Borghi, N., Sorokina, M., Shcherbakova, O.G., Weis, W.I., Pruitt, B.L., Nelson, W.J., Dunn, A.R., 2012. E-cadherin is under constitutive actomyosin-generated tension that is increased at cell-cell contacts upon externally applied stretch. Proc. Natl. Acad. Sci. USA 109, 12568–12573.

Brodland, G.W., Veldhuis, J.H., Kim, S., Perrone, M., Mashburn, D., Hutson, M.S., 2014. CellFIT: a cellular force-inference toolkit using curvilinear cell boundaries. PloS one 9, e99116.

Buchmann, A., Alber, M., Zartman, J. J., 2014. Sizing it up: the mechanical feedback hypothesis of organ growth regulation. Seminars in cell & developmental biology 35, 73–81.

Campas, O., 2016. A toolbox to explore the mechanics of living embryonic tissues. Seminars in cell & developmental biology.

Campinho, P., Behrndt, M., Ranft, J., Risler, T., Minc, N., Heisenberg, C.-P., 2013. Tension-oriented cell divisions limit anisotropic tissue tension in epithelial spreading during zebrafish epiboly. Nature cell biology 15, 1405–1414.

Chen, C.S., 1997. Geometric Control of Cell Life and Death. Science 276, 1425–1428.

Cheng, G., Tse, J., Jain, R.K., Munn, L.L., 2009. Micro-environmental mechanical stress controls tumor spheroid size and morphology by suppressing proliferation and inducing apoptosis in cancer cells. PloS one 4, e4632.

Chiou, K.K., Hufnagel, L., Shraiman, B.I., 2012. Mechanical stress inference for two dimensional cell arrays. PLoS computational biology 8, e1002512.

Christen, P., Ito, K., Ellouz, R., Boutroy, S., Sornay-Rendu, E., Chapurlat, R.D., van Rietbergen, B., 2014. Bone remodelling in humans is load-driven but not lazy. Nature communications 5, 4855.

Conlon, I., Raff, M., 1999. Size Control in Animal Development. Cell 96, 235–244.

Crockett, J.C., Rogers, M.J., Coxon, F.P., Hocking, L.J., Helfrich, M.H., 2011. Bone remodelling at a glance. Journal of cell science 124, 991–998.

Day, S.J., Lawrence, P.A., 2000. Measuring dimensions: the regulation of size and shape. Development (Cambridge, England) 127, 2977–2987.

Egginton, S., 2011. In vivo shear stress response. Biochemical Society transactions, 39, 1633–1638.

ACCEPTED MANUSCRIPT

Fernandez, B.G., Gaspar, P., Bras-Pereira, C., Jezowska, B., Rebelo, S.R., Janody, F., 2011. Actin-Capping Protein and the Hippo pathway regulate F-actin and tissue growth in Drosophila. Development (Cambridge, England) 138, 2337–2346.

Geiger, B., Spatz, J.P., Bershadsky, A.D., 2009. Environmental sensing through focal adhesions. Nature reviews. Molecular cell biology 10, 21–33.

Grashoff, C., Hoffman, B.D., Brenner, M.D., Zhou, R., Parsons, M., Yang, M.T., McLean, M.A., Sligar, S.G., Chen, C.S., Ha, T., Schwartz, M.A., 2010. Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. Nature 466, 263–266.

Green, K.J., Simpson, C.L., 2007. Desmosomes: new perspectives on a classic. The Journal of investigative dermatology 127, 2499–2515.

Guilak, F., Cohen, D.M., Estes, B.T., Gimble, J.M., Liedtke, W., Chen, C.S., 2009. Control of stem cell fate by physical interactions with the extracellular matrix. Cell stem cell 5, 17–26.

Hamaratoglu, F., Lachapelle, A.M. de, Pyrowolakis, G., Bergmann, S., Affolter, M., 2011. Dpp signaling activity requires Pentagone to scale with tissue size in the growing Drosophila wing imaginal disc. PLoS biology.

