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The cellular dynamics and epithelial morphogenesis in *Drosophila* wing development

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Department of Developmental Biology, Institute of Molecular and Cell Biology, University of Tartu, Estonia

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"Tell me and I forget, teach me and I may remember, involve me and I learn."

Benjamin Franklin

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications. The figures and content are referred to according to the Roman numerals. The articles are re-printed with the kind permission from the publishers.

Ref I
Tran NV, Montanari MP, Gui J, Lubenets D, Fischbach LL, Antson H, Huang Y, Brutus E, Okada Y, Ishimoto Y, Tõnissoo T, Shimmi O. Programmed disassembly of a microtubule-based membrane protrusion network coordinates 3D epithelial morphogenesis in *Drosophila*. EMBO J. 2024 Feb; 43(4):568–594. doi: 10.1038/s44318-023-00025-w. Epub 2024 Jan 23. PMID: 38263333.

Ref II
Tran NV, Montanari MP, Lubenets D, Fischbach LL, Antson H, Okada Y, Ishimoto Y, Tõnissoo T, Shimmi O. α-Spectrin regulates cell shape changes during disassembly of microtubule-driven protrusions in *Drosophila* wings. MicroPubl Biol. 2024 Apr 15;2024. doi: 10.17912/micropub.biology.001169. PMID: 38690064; PMCID: PMC11058509.

Ref III
Toddie-Moore DJ, Montanari MP, **Tran NV**, Brik EM, Antson H, Salazar-Ciudad I, Shimmi O. Mechano-chemical feedback mediated competition for BMP signaling leads to pattern formation. Dev Biol. 2022 Jan; 481:43–51. doi: 10.1016/j.ydbio.2021.09.006. Epub 2021 Sep 20. PMID: 34555363.

The author's contributions to the listed articles were as follows:

Ref I: Tran NV conceptualized, investigated experiments, visualized results and methodology. Tran NV co-wrote the manuscript with Montanari MP and Shimmi O.

Ref II: Tran NV conceptualized, investigated experiments, visualized results and methodology. Tran NV co-wrote the manuscript with Shimmi O.

Ref III: Tran NV investigated experiments for Figures 3G, 3I, and Figure 4G and performed related statistical analyses.

ABBREVIATIONS

2D	Two Dimension
3D	Three Dimension
APF	After Puparium Formation
A-P	Anterior-Posterior
BM	Basement Membrane
BMP	Bone Morphogenetic Protein
CI	Confidence Intervals
CVs	Cross Veins
DLG1	Discs Large Homolog 1
Dgo	Diego
DN	Dominant Negative
DP	Disc Proper
Ds	Dachsous
Dsh	Dishevelled
ECM	Extracellular Matrix
EMT	Epithelial-Mesenchymal Transition
Fmi	Flamingo
Fj	Four-joints
Fz	Frizzled
Ft	Fat
GFP	Green Fluorescent Protein
GIANI	General Image Analysis Of Nuclei-Based Images
GSC	Germline Stem Cell
GTP	Nucleotide Guanosine Triphosphate
IFT-B	Intraflagellar Transport-B
IPAN	Interplanar Amida Network
wL3	wing imaginal disc from late larval stage
LVs	Longitudinal Veins
MAP2	Microtubule-Associated Protein 2
MF	Lateral Microfilament
MT	Microtubule
MTOC	Microtubule Organizing Centers
MYO10	Motor Myosin 10
P-D	Proximal-Distal
PC	Pheochromocytoma
PCM	Pericentriolar Material
PCP	Planar Cell Polarity
PCV	Posterior Crossvein
Pk	Prickle
PM	Peripodial Membrane
Tkv	Thickveins
RFP	Red Fluorescent Protein

RNAi	RNA interference
ROI	Region Of Interest
Stbm	Strabismus
TCA	Transalar Cytoskeletal Arrays
TNT	Tunneling Nanotube
UAS	Upstream Activating Sequence

ABSTRACT

Epithelial cells play a fundamental role in the formation of organs during animal development. To form three-dimensional (3D) tissue structures and organs from their progenitors, cells must adapt their shape appropriately. However, the relationship between cellular changes and dynamic tissue morphogenesis is not well understood. Therefore, it is crucial to establish a system that allows us to observe real-time changes in cell shape to gain a better understanding of this process.

