# Introduction

## Epithelial Morphogenesis

Epithelial tissues are amongst the most abundant tissues in the animal, forming continuous sheets at external and internal interfaces. Main characteristics of epithelia are an apical-basal polarity and lateral coherency mediated by cell junctions which form tight connections between cells. Epithelia are often surrounded by an extracellular matrix (ECM). Although Epithelia are formed by a single layer of interconnected cells, they generate transient or permanent 3D geometries during development.

### Mechanical properties of epithelia

#### Cellular geometry and forces (and molecular machinery)

Lateral coherency is mediated by adherence junctions that are located towards the apical side of the epithelium. Adherence junctions are multiprotein cell-cell adhesion complexes that form extracellular interactions, maintaining tissue integrity whilst allowing cell junctional remodeling and cell movement. The main structural components of adherence junctions are conserved along metazoan organisms and include a diverse set of proteins. Cadherins form homophilic interaction though their extracellular domain, and interact intracellular with different catenin proteins, such as p120, α- and β-catenin, to mediate cell-cell adhesion to the cytoskeleton and though their apical location also provides an apical-basal polarity cue (Harris & Tepass, 2010).

At the basal side focal adhesions connect the cytoskeleton with the basement membrane, thereby coupling them mechanically and allowing for force generation and transmission. The coupling between ECM and cytoskeleton is achieved via clusters of integrin transmembrane receptors which bind to the basement membrane and actin binding proteins that convey and modulate force transmission (Ciobanasu et al., 2013). Inside the cell apical, basal and lateral forces are mechanically coupled via the actomyosin network of the cell cortex and medial and lateral microtubules.

#### Tissue material properties

Epithelial tissues can be described as viscoelastic, active materials, meaning they combine elastic and viscous behaviors. Over short timescales the deformation in response to physical stress is relaxed as soon as the stress is removed and on longer timescales epithelia remodel their internal structure, thereby relaxing internal stresses fluid like (Forgacs et al., 1998; Banks et al., 2011). Tissue material properties can be determined by cytoskeletal elements as well as collective effects of cell-cell adhesion and dynamics (Iyer et al., 2019). Mechanical forces are transmitted between neighboring cells though adherence junctions and their linkage to the underlying actomyosin cytoskeleton. Thus, internal or external forces can propagate at multicellular length scales as it has been demonstrated using laser ablation studies (Fernandez-Gonzalez et al., 2009) or on the orientation of cell division axis (Fink et al., 2011). Large scale propagation of mechanical stress is perhaps best demonstrated by the polarizing action of hinge contraction on cell elongation, division and rearrangements and planar polarity in the drosophila pupal wing blade (Etournay et al., 2015; Iyer et al., 2019).

Whether a tissue behaves more liquid like or solid like is a consequence form cell mechanics, adhesion, and cortical contractility, and state transitions are reflected in cell area variability, shape anisomery and packing topology (Farhadifar et al., 2007; Atia et al., 2018). Tissue fluidization involves topological changes by cell rearrangements, generated though T1 transitions, cell division and cell death. The number of rearrangements is dependent on cell shape, size contractility and motility. Thus cell packing geometry can be an indicator of unjamming (Bi et al., 2015; Atia et al., 2018). Moreover cell rearrangements can be active or passive and depend on stochastic effects on bond tension fluctuation and can have intrinsic anisotropy, thereby modulating tissue material properties (Duclut et al., 2021, 2022).

\*\*\*ECM?

#### Geometric considerations

Epithelial packing geometry can be described by apical cell shapes such as cell area variability, cell shape anisotropy and nematic order and polygonal cell shapes. These properties are tightly intertwined and are not only influences by cell mechanics, but also follow a geometrical logic. Cell area is often tightly linked with neighbor numbers, the Lewis’ law states that the average area of an n-sided cell increases linearly with n, the Aboav-Weaire Law states that cells with a higher number of sides have few-sided neighbors and the Euler’s theorem states that the average number of neighbors in a tissue is six. The optimal order of a flat tissue can be achieved with isotopically shaped hexagons of uniform size, a packing that is achieved at the end of pupal wing morphogenesis (Classen et al., 2005). Growing tissue differ from hexagonal packing, instead they exhibit a specific and robust distribution of cell neighbor numbers that is highly conserved across species and is a consequence of cell divisions and rearrangements (Gibson et al., 2006).