Handke, B., Szabad, J., Lidsky, P.V., Hafen, E., Lehner, C.F., 2014. Towards long term cultivation of Drosophila wing imaginal discs in vitro. PloS one 9, e107333.

Harmansa, S., Hamaratoglu, F., Affolter, M., Caussinus, E., 2015. Dpp spreading is required for medial but not for lateral wing disc growth. Nature 527, 317–322.

Harris, A.R., Peter, L., Bellis, J., Baum, B., Kabla, A.J., Charras, G.T., 2012. Characterizing the mechanics of cultured cell monolayers. Proceedings of the National Academy of Sciences of the United States of America 109, 16449–16454.

Heath, J.P., Dunn, G.A., 1978. Cell to substratum contacts of chick fibroblasts and their relation to the microfilament system. A correlated interference-reflexion and high-voltage electron-microscope study. Journal of cell science 29, 197–212.

Heemskerk, I., Lecuit, T., LeGoff, L., 2014. Dynamic clonal analysis based on chronic in vivo imaging allows multiscale quantification of growth in the Drosophila wing disc. Development (Cambridge, England) 141, 2339–2348.

Helmlinger, G., Netti, P.A., Lichtenbeld, H.C., Melder, R.J., Jain, R.K., 1997. Solid stress inhibits the growth of multicellular tumor spheroids. Nature biotechnology 15, 778–783.

Hoefer, I.E., den Adel, B., Daemen, Mat J A P, 2013. Biomechanical factors as triggers of vascular growth. Cardiovascular research 99, 276–283.

Huber, F., Boire, A., Lopez, M.P., Koenderink, G.H., 2015. Cytoskeletal crosstalk: when three different personalities team up. Current opinion in cell biology 32, 39–47.

Hufnagel, L., Teleman, A.A., Rouault, H., Cohen, S.M., Shraiman, B.I., 2007. On the mechanism of wing size determination in fly development Proc. Natl. Acad. Sci. USA 104, 3835–3840.

Ishihara, S., Sugimura, K., 2012. Bayesian inference of force dynamics during morphogenesis. Journal of theoretical biology 313, 201–211.

Izzard, C.S., Lochner, L.R., 1976. Cell-to-substrate contacts in living fibroblasts: an interference reflexion study with an evaluation of the technique. Journal of cell science 21, 129–159.

Jacobs, C.R., Temiyasathit, S., Castillo, A.B., 2010. Osteocyte mechanobiology and pericellular mechanics. Annual review of biomedical engineering 12, 369–400.

Klein-Nulend, J., Bacabac, R.G., Bakker, A.D., 2012. Mechanical loading and how it affects bone cells: the role of the osteocyte cytoskeleton in maintaining our skeleton. European cells & materials 24, 278–291.

LeGoff, L., Rouault, H., Lecuit, T., 2013. A global pattern of mechanical stress polarizes cell divisions and cell shape in the growing Drosophila wing disc. Development (Cambridge, England) 140, 4051–4059.

LeGoff, L., Lecuit, T., 2015. Mechanical forces and growth in animal tissues. Cold Spring Harbor perspectives in biology.

Levayer, R., Dupont, C., Moreno, E., 2016. Tissue Crowding Induces Caspase-Dependent Competition for Space. Current biology : CB 26, 670–677.

Mao, Y., Baum, B., 2015. Tug of war--the influence of opposing physical forces on epithelial cell morphology. Developmental biology 401, 92–102.

ACCEPTED MANUSCRIPT

Mao, Y., Tournier, A.L., Hoppe, A., Kester, L., Thompson, B.J., Tapon, N., 2013. Differential proliferation rates generate patterns of mechanical tension that orient tissue growth. The EMBO journal 32, 2790–2803.

Martz, E., Steinberg, M.S., 1972. The role of cell-cell contact in "contact" inhibition of cell division: a review and new evidence. Journal of cellular physiology 79, 189–210.

Metcalf, D., 1963. THE AUTONOMOUS BEHAVIOUR OF NORMAL THYMUS GRAFTS*. Immunol Cell Biol 41, 437–447.