This thesis investigates how changes in cell shape coordinate 3D morphogenesis during two successive developmental stages of *Drosophila melanogaster* (referred to as *Drosophila* throughout the thesis) pupal wing: inflation and second apposition (or reapposition).

In the inflation stage, the dynamics of microtubule-based membrane protrusions were elucidated, leading to the identification of cellular structure termed the Interplanar Amida Network (IPAN). To better understand cellular dynamics, advanced bio-imaging protocols were developed, encompassing *in vivo* live imaging and five-dimensional (5D) dataset analysis. The findings indicate that IPAN constitutes a 3D meshwork structure formed by dorsal and ventral cells, characterized by vertical microtubule (MT) protrusions and lateral microfilament (MF) extensions. The programmed disassembly of IPAN involves the loss of cell-cell contact and the degeneration of aligned microtubules, which is necessary for mitosis and coordinated tissue growth. In addition, it was observed that cell shape changes led to a transition from non-centrosomal to centrosomal microtubule-organizing centers, underscoring the significance of IPAN in providing a unique framework for 3D morphogenesis.

The research also focuses on cell shape changes during the disassembly of microtubule protrusions. It was found that α-Spectrin plays an essential role in regulating cell shape changes through actomyosin networks. α-Spectrin is also crucial for apical relaxation, which is important for robust 3D tissue morphogenesis. When the function of α-Spectrin is lost, the cell membrane becomes more relaxed, directly affecting the temporal sequence of microtubule structure changes and the robustness of IPAN structure dynamics.

In the second-apposition stage, the study aimed to understand the relationship between cell shape alteration and Bone Morphogenetic Protein (BMP) signaling in posterior crossvein (PCV) morphogenesis in the *Drosophila* pupal wing. The results show that apical constriction facilitates BMP signal refinement to differentiate wing progenitor cells into veins or intervein cells.

Overall, this study revealed that the development of 3D structures in *Drosophila* pupal wings involves different types of cell shape changes. Live imaging using confocal microscopy revealed that loss of cell-cell contact regulates mitosis, supporting 3D tissue growth in both epithelium layers. Additionally, the study found that cell shape changes during IPAN disassembly are regulated by α-Spectrin. Furthermore, a feedback loop between changes in cell shape and BMP signaling was observed, leading to self-organizing refinement of the developmental PCV field during pattern formation.

1. REVIEW OF THE LITERATURE

In my literature review, I discuss the changes in cell shape that occur during epithelial morphogenesis. These changes are caused by several distinct processes including apical constriction and convergent extension. If these processes fail, it can disrupt the overall morphology of the 3D functional tissue. To better understand how these different processes are utilized for accomplishing various dynamic morphogenesis events, I analyze cell behaviors during three successive developmental stages of *Drosophila* pupal wing development: first apposition, inflation, and second apposition (or reapposition).

During the early stages of wing development in a pupa, the wings undergo elongation through convergent extension, which changes the shape of the cells from columnar to cuboidal. In the inflation stage, cells continue to interact with each other even when the two epithelial layers are separated. While protruding cells have been observed during this stage, the mechanisms of intercellular communication remain unclear. Finally, in the second apposition stage, apical constriction and elongation are observed, which reshape the wing's final form and define the pattern of its veins.

In the following section, I discuss the interactions between cell shape changes and morphogenesis in *Drosophila*. One of the focuses is on the role of cellular protrusions in different contexts, and their function in long-distance cellular interactions. These protrusions serve as a unique communication mechanism that allows cells to come into direct physical contact with each other, leading to specificity in signaling.

Lastly, I provide a brief summary of the key features of refining the signaling of Bone Morphogenetic Protein (BMP) and the process of wing vein differentiation in the second apposition stage of pupal wing development. This coupling is crucial for the wing vein morphogenesis of the posterior crossvein.