Pseudostratified epithelial also experience a characteristic 3D organization, each cell spans the entire apical-basal axis, but due to their tight packing, cells are not circumferentially homogenous along their apical-basal axis. While following the same geometric laws as apical cell shapes, they exhibit multiple lateral neighbor exchanges, so-called lateral T1s, and the cross-sectional area varies depending on the position of the nucleus (Gómez et al., 2021).

Apical cell shape anisotropy can also generate a global nematic order as observed in the wing imaginal disc (Dye et al., 2021). Topological defects can play a role in tissue development by driving cell apoptosis and topological mechanisms could be able to facilitate morphogenesis (Saw et al., 2017; Guillamat et al., 2022). \*\*\*does that go into morphogenesis?

### 2D and 3D morphogenesis:

In-plane morphogenesis of epithelial sheets can be mediated by cell behaviors such as cell rearrangements, oriented cell division, cell death or cell shape changes (Aigouy et al., 2010; Merkel et al., 2017).

The ability remodel tissue shape though coordinated neighbor exchanges has been described in various systems and is not unique to epithelia (Warga & Kimmel, 1990; Irvine & Wieschaus, 1994; Keller et al., 2000; Wallingford & Harland, 2002). Cell intercalations in epithelia require the remodeling of cell-cell junctions, a process that is characterized by the shrinkage of a bond between two neighboring cells, a transient stage 4-fold vertex stage and the formation of a new bond perpendicular to the lost bond. This process is referred to as a T1 transition (Bertet et al., 2004). A more complex form of rearrangements during large scale tissue elongation and thinning movements are rosette based intercalations (Blankenship et al., 2006). Cell rearrangements require contractile and adhesive properties between cells. In Drosophila contraction is mediated by the action of nonmuscle myosin II on actin filaments and adhesive function is sustained at the cell interfaces though adherence junction proteins, such as E-cadherin, α-catenin and β-catenin (reviewed by Paré & Zallen, 2020). Directionality of cellular rearrangements is generally attributed to asymmetrical myosin localization and activity and modulation on cell-cell adhesion (Fernandez-Gonzalez et al., 2009; Rauzi et al., 2008). Planar polarized contractility and adhesion are mediated by Rho kinase localization and can also be modulated by turnover and composition of adherents junctions as well as feedback and plasticity between adhesive and contractile components (Simões et al., 2010; Paré & Zallen, 2020).

Similar to oriented rearrangements, the orientation of cell divisions modifies tissue shape though their effects on tissue topology. Thus, cell divisions are not a mere byproduct of tissue growth but have been shown to contribute to tissue morphogenesis by division along the axis of tissue extension (da Silva & Vincent, 2007; Gong et al., 2004). The orientation of cell division can often be predicted by cell shape before division as cells tend to divide along the long axis (Tsou et al., 2003), there are however noticeable exceptions that suggest a mechanical control of the cell division axis (Campinho et al., 2013; Wang et al., 2017). An interesting example for the role of programmed cell death during tissue morphogenesis comes from the dorsal closure in Drosophila, where apoptotic cells induce cell shape changes and accelerates tissue dynamics (Toyama et al., 2008; Gorfinkiel et al., 2009).

As opposed to the changes in tissue topology though cell rearrangements cell divisions and cell death, cell shape changes deform tissues without effects on tissue topology. Changes in cell area lead to isotropic increase or reduction in size in an epithelium. Anisotropic cell shape changes, such as changes in the direction or total amount of cell elongation, cause anisotropic effects on the tissue. Remarkably, highly anisotropic cell shapes are often observed transiently during morphogenesis and reflect global tissue strain (Butler et al., 2009; Etournay et al., 2015; Lye et al., 2015).