Milan, M., Campuzano, S., Garcia-Bellido, A., 1996. Cell cycling and patterned cell proliferation in the wing primordium of Drosophila. Proc. Natl. Acad. Sci. USA 93, 640–645.

Nelson, C.M., Jean, R.P., Tan, J.L., Liu, W.F., Sniadecki, N.J., Spector, A.A., Chen, C.S., 2005. Emergent patterns of growth controlled by multicellular form and mechanics. Proc. Natl. Acad. Sci. USA 102, 11594–11599.

Neufeld, T.P., de la Cruz, Aida Flor A, Johnston, L.A., Edgar, B.A., 1998. Coordination of Growth and Cell Division in the Drosophila Wing. Cell 93, 1183–1193.

Nienhaus, U., Aegerter-Wilmsen, T., Aegerter, C.M., 2009. Determination of mechanical stress distribution in Drosophila wing discs using photoelasticity. Mechanisms of development 126, 942–949.

Nienhaus, U., Aegerter-Wilmsen, T., Aegerter, C.M., 2012. In-vivo imaging of the Drosophila wing imaginal disc over time: novel insights on growth and boundary formation. PloS one 7, e47594.

Nowotarski, S.H., Peifer, M., 2014. Cell biology: a tense but good day for actin at cell-cell junctions. Current biology : CB 24, R688-90.

Pelham, R. J., Wang, YL., 1997. Cell locomotion and focal adhesions are regulated by substrate flexibility. Proc. Natl. Acad. Sci. USA, 94, 13661-13665

Pope, K.L., Harris, T.J.C., 2008. Control of cell flattening and junctional remodeling during squamous epithelial morphogenesis in Drosophila. Development (Cambridge, England) 135, 2227–2238.

Puliafito, A., Hufnagel, L., Neveu, P., Streichan, S., Sigal, A., Fygenson, D.K., Shraiman, B.I., 2012. Collective and single cell behavior in epithelial contact inhibition. Proc. Natl. Acad. Sci. USA 109, 739–744.

Raff, M., 1998. Cell suicide for beginners. Nature 396, 119–122.

Rauskolb, C., Sun, S., Sun, G., Pan, Y., Irvine, K.D., 2014. Cytoskeletal tension inhibits Hippo signaling through an Ajuba-Warts complex. Cell 158, 143–156.

Restrepo, S., Zartman, J.J., Basler, K., 2014. Coordination of patterning and growth by the morphogen DPP. Current biology : CB 24, R245-55.

Roman, B.L., Pekkan, K., 2012. Mechanotransduction in embryonic vascular development. Biomechanics and modeling in mechanobiology 11, 1149–1168.

Rooij, J. de, 2014. Cadherin adhesion controlled by cortical actin dynamics. Nature cell biology 16, 508–510.

Roth, K.A., D'Sa, C., 2001. Apoptosis and brain development. Mental retardation and developmental disabilities research reviews 7, 261–266.

Salbreux, G., Charras, G., Paluch, E., 2012. Actin cortex mechanics and cellular morphogenesis. Trends in cell biology 22, 536–545.

Sansores-Garcia, L., Bossuyt, W., Wada, K.-I., Yonemura, S., Tao, C., Sasaki, H., Halder, G., 2011. Modulating F-actin organization induces organ growth by affecting the Hippo pathway. The EMBO journal 30, 2325–2335.

Schluck, T., Nienhaus, U., Aegerter-Wilmsen, T., Aegerter, C.M., 2013. Mechanical control of organ size in the development of the Drosophila wing disc. PloS one 8, e76171.

Schulte, F.A., Ruffoni, D., Lambers, F.M., Christen, D., Webster, D.J., Kuhn, G., Muller, R., 2013. Local mechanical stimuli regulate bone formation and resorption in mice at the tissue level. PloS one 8, e62172.

Schwank, G., Basler, K., 2010. Regulation of organ growth by morphogen gradients. Cold Spring Harbor perspectives in biology 2, a001669.

Shraiman, B.I., 2005. Mechanical feedback as a possible regulator of tissue growth. Proc. Natl. Acad. Sci. USA 102, 3318–3323.