1.1 Cellular dynamics processes in epithelial morphogenesis

Epithelial morphogenesis is a crucial process in multicellular organisms. During morphogenesis, planar sheets of epithelial cells transform into complex structures that shape the body during development and organ formation. The formation of functional 3D structures requires coordinated space and time interactions between cells and the extracellular matrix (ECM) (Dickinson, 2020; Martín-Blanco & Knust, 2001; Osterfield et al., 2017). To better understand how these dynamic processes interact at scale to form functional 3D structures, it is necessary to image and manipulate them in real-time in living models. Recent advancements in high-resolution live imaging across various organisms help us comprehend the cellular dynamics in 3D epithelial formation under specific circumstances.

Epithelial morphogenesis involves three primary processes. The first step includes morphological gradients or fate-determining genes that act early, imparting different transcriptional programs to cells and resulting in different fates. In the second step, cells interpret these spatial signals and are activated into specific morphogenetic events, leading to unique cell behaviors within the entire epithelial sheet or a particular subset of cells. Finally, cells attain a specific morphogenetic program that requires the precise coordination of cell shape and cell-cell adhesion properties. This process may also involve proliferation and cell death patterns, making it a complex route that plays an essential role in developing the final epithelial tissue structure (Gillard & Röper, 2020; Kiehart et al., 2017; Osterfield et al., 2017).

Although the morphogenetic events vary across different animal species and at various times, two common events take place in epithelial morphogenesis: apical constriction and convergent extension. These changes in cell shape are commonly observed throughout the animal kingdom.

1.1.1 Apical constriction

Apical constriction is a process that involves the contraction of the apical part of a cell, which causes the nucleus to move downwards. This process has been extensively studied in fly embryos during ventral furrow formation over the past years (Holcomb et al., 2021; Leptin, 1999). The process starts with the accumulation of activated myosin II in the apical compartment of the cell. Then, it binds to actin filaments at adhesion junctions and induces the reduction of the actomyosin cable. These networks undergo pulsatile contractions that narrow the cell's apical surface, transforming it from a columnar shape with roughly equal top and bottom surfaces to a wedge shape with a narrow top surface and a wide bottom surface. These pulsatile contractions are stabilized in the cell and maintain tension and tissue integrity (Figure 1A), (Le & Chung, 2021; Perez-Vale & Peifer, 2020; van der Spuy et al., 2023).

Epithelial cells use apical constriction to form various structures, which is a crucial process in morphogenetic events like gastrulation in invertebrates. In *Drosophila*, several processes were reported to fulfill apical constriction. For example, individual cells employ it during neural stem cell invasion (Shard et al., 2020). A small group of cells can also use it to produce invagination during salivary duct formation (Figure 1B), (Girdler & Röper, 2014). Similarly, apical constriction is also utilized by cell groups programmed along the body axis during Drosophila mesoderm invasion (Osterfield et al., 2013; Perez-Vale & Peifer, 2020).