Importantly in developing epithelia cell rearrangements, division, death and shape changes directly feed back onto one another and affect the global packing geometry of the tissue. Topological changes though rearrangements, death or division lead to changes in cell shape anisotropy and polygonal cell shape (FIGURE). In turn, cell shape anisotropy can affect the axis of cell division and cells often reach a critical size before they divide. Anisotropic bond lengths also provides a bias for the direction of T1 transitions. (Figure)

An interesting question remains what causes cell behaviors, active cell driven behaviors mediated by the cellular cytoskeleton can collectively transform epithelial shape but in turn epithelia can remodel in in response to stress imposed by external forces. Notably externally motivated cell shape changes still require the remodeling of the cellular cytoskeleton but underlying molecular and energetic behaviors as well as topological outcomes can very different\*\*more citations here (Dye et al., 2021; Duclut et al., 2022).

However, cell shape anisotropy is not only a result of tissue strain or rearrangements but in itself it could provide instructive cues for tissue morphogenesis as hypothesized in 1991 by Condic and colleagues (Condic et al., 1991). More recently a role for nematic cell polarity and nematic order has been discussed for 3D tissue morphogenesis.

3D aspects of tissue morphogenesis.

Theoretical and technological advances have recently shifted the focus towards 3D tissue morphogenesis.

#### Tissue morphogenesis through changes in 3D cell geometry

Out of plane morphogenesis is often attributed to differential changes in 3D cell geometry. A well characterized morphological change is the formation of epithelial folds, that generated though a unidirectional change epithelial curvature. Folding can be achieved though differential expansion or contraction along apical, basal and lateral surfaces of cells in an epithelial sheet. A frequent force generating mechanism for folding morphogenesis is the contraction of the apical actomyosin network as for example observed in the ventral furrow formation during Drosophila embryogenesis (Sweeton et al., 1991; Martin et al., 2009). Noticeably, the cellular volume remains conserved, which propagates forces generated by apical constriction into changes in aspect ratio of cell surfaces and thus 3D cell shape changes (Polyakov et al., 2014). Recently it became evident that apical constriction is not the only active process that leads to apical inwards folding, changes in basal or lateral cortex tension can induce folds without apical constriction in the Drosophila wing disc while keeping constant cell volume (Sui et al., 2018). Opposite curvature can be achieved by basal constriction and resulting apical expansion, as observed during optic cup formation in medaka and zebrafish or the formation of the zebrafish midbrain hindbrain boundary (Gutzman et al., 2008; Martinez-Morales et al., 2009; Sidhaye & Norden, 2017). Cell shape changes mediate not only folding morphogenesis but have also been shown to lead to more complex changes in curvature such as the formation of intestinal crypts though apical constriction (Sumigray et al., 2018) or the formation epithelial cysts by a combination of apical and lateral tension (Bielmeier et al., 2016).

#### Cellular effects on large scale tissue deformation

Differential changes in cell shapes and their effects on tissue shape changes are observed at short length scales by the collective action of a few cells along the direction of curvature. To understand large scale deformations in tissue shape, supracellular effects and external contributions to shape must be disentangled. In the case of ventral furrow formation in the Drosophila embryo, in silico together with in vivo analysis shows that that different tissue material properties, mediated by spatial differences in the cellular actomyosin network affect furrow internalization (Conte et al., 2012; Rauzi et al., 2015). Highlighting how robustness and temporal regulation of large-scale tissue deformation involves a combination of tissue level effects in addition to localized cell shape changes and how the cell and tissue level forces influence each other in a bidirectional way (Fernandez-Gonzalez et al., 2009; Martin et al., 2010).

#### Large scale mechanisms for epithelial shape changes - in plane stresses

Change in cell aspect ratio is not the only potential driver for changes in 3D tissue architecture.

Large epithelia can be mechanically described thin sheets and as such they can release in plane stresses by out of plane deformations such as wrinkling or bucking. The nature of the compression can be external confinement in combination with growth (Trushko et al., 2020) or growth mismatch with adjacent layers as suggested for the morphogenesis of several tissues, such as intestine, cerebral cortex, teeth or lung (Nelson, 2016). Strikingly in vitro experiments demonstrate, that epithelial pre-tension can resolve compression induced buckling on short time scales though actomyosin rearrangements. However, if buckling exceeds a certain threshold, the curvature changes persist. These experiments highlight how viscoelastic properties of epithelia impact buckling induced curvature change (Wyatt et al., 2020). Epithelial buckling could however also arise from tissue intrinsic stresses, as a consequence of differential growth and tissue geometry. Such a combination of tissue intrinsically generated compressive forces and buckling transitions could lead to, or aid out of plane tissue deformations. A potential role for differential growth could be to position the developing folds in the Drosophila wing disc, as in silico analysis suggests (Tozluoǧlu et al., 2019). However, the inhibition of cell divisions prior to folding does not affect the formation of folds (Sui et al., 2018), thus if differential growth indeed affects fold positioning in vivo needs to be tested and the morphogenetic mechanisms are still unclear.