Smith, M.A., Hoffman, L.M., Beckerle, M.C., 2014. LIM proteins in actin cytoskeleton mechanoresponse. Trends in cell biology 24, 575–583.

ACCEPTED MANUSCRIPT

Streichan, S.J., Hoerner, C.R., Schneidt, T., Holzer, D., Hufnagel, L., 2014. Spatial constraints control cell proliferation in tissues. Proc. Natl. Acad. Sci. USA 111, 5586–5591.

Sugimura, K., Lenne, P.-F., Graner, F., 2016. Measuring forces and stresses in situ in living tissues. Development (Cambridge, England) 143, 186–196.

Takai, Y., Miyoshi, J., Ikeda, W., Ogita, H., 2008. Nectins and nectin-like molecules: roles in contact inhibition of cell movement and proliferation. Nature reviews. Molecular cell biology 9, 603–615.

Tatsumi, S., Ishii, K., Amizuka, N., Li, M., Kobayashi, T., Kohno, K., Ito, M., Takeshita, S., Ikeda, K., 2007. Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. Cell metabolism 5, 464–75.

Tepass, U., 2012. The apical polarity protein network in Drosophila epithelial cells: regulation of polarity, junctions, morphogenesis, cell growth, and survival. Annual review of cell and developmental biology 28, 655–685.

Thompson, D.W., 1917. On Growth and Form. [With illustrations.]. University Press, Cambridge.

Twitty, V.C., Schwind, J.L., 1931. The growth of eyes and limbs transplanted heteroplastically between two species of Amblystoma. J. Exp. Zool. 59, 61–86.

Uyttewaal, M., Burian, A., Alim, K., Landrein, B., Borowska-Wykręt, D., Dedieu, A., Hamant, O., 2012. Mechanical stress acts via katanin to amplify differences in growth rate between adjacent cells in Arabidopsis. Cell, 149, 439–51.

Vico, L., Collet, P., Guignandon, A., Lafage-Proust, MH., Thomas, T., Rehaillia, M., Alexandre, C., 2011.
Effects of long-term microgravity exposure on cancellous and cortical weight-bearing bones of cosmonauts. Lancet 355, 1607–1611

Wartlick, O.; Mumcu, P.; Kicheva, A.; Bittig, T.; Seum, C.; Julicher, F.; Gonzalez-Gaitan, M., 2011. Dynamics of Dpp signaling and proliferation control. Science 331, 1154–1159.

Wartlick, O., Mumcu, P., Julicher, F., Gonzalez-Gaitan, M., 2011. Understanding morphogenetic growth control -- lessons from flies. Nature reviews. Molecular cell biology 12, 594–604.

Weigmann, K., Cohen, S. M., Lehner, C. F., 1997. Cell cycle progression , growth and patterning in imaginal discs despite inhibition of cell division after inactivation of Drosophila Cdc2 kinase. Development 124, 3555–3563.

Wyatt, T.P.J., Harris, A.R., Lam, M., Cheng, Q., Bellis, J., Dimitracopoulos, A., Kabla, A.J., Charras, G.T., Baum, B., 2015. Emergence of homeostatic epithelial packing and stress dissipation through divisions oriented along the long cell axis. Proceedings of the National Academy of Sciences of the United States of America 112, 5726–5731.

Wyatt, T., Baum, B., Charras, G., 2016. A question of time: tissue adaptation to mechanical forces. Current opinion in cell biology 38, 68–73.

You, L., Cowin, S.C., Schaffler, M.B., Weinbaum, S., 2001. A model for strain amplification in the actin cytoskeleton of osteocytes due to fluid drag on pericellular matrix. Journal of Biomechanics 34, 1375–1386.

Zartman, J., Restrepo, S., Basler, K., 2013. A high-throughput template for optimizing Drosophila organ culture with response-surface methods. Development 140, 667–674.

Zecca, M., Basler, K., Struhl, G., 1995. Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the Drosophila wing. Development 121, 2265–2278.