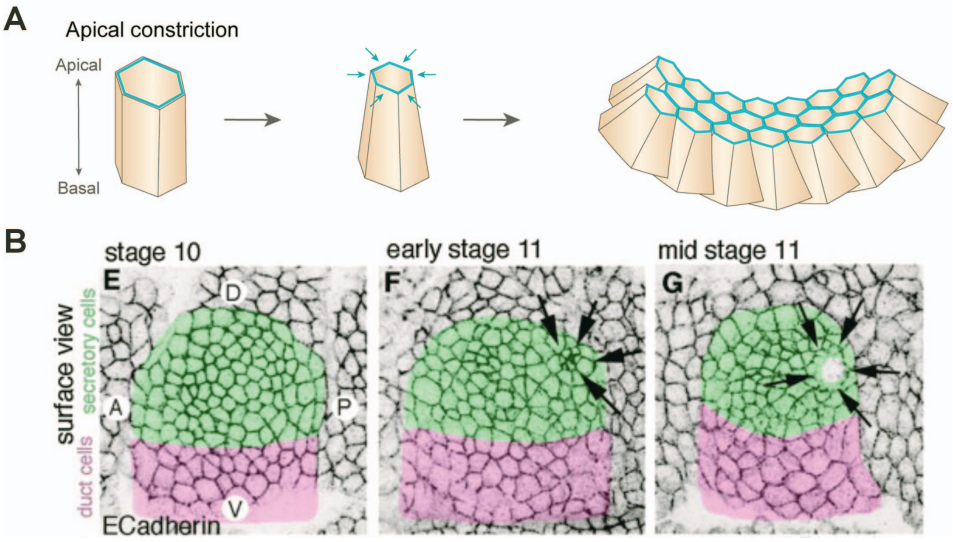

Figure 1. Apical constriction process. **A.** Schematic showing how apical actomyosin (blue) contraction causes apical constriction, which reduces cell surface area and bends epithelial tissue. **B.** Time lapses of apical constriction during salivary gland placode development in *Drosophila*. At stage 10, apices in the placode are not constricted. At early stage 11, apices in the dorsal-posterior corner start to narrow, but no invagination occurs yet. At mid-stage 11, a small invaginating pit forms. E-Cadherin marks the apical surface during stages 10 to mid-stage 11. Secretory cells are green, and duct cells are magenta. The arrows indicate the cells that are undergoing apical constriction. (**A**) modified from (van der Spuy et al., 2023), (**B**) modified from (Girdler & Röper, 2014).

1.1.2 Convergent extension

A convergent extension is the second type of cellular shape change that occurs during morphogenesis in many animals. During this process, cells sense planar polarization at the global tissue level, causing the tissue to elongate (extend) along one axis and narrow (converge) in one or both of the other axes (Figure 2A) (Shindo, 2018; van der Spuy et al., 2023). This phenomenon was first observed during the formation of the notochord in the African-clawed frog (*Xenopus laevis*) embryos (Keller et al., 1985; Winklbauer & Nagel, 1991). Since then, it has been extensively researched in various contexts, such as epithelial cells during *Drosophila* germband expansion (Irvine & Wieschaus, 1994; Lecuit & Lenne, 2007), *Drosophila* wing development (Diaz-de-la-Loza et al., 2018; Etournay et al., 2015), and a region of the *Drosophila* leg imaginal disc during its evagination (Condic et al., 1991).

In the development of *Drosophila* wings, the process of polarized junction remodeling is crucial. This process enables the rearrangement of cells through convergence and extension. The junctions that connect neighboring cells shorten and eventually fuse two adjacent cells together. As a result, actomyosin accumulates on the proximal and distal cell surfaces, providing the contractile force

required to elongate the wings along the proximal-distal axis. This extension process leads to wings that are twice as long as they are wide in just about 5 hours after pupariation (Figure 2B) (Diaz-de-la-Loza et al., 2018).

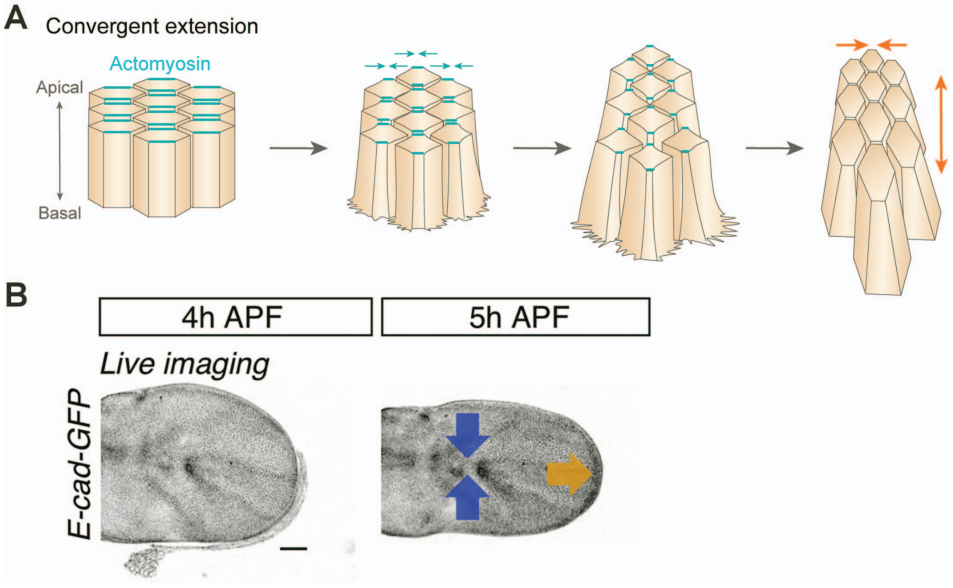

Figure 2. Convergent extension process. **A.** Schematic illustrating how the process of convergent extension is responsible for the narrowing and lengthening of the tissue. **B.** During 4–5 h after puparium formation (APF), the *Drosophila* pupal wing narrows (blue arrow) and lengthens (orange arrow) due to convergent extension. (**A**) modified from (van der Spuy et al., 2023), (**B**) modified from (Diaz-de-la-Loza et al., 2018).