As deformations in 3D shape usually necessitate localized tissue expansion and cell shape changes and growth can both induce as well as respond to tissue strain, it remains challenging to disentangle active and passive contributions to tissue shape changes. Moreover, to understand local deformations the respective mechanical and geometrical properties of the tissue environment need to be taken into account.

#### ECM

An important aspect for epithelial mechanics is the composition and mechanical properties of the ECM. Epithelial tissues can have an apical and a basal ECM, which are of very different composition and can be highly diverse between species. The basement membrane is the basal layer of ECM proteins in Drosophila. It is evolutionary conserved and consist of collagen IV, perlecan, nidogen and laminin and is linked to the cellular cytoskeleton via integrins (Hynes & Zhao, 2000). In the Drosophila wing disc the collagen IV is required to maintain the 3D shape of the larval wing imaginal disc and ECM stiffness affects cell height (Pastor-Pareja & Xu, 2011). Changes in ECM stiffness have also been shown to affect the shape of the Egg Chamber or cell migration in the ventral nerve cord in *Drosophila* (Ramos-Lewis & Page-McCaw, 2019). Localized reduction in collagen IV has a morphogenetic role leading to a reduction in basal tension the formation of the HH fold of the *Drosophila* wing disc. Moreover ectopic degradation of basal ECM generates ectopic folds (Sui et al., 2018). ECM dependent basal contraction plays a role in the apical outwards folding of the zebrafish midbrain - hindbrain boundary as folding is disrupted in laminin mutants (Gutzman et al., 2008). Together, these experiments demonstrate that the basal ECM constitutes an important mechanical environment, maintaining tissue shape as well as enabling cell shape changes though changes in ECM stiffness and interactions with the basal cytoskeleton.

Apical ECM is generally less conserved between species and its role for tissue shape and morphogenesis is less well understood. During late development apical ECM forms rigid protective layers such as the coticule in various organisms or barriers along the skin or internal tubular organs (Li Zheng et al., 2020). During early stages the apical ECM consists largely of zona pellucida‑domain proteins (ZP-proteins). Insights on the role of apical EM in tissue morphogenesis comes from branching morphogenesis of tracheal tubes in *Drosophila*, as during branching, the ZP-proteins Dumpy (Dp) and Piopio (Pio) a required for junctional remodeling leading to cell rearrangements and intercalation (Jaźwińska et al., 2003). Dumpy has also a critical role in pupal wing morphogenesis, providing a physical constraint to maintain epithelial tension in response to hinge contraction (Etournay et al., 2015). The complete removal of apical and basal ECM drives columnar-to-cuboidal shape changes and subsequent tissue flattening during Drosophila leg and wing elongation (Diaz-de-la-Loza et al., 2018)

## drosophila wing disc eversion

Research on 3D epithelial shape changes is often challenged by challenges in live imaging, genetic accessibility and computational tools. These challenges have been overcome in various vertebrate and invertebrate organisms which revealed individual contributors of 3D morphogenesis, such as actomyosin, ECM and tissue geometric effects. However, we still lack understanding of the quantitative, collective effects for 3D tissue shape and understanding relative contributions to shape requires holistic analysis of cell and tissue shape changes throughout development. A model that allows such in-depth analysis comes from the *Drosophila* wing development. The Drosophila wing imaginal disc presents an excellent model to investigate the generation and function of three-dimensional epithelial shape, as seen for example in the case of fold formation during wind disc growth (Sui et al., 2018).