Zecca, M., Basler, K., Struhl, G., 1996. Direct and long-range action of a wingless morphogen gradient. Cell 87, 833–844.

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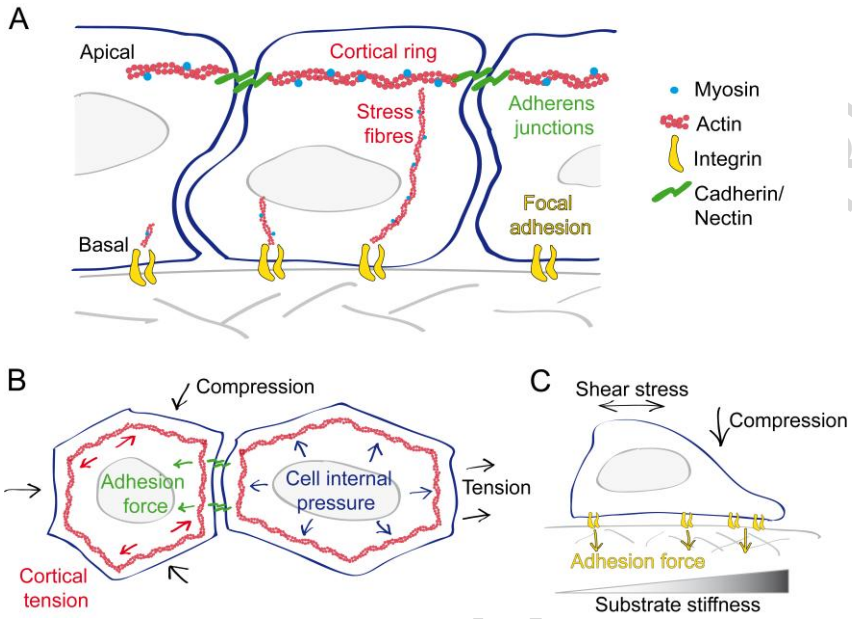

Fig.1. Schematic representation of an epithelial cell and its mechanical environment. (A) A polarized epithelial cell is connected via adherens junctions to its neighboring cells and via focal adhesion to the substrate. Desmosomal, septate and tight junctions are not shown because they do not appear in all epithelial cell types. (B, C) Epithelial cells are exposed to various types of mechanical stresses from intrinsic forces, neighboring cells and the extracellular space. (C) Cells respond to increasing substrate stiffness by reinforcing adhesion and active spreading on the substrate in an actomyosin- dependent process.

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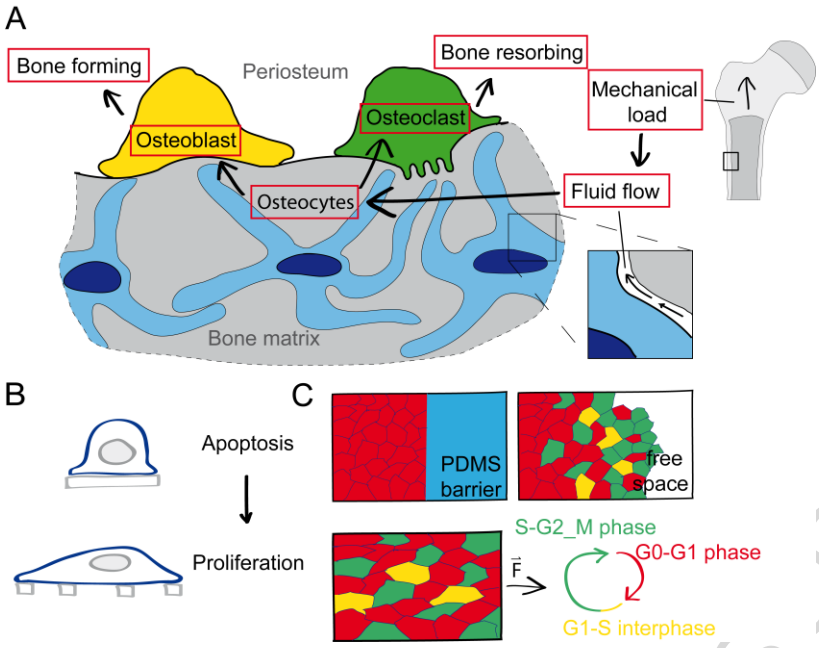