1.2 *Drosophila* pupal wing morphogenesis

1.2.1 The pupal wing is a model system for studying 3D morphogenesis

The pupal wing of *Drosophila* is an excellent model for studying 3D epithelial morphogenesis and organogenesis due to its unique characteristics. The pupae have a high resistance to phototoxicity. The pupal outer cases could be removed without interfering with normal growth, making it possible to apply modern microscopy techniques. Although technically challenging, spinning disk confocal microscopy, high-resolution laser scanning microscopy, and light sheet microscopy can be used to detect the natural wing development process for several hours (Classen et al., 2008; Etournay et al., 2015; Gui et al., 2019; T. Sun et al., 2021).

In 2010 and 2013, two studies were conducted to characterize the tissue dynamics involved in converting an imaginal disc into a 3D pupal wing via an *ex vivo* approach. These studies helped reveal the mechanisms and processes of *Drosophila* imaginal wing disc inversion, paving the way for further research in

this field (Aldaz et al., 2010, 2013). Nowadays, it is widely agreed that the development processes that occur within the first 24 hours after puparium formation (hAPF) can be divided into three stages: first apposition (0 h – 10 h APF), inflation (10 h – 20 h APF), and second apposition (after 20 h APF) (Fuhrmann et al., 2024; Gui et al., 2019; Montanari et al., 2022; Ray et al., 2015; Waddington, 1940) (Figure 3A). In detail, during the initial stages of development, the wings increase in size through first apposition and inflation. In the second-apposition stage of development, the wings undergo differentiation and patterning to form the veins. An adult *Drosophila* wing typically features five longitudinal veins (LVs) and two cross veins (CVs), which are essential components for a normal wing (Figure 3B, C).

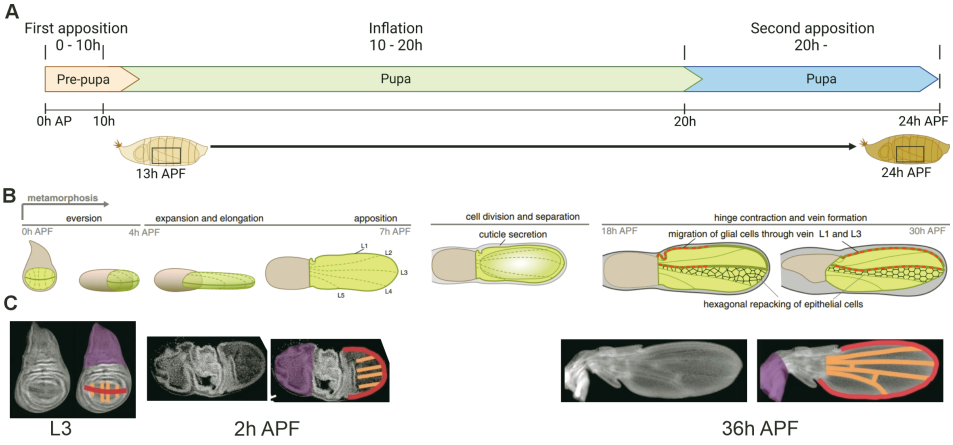

Figure 3. *Drosophila* pupal wing development in a nutshell. **A.** The pupal wing (in black box) development timeline from 0 h to 24 h APF with accompanying schematics in **(B)** and actual tissue samples in **(C)**. The orange line marks the wing veins, the red marks the wing blade, the magenta marks the wing notum. Modified from (Diaz de la Loza & Thompson, 2017; Sobala & Adler, 2016).