#### Wing disc patterning

The wing imaginal disc wing develops into the adult wing blade, the wing hinge and into the mesothorax (Bryant, 1975). It is formed during embryonic development containing ~24-30 cells, which proliferate throughout larval development to reach a final size of ~30.000-40.000 cells (Bate & Arias, 1991; Cohen et al., 1993; Martín et al., 2009; McClure & Schubiger, 2005). Wing disc morphology was first described in 1935 by Auerbach and our morphological and molecular knowledge has since been further expanded (Auerbach, 1935). Initially the wing disc forma as a flat epithelial sac with the apical side pointing inwards, during larval development the wing disc refines its morphology, by the end of larval development the wing discs consists of a tall pseudostratified layer, the disc proper layer (DP), and a squamous cell layer, the peripodial epithelium (PE) (McClure & Schubiger, 2005). The disc proper is subdivided into three distinct domains: the notum, the hinge and the dome shaped wing pouch. The disc forms three main folds, between the notum and the hinge (NH-fold), inside the hinge (HH-fold) and between hinge and pouch (HP-fold). The wing pouch gives rise to the blade and part of the hinge, the folded hinge region gives rise to the wing hinge and the base of the wing, and the notum which forms the adult thorax (Bryant, 1975).

The wing is early on compartmentalized into anterior and posterior compartments. The anterior-posterior (AP) compartment boundary is established in the embryo, the second compartment boundary, the dorsal-ventral (DV) boundary is established around 60 hAEL (2nd instar larva) and later gives rise to the wing margin. Interestingly the AP compartment boundary but does not have a morphological equivalent in the adult disc. Both, AP and DV-boundary are cell lineage boundaries (Bryant, 1970; Garcia-Bellido et al., 1973, 1976). Maintenance and of DV and AP boundary quires signaling pathways and selector genes such as Engrailed (En) for the AP axis, and Vein (Vn), Wingless (Wg) and Decapentaplegic (Dpp) for DV and PD axis. Similarly PE versus DP are specified early on (Tripathi & Irvine, 2022).

#### Growth and packing geometry

Research on wing disc during larval stages has much concerned with the mechanisms of growth given its more than 1000x fold increase in size from embryo to late larva, corresponding to more than 10 cell division cycles. The average cell cycle time is around 8 hr at 90 hAEL but growth rates decline as the wing disc ages (Garcia-Bellido & Merriam, 1971; Schubiger & Palka, 1987; Martín et al., 2009; Bittig et al., 2009). Growth rates are mostly homogeneous, although a zone of non-proliferating cells is established at the DV boundary in late 3rd instar wing discs. This non-proliferative region is regulated by wingless and notch signaling and is first observed at 96 hAEL it increases subsequently up 120 hAEL, corresponding to 10 rows of cells (O’Brochta & Bryant, 1985; Schubiger & Palka, 1987; Johnston & Edgar, 1998). Growth anisotropy has been extensively studied in the wing pouch, and increased proliferation has been observed in the center of the pouch at 48–72 hAEL as compared to the periphery but is uniform afterwards (Mao et al., 2013). The decrease in proliferation at late larval stages could be due to a mechanical feedback mechanism as a consequence of a change in the compression gradient (Aegerter-Wilmsen et al., 2012). Moreover there are geometric and implications for anisotropic growth, it has been suggested that differential growth leads to anisotropies in tissue compression, which could persists and explain tissue shape and epithelial packing during larval growth even at later development (Mao et al., 2013). This view has however been challenged as rearrangements in the tissue can resolve the early patterns of differential tension (Heller et al., 2016; Dye et al., 2017). Interestingly in later developmental stages (96 hAEL -109 hAEL) and in the absence of differential growth, tangential cell elongation further increases and area gradients persist. The increase in tangential elongation can be accounted for by an active patterning of radial cell neighbor exchanges which is driven by a mechanosensitive feedback mechanism (Dye et al., 2021).