Fig.2. Model systems for mechanical growth control. (A) Mechanical load is driving bone remodeling in a multi-scale process: Mechanical load on the bone leads to fluid flow surrounding the osteocytes. Subsequently, shear-stress induced activation of osteocytes stimulates osteoblasts or osteoclasts to form and resorb the bone matrix, respectively. (B) Endothelial cells switch from proliferation to apoptosis when cell size is decreased by using micropatterned islands of adhesive substrate (Chen, 1997). (C) Proliferation patterns of MDCK cells analyzed with Fucci cell cycle marker. Cells constrained with a PDMS barrier do not proliferate, but progress in cell cycle when the barrier is removed. Similarly, when cell area is increased by stretching the substrate, cells continue in the cell cycle (Streichan et al., 2014).

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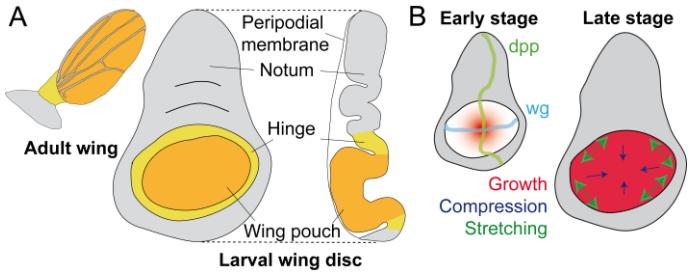

Fig.3. *Drosophila* wing imaginal disc. (A) Top and lateral view of the wing imaginal disc at third instar. The wing pouch will develop into the wing blade of the adult fly. (B) The concentration of morphogens is highest in the center of the wing pouch. Growth models suggest that the morphogen distribution promotes growth in the center, resulting in a growth gradient at early stages (Aegerter-Wilmsen et al., 2007; Hufnagel et al., 2007). This gradient changes the global tensions in the wing pouch which in turn stimulates proliferation at the periphery and suppresses proliferation at the center. This feedback loop leads to homogenous growth throughout the wing pouch at later stages as well as controlling size.

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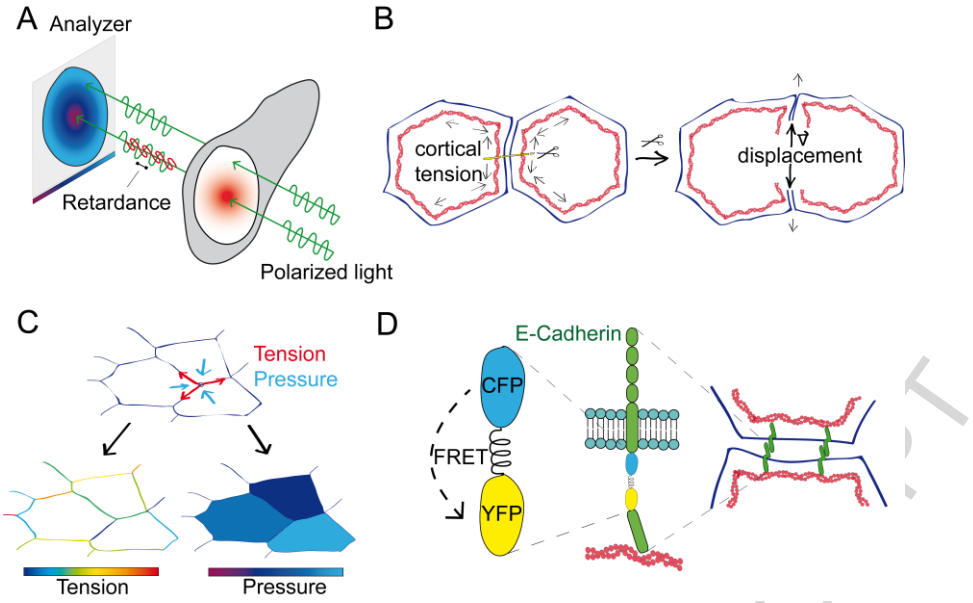