Fluorescence microscopy is an incredibly useful tool by itself. However, its advantages could be greatly increased by linking target proteins to fluorescent tags via genetic manipulations. Nowadays, scientists are able to manipulate various tissues in *Drosophila*, create transgenic lines, and modify gene function with precision. This approach also involves combining expression systems with optogenetic techniques and nanobody trapping methods, which allows researchers to study gene expression and protein function at different stages of development with great accuracy (Alessandra Vigano et al., 2021; Del Valle Rodríguez et al., 2012; Gyoergy et al., 2018; Honda, 2022; Matsuda et al., 2022; Riemensperger et al., 2016).

Combining high-resolution time-lapse 3D imaging with genetic manipulations allows scientists to visualize the internal structures of living cells and track their movement and shape changes. Various fluorescent protein tools can image cellular components such as membranes, nuclei, and cytoskeletal markers. For instance,

advances in quantitative analysis enable comprehensive characterization of apical cell shape changes, tissue movements, and characterization of morphogenetic events using *Drosophila* wings with GFP-tagged E-Cadherin (Etournay et al., 2015; Stegmaier et al., 2016).

Open-source software tools have been developed to support the management of 5D datasets, including the x, y, and z dimensions, as well as the t (time) and λ (wavelength) dimensions. These tools significantly simplify the scientific analysis of large datasets. For example, Generic Image Analysis of Nuclear-Based Images (GIANI) is an open-source software that can facilitate the segmentation of nuclei and cells in 3D microscope images using an ImageJ plugin. Another ImageJ plugin, Tissue Analyzer (formerly Packaging Analyzer), can detect cell borders automatically. Users can adjust parameters for automatic segmentation and manually correct cell borders as needed. On the other hand, IMARIS is a commercial platform for 3D dataset management and offers various powerful tools for 3D visualization, feature recognition, and tracking (Barry et al., 2022; Tinevez et al., 2017).

1.2.2 Transition from larval to pupal stage: Extracellular matrix remodelling and Myosin II contractility control morphogenesis

During the third larval instar, wing discs appear flat compared to the 3D shape of wings in the pupal stage. At this moment, the wing imaginal disc forms a circular cluster of epithelial cells called the wing pouch, which consists of thin layers (Fuhrmann et al., 2024).

During the metamorphosis, the wing discs undergo an inversion, causing the wing pouch region to turn outward and form a double-layered pupal wing comprised of dorsal and ventral layers attached to each other. This stage takes place at approximately 8h APF and is known as the first apposition stage (Figure 4) (Fristrom et al., 1993). As a result, these tissues' elongation occurs coordinately through a process of convergent extension and expansion. During this stage, the size and shape of the tissues are remodeled, depending entirely on changes in cell shape and the direction of cell rearrangement (Aldaz et al., 2010; Diaz-de-la-Loza et al., 2018; Matamoro-Vidal et al., 2015).