The relative size differences of apical cell areas: smaller in, larger out, that is observed in the wing pouch has first been described in 1982 by Brower and colleagues and in 1991 a similar pattern was observed in leg discs (Brower et al., 1982; Condic et al., 1991). Moreover the novel methods at the time revealed the tangential elongation patterns and lateral T1s in the tissue (Condic et al., 1991). These cell shape patterns have since been gaining a lot of interest to understand mechanical mechanisms of tissue growth and pattering (Aegerter-Wilmsen et al., 2010; Legoff et al., 2013; Mao et al., 2013; Dye et al., 2021). However less though has been given to their implications for subsequent development …. Expand and follow up to eversion

At the prepupal stage, which marks the larval to pupal period and takes from puparium formation, the so-called *white Pupa* stage, to about 12 hours after puparium formation (hAPF), the wing undergoes a morphogenetic transformation that leads to the formation of the wing bilayer. The prepupal period is initiated after approximately 6 hours of exposure to by high levels of the molting hormone ecdysone. At the white Pupa stage (0 hAPF) the larva stops crawling and everts its spiracles. Approximately 1hr after white pupa formation, the pupal case starts tanning to a yellow, brown color (D. K. Fristrom & J. W. Fristrom, 1993). This color change makes the white pupa stage easy to identify and allow an easy visual way of pupal staging by hAPF.

During the prepupal stage the wing disc undergoes a dramatic morphogenetic change that is often called evagination or eversion. These processes are often described by two discrete stages: eversion which describes the removal of the PE but often disregards the DP, and elongation which describes the flattening of the DP cells. These processes together are called evagination. As the developmental processes that lead to the removal of the PE also involve changes in the DP and they happen simultaneously, I will from now refer to the shape changes in the whole wing disc until the PE is removed (4 hAPF) as eversion, and subsequent morphogenesis, which is characterized by apio-basal flattening as expansion, I won’t use the term ‘elongation’ to avoid confusion with cell elongation and implicit statements on the unidirectionality of tissue expansion. ‘Evagination’ is often used interchangeably with eversion, but sometimes also means the combination of eversion and expansion and I will only use it as such. (D. Fristrom & Fristrom, 1975; D. K. Fristrom & J. W. Fristrom, 1993; Taylor & Adler, 2008).

#### The peripodial membrane during eversion

The PE contracts and breaks at 3-4 hAPF and it essential to place the wing disc to the outside of the epidermis. Around that time, cells of the PE and the disc stalk, a structure at the most proximal disc, connecting the wing disc and the epithelium, invade the larval epidermis and the PE ruptures at the distal end. (Pastor-Pareja et al., 2004). Moreover, a wave of programmed cell death occurs in most of the PE and only few, if any PE cells will end up in the epidermis (Aldaz et al., 2010). Interestingly, as the wing begins to evert, the central PE cells are under tension and expand, while actomyosin accumulates primarily in a ring at the periphery of the PE. However, if myoII is knocked down in the central region of the PE, the cells fail to expand and PE retraction is prevented, while the DP develops relatively normal. Similarly, when myoII is knocked down in the periphery surrounding the PE, the dorsal-ventral apposition of the DP is correctly executed but the PE is not removed and the DP does not expand and the disc does not bend ventrally as observed for the wt (Aldaz et al., 2013). These experiments suggest that actomyosin contraction in the PE is necessary to retract the PE and has implications for the 3D morphology of the whole wing disc but it does not interfere with the formation of the wing bilayer during eversion. These observations oppose the concept that evagination is accomplished by a continuous circumferential constriction, but rather point towards a necessity for complete removal of the PE, in order to achieve evagination. This view is further supported by the observation that cut wing and leg discs evaginate, even when cut in half. Which takes care of PE removal but prevents circumferential force generation (D. Fristrom & Chihara, 1978). Research by Milner et al., in 1984 supported this idea by suggesting that the main role of the PE is not a force generating one but to stretch to accommodate elongation and contract to allow the placement of the disc outside the body (Milner et al., 1984).

#### The Disc Proper during evagination

To further dissect the morphogenetic contributions to evagination, the DP needs to be considered. Upon inhibition of actomyosin with Cytochalasin B discs still undergo some reduction in cell height but evagination is inhibited completely. Destabilization or removal of microtubules does not prevent evagination, unlike stabilization of microtubules, which is likely to interfere with flattening and rearrangements and inhibits evagination. Thus, role for actomyosin and cell shape changes in the DP can be assumed (D. Fristrom & Fristrom, 1975).