Fig.4. Methods to measure forces in the wing disc. (A) Birefringence measurement: The polarization state of a linearly polarized beam of light is changed when passing through a birefringent material. This is described by a phase difference in the different states called retardance and can be due to stress anisotropies in the material. (B) Laser ablation: When the actomyosin cortex is cut with a focused laser beam, the remaining edges retract, indicating a positive tension at the cortex. The velocity of the displacement provides a measure for this tension. (C) Force inference: The cell geometries of the input image reflect the balance between internal pressure and apical tensions. Solving force balance equations returns maps of cell pressure and tensions at cell-cell junctions. (D) FRET tension sensor: Sensor module is composed of two fluorophores connected by an elastic linker. The module is genetically integrated to a protein of choice, here E-cadherin. E-cadherin mechanically connects adjacent cells, thus tension between neighboring cells is transduced via E-cadherin. This moves the two fluorophores apart and can be measured by FRET efficiency.

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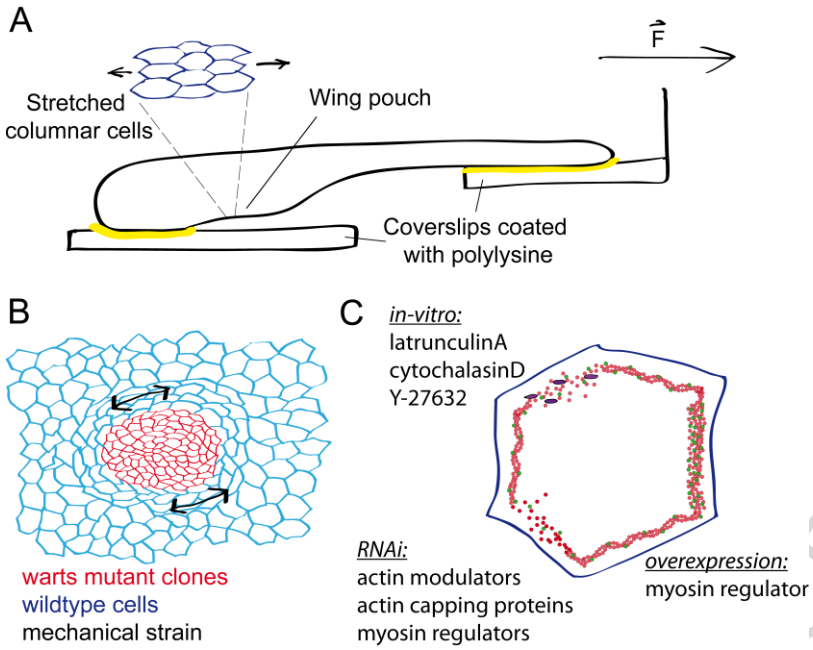

Fig.5. Methods to manipulate forces in the wing disc. (A) Stretching device: The wing disc is attached *in vitro* onto two flexible coverslips with poly-lysine. The coverslips can be moved apart with a defined force to stretch the wing disc tissue. (B) Overproliferating clone: Clonal manipulation of the Hippo pathway stimulates a patch of cells to overproliferate. Cells within the clone are compressed and the surrounding tissue is stretched tangentially. (C) Cytoskeletal modification: Pharmacological drugs applied *in vitro* or RNAi against actin or myosin regulators reduces tension of the actomyosin network, especially at the apical cortex. Overexpression of myosin regulators increases the tensional state.

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Highlights

- Epithelial cells are embedded in a biophysical microenvironment which influences cellular behavior.
- Mammalian bones adapt to mechanical loads in size and shape.
- *In vitro* studies on epithelial monolayers revealed that mechanical constraints, resulting from tissue dynamics, feed-back on tissue growth.
- Growth models for the *Drosophila* wing imaginal disc incorporated mechanical feedback loops to modulate growth and final size.
- *In vivo* measurements and manipulations of tissue mechanics remain challenging.

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