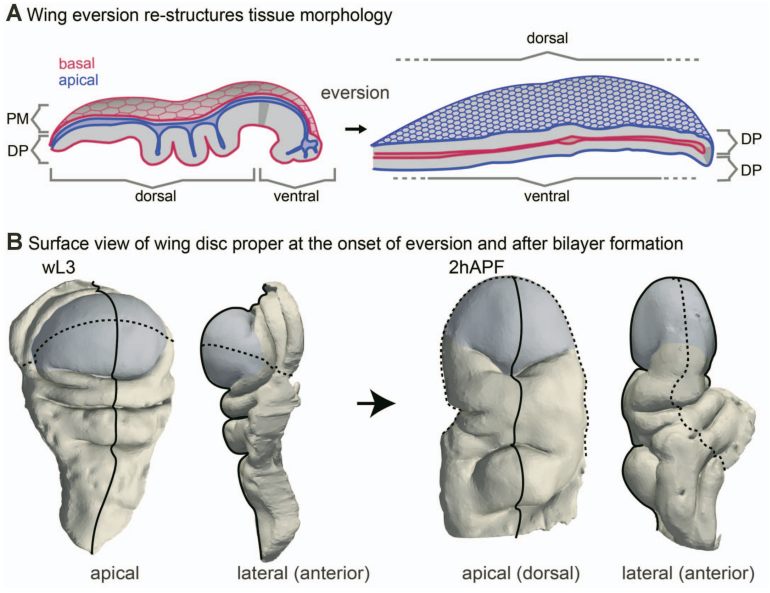

Figure 4. The wing imaginal disc undergoes eversion during pupal wing formation, turning inside out. **A.** The cross-section of wing imaginal disc and pupal wing (2 h APF) after eversion. Wing imaginal disc tissue has two layers: Disc Proper (DP) – thick, folded, pseudo-stratified monolayer and Peripodial Membrane (PM) – thin, squamous monolayer. **B.** The 3D view of the wing imaginal disc from the late larval stage (wL3) can be viewed at the start of the eversion (left) and the pupal wing after bilayer formation (right). This figure was originally presented in (Fuhrmann et al., 2024).

1.2.2.1 Cell shape shange from a columnar to cuboidal shape

During the first apposition stage, there are two distinct phases: the convergent extension phase (4 h–6 h APF) and the expansion phase (6 h–8 h APF). The size and shape of the cell change significantly during these two stages, with cells transitioning from a columnar to a cuboidal shape. This leads to decreased cell height and increased cell base area (Figure 5), (Lemke & Nelson, 2021). But what causes these significant cellular changes during this period?

The changes in cell shape during this stage are primarily caused by extracellular matrix (ECM) remodeling. When studying the ECM in pupal wings from 4 h to 8 h APF, four major matrix components (Collagen IV, Laminin, Perlecan, and Nidogen) were significantly reduced compared to their amounts at the onset of metamorphosis. Additionally, preventing ECM degradation is sufficient to avoid wing elongation (T. Sun et al., 2021). Furthermore, interfering with Integrins, which are distributed in the basolateral membranes of the cells in the early stages of the evagination process, fails to achieve proper contact between the dorsal and ventral epithelium during their apposition (Brabant et al., 1996; Diaz-de-la-Loza et al., 2018; Domínguez-Giménez et al., 2007; Fernandes et al., 2014; Fristrom et al., 1993). This suggests that the temporal control of ECM degradation is necessary to facilitate cell shape change during wing morphogenesis.

The transformation from columnar to cuboidal shape is responsible for the increase in the wing surface. However, this does not fully explain how the wing can double in length along the P-D axis while contracting along the anterior-posterior (A-P) axis in just a few hours of first apposition. During the pupal wing's convergent extension and expansion phase, there is a phenomenon known as planar polarization of non-muscle myosin IIA (MyoII) activity. This was first observed during gastrulation in the *Drosophila* germband extension (Bertet et al., 2004). In the wing, cells have varying levels of MyoII localization along their A-P and P-D boundaries. At 4 h – 5 h APF, MyoII becomes concentrated at the P-D boundary, with a 2-fold increase in fluorescence intensity along the P-D axis compared to the A-P axis. As the tissue expands isotropically from 6 h to 8 h APF, it relocates from the apical to the lateral compartments of the cell. Furthermore, Rho kinase (Rok), which generally phosphorylates and activates MyoII, localizes at the same site as MyoII at 4 h APF, and by 7 h APF, it disperses apically. Inhibition of Rok severely affected the elongation of the wing between 5.5 h and 7 h APF. Therefore, MyoII polarization activity plays a vital role in causing the constriction of cell-cell contacts between P-D neighbors and drives axial convergent extension, ultimately leading to cell shape change and tissue elongation (Athilingam et al., 2021; Diaz-de-la-Loza et al., 2018).