The localization of actomyosin in the cells changes after 4 hAPF, supporting the idea of a major transition in cellular behaviors at this stage. Up to 4 hAPF Myosin II locates on the apical part of the cell, but it relocates afterwards towards the lateral membranes and it becomes homogeneously distributed at 7-8 hAPF (Diaz-de-la-Loza et al., 2018). Given the necessity for actomyosin activity in the DP, a range of cellular behaviors could be required for eversion. One potential candidate are oriented cell divisions, however evagination can take place in vitro and in the absence of cell divisions and also involves little cell death (J. W. Fristrom et al., 1973; D. Fristrom & Fristrom, 1975; J. W. Fristrom et al., 1977). These results are consistent with the observation that the number of cell divisions are drastically reduced in vivo at puparium formation (Schubiger & Palka, 1987). Nevertheless approximately 40% of cells in the future wing blade divide between 0 hAPF and 12 hAPF (Taylor & Adler, 2008). These experiments however span a large developmental period and do not reveal any necessity of cell divisions for morphogenesis. On the contrary, most cells are arrested in G2 phase by 3 hAPF and only cells in the presumptive vein region undergo DNA replication from 0-2 hAPF and cells around the anterior margin undergo DNA replication at 4-6 hAPF, where they might be involved in the generation of differentiated cell lineages such as neuronal precursors (Schubiger & Palka, 1987).

Other cellular contributions to tissue shear involve cell rearrangements and changes in cell elongation. Early research suggested a role for rearrangements during leg and wing disc evagination (Fekete et al., 1975; D. Fristrom, 1976). This has been challenged by the idea that in the leg disc changes in apical cell shape could drive evagination. In the leg, similar to the wing disc, cells are elongated circumferentially. After evagination, these patterns of elongation resolve, providing a unidirectional extension mechanism without the need for rearrangements (Condic et al., 1991). Interestingly cells are more elongated apically than basally, suggesting that this mechanism is executed at the apical junctional network. The same laboratory suggested also an involvement for inhomogeneous cell area increase in in the wing pouch during morphogenesis (D. K. Fristrom & J. W. Fristrom, 1993). However, these novel morphogenetic concepts have not been further explored and more recent research has focused on rearrangements and cell divisions in the future blade region. Notably research on evagination has mostly been focusing on the expansion of the wing from 4 hAPF up to 8 hAPF. From 4.5 to 5 hAPF Diaz de la Loza et al., observe a decrease in cell shape anisotropy and cell rearrangements, whereas from 6 hAPF onwards, no rearrangements but an increase in cell area is observed (Diaz-de-la-Loza et al., 2018). Accordingly, Diaz de la Loza et al. suggest a transition from a convergent-extension mechanism at 4-5 hAPF to a homogeneous expansion mechanism at 6-7 hAPF. These results contradict earlier work form Taylor and Adler, who suggest that cell divisions are driving extension from 4-8 hAPF. Taylor and Adler also suggest a role for convergent extension for eversion from 2-4 hAPF (Taylor & Adler, 2008).

Less attention has been given to the mechanisms that lead to the formation of the bilayer and the folding along the DV boundary. This process has been shown to be largely independent of the PE as mentioned above (Aldaz et al., 2013; Milner et al., 1984), but constitutes the most dramatic change in 3D morphology during evagination. Fristrom and Fristrom suggest that the DV-boundary performs ‘hinging’ action though wedge shaped cells to bring dorsal and ventral together, but also point out that this must be accommodated by differential expansion in the tissue due to its 3D geometry. Moreover, unfolding of the wing disc folds could substantially contribute to the process. Lastly a potential role for basal-basal junction in formation of the double layer is ruled out buy the observation that basal junctions do not form until 9-10 hAPF (D. Fristrom & Fristrom, 1975; D. K. Fristrom & J. W. Fristrom, 1993). Generally, the complex change in 3D geometry of the wing pouch necessitates an in-depth temporal and spatial analysis to dissect cellular contributions to tissue shape during eversion.