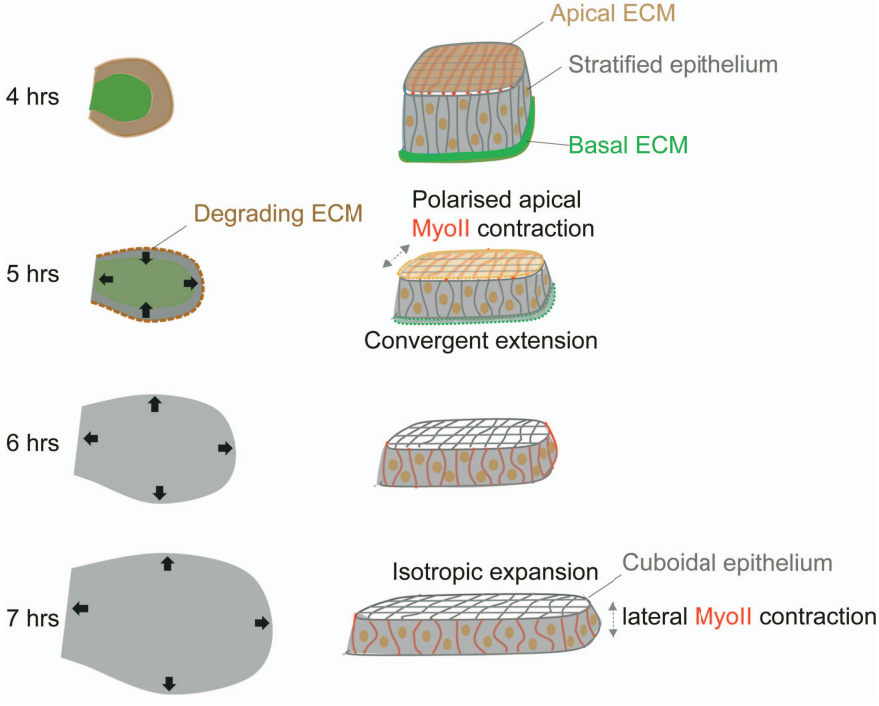

Figure 5. Cells change their shape from columnar to cuboidal during the first apposition stage. This transformation is brought about by the degradation of the extracellular matrix (ECM) and polarized apical non-muscular Myosin IIA (MyoII) contraction. The left panel (top view) represents wing blade structures, whereas the right panel (side view) depicts cell-shaped structures. This figure was originally presented by (Athilingam et al., 2021).

1.2.2.2 Oriented cell rearrangements play a major role during first apposition

During the transition from the late third instar larval stage to the pupal stage, the elongation of wings involves various rearrangement events among cells. Two separate studies examined the development of cell clones. They found that during the early pupal stages, the cell population in a patched domain (a marker for cells in the center of the wing) and Raeppli clones (a whole-tissue labeling tool for live imaging) rearrange to become narrower along the A-P axis and more elongated along the P-D axis compared to their larval phase (Kanca et al., 2014; Taylor & Adler, 2008). In a recent study, researchers used GFP-tagged E-Cadherin live imaging to observe cell rearrangements during convergent extension and expansion periods. The study revealed that during convergent extension, the epithelium contracts along the A-P axis through cell rearrangements, transitions (T1, T2), the formation of rosettes, and changes in cell shape. They also found that cell rearrangement only occurs during convergent extension and contributes to wing elongation only for a short and specific period (Figure 6), (Diaz-de-la-Loza et al., 2018).

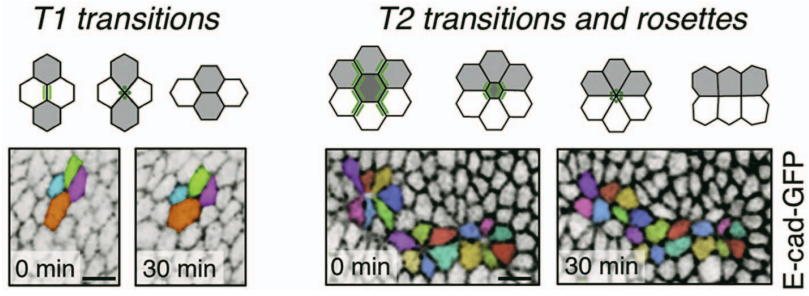

Figure 6. During convergent extension (4 h 30min to 5 h APF), epithelia contract along the A-P axis by cell rearrangements (T1 in the left panel, T2 transitions, and rosettes in the right panel). Cell tracking labeling by colored cells demonstrates these cell shape changes. Scale bar: 10 μm. This figure was originally presented in (Diaz-de-la-Loza et al., 2018).

To elaborate, tissue elongation through T1 transition is achieved by the contraction and growth of new cell boundaries. In a four-cell array, neighboring cells in the new axis have an extended range of cell-cell contact while neighboring cells in the old axis remain separate, leading to cell-cell interphase events and, ultimately, tissue elongation in a new axis. During the T2 transition, cells are eliminated from the monolayer through extrusion