#### The role of ECM for evagination

An important mechanical and property of epithelia is the extracellular matrix. Wing disc evagination is accelerated ex vivo if ECM is removed by trypsin. Yet, trypsination only leads to successful accelerated evagination if discs had minimum 5h of prior exposure to β‑ecdysone, corresponding to 0 hAPF. Larval wing discs that were not exposed to β‑ecdysone prior to trypsin exposure do not evaginate, but flatten in their apical-basal height. Any changes in cell height, no matter if their occur during evagination or not, appear to be energy dependent and involve myosin (Fekete et al., 1975). These results have two important consequences. First, maintenance of larval wing disc shape, especially apicobasal height is ECM dependent and second temporally regulated degradation of ECM might play a role for evagination.

Knockdown of collagen IV produced in the fat body leads to the same flattening in larval wing discs that is observed with trypsin treatment, indicating that collagen is synthesized by the fat body, and incorporated into the basement membrane and that the basement membrane is necessary for proper wing disc shape (Pastor-Pareja & Xu, 2011). Consequentially, the role of basal ECM to maintain and generate tissue shape has recently gained attention. As the wing disc grows, the cells of the wing pouch extent their height and the curvature of the wing pouch increases, forming a thick dome. Computational approaches suggest that while the cellular actomyosin is required for generating epithelial bending, ECM pre‑strain maintains the shape. Moreover, acute inhibition of myosin contractility, unlike ECM degradation, does not impact tissue shape (Nematbakhsh et al., 2020). A recent preprint addresses the interaction between cell layer and ECM during growth and suggest that spatial growth anisotropy between ECM and the cell layer lead to epithelial doming (Harmansa et al., 2022). These intriguing, preliminary results highlight another potential role of the basal ECM for wing disc shape which is complementary to the role of basal relaxation during fold formation (Sui et al., 2018).

Collagen IV is cleaved after 2hr of exposure to 20-hydroxyecdysone, its cleavage products can be detected by western blot in early prepupa stages, increase up to 2-3 days APF and only decrease at eclosion (Fessler et al., 1993). ECM dynamics and cleavage are regulated by different proteases, which are specific to basal or apical ECM. Collagen degradation is regulated by Matrix metalloproteinase 1 and 2 (Mmp1/2) and the Tissue inhibitor of metalloproteinases (Timp). Both Mmps have membrane tethered and secreted isoforms and are inactive until an autoinhibitory region is cleaved (LaFever et al., 2017). Mmp1 is necessary for collagen removal around 3 hAPF in the wing disc, as the pouch forms a wing bilayer (De las Heras et al., 2018). Moreover, *Mmp1* and *Mmp2* are both expressed in the stalk of wing imaginal discs and are critical for disc eversion and invasion of the larval epithelium (Srivastava et al., 2007).

In 1993 Appel et al., showed that the apical ECM protease *Stubble (Sb)* is expressed at puparium formation and Sb mutant leg discs are delayed in evagination. This early expression of an apical protease is in accordance with reports that both apical and basal matrix are remodeled during the columnar-to-cuboidal cell shape changes at wing expansion (5-7 hAPF). Interestingly the apical ECM, visualized by the large ZP-domain protein Dumpy (DP-YFP), is removed completely, while the basal ECM, visualized by *Drosophila* Collagen IV (Collagen IV α2-subunit, encoded by *viking*, Vkg-GFP) remains partially in place (Diaz-de-la-Loza et al., 2018). The inhibition apical and basal proteases or knockdown of *Stubble* and overexpression of *Timp* blocks the elongation of the wing (Diaz-de-la-Loza et al., 2018). Interestingly the morphogenetic transformation between wing and haltere can be attributed by Ultrabithorax (Ubx) mediated inhibition of apical proteases *Sb* and *Notopleural* (*Np*) and overexpression of *Timp*. Both Sb, Np, Mmp1 and Mmp2 are abundant at 4 hAPF wings but, with exception of Mmp2, not in halteres (Diaz-de-la-Loza et al., 2020).

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finish with summary of what we understand and what not (eversion)

How there are novel mechanisms and general mechanisms of 3D shape deformation…

Evagination marks the transition from the wing disc to the future evaginated wing that consist of distinct blade, hinge and thorax. For simplicity I will continue to refer to the wing disc at prepupal stages if the entire structure is concerned until thorax fusion around 6 hAPF (Martín-Blanco et al., 2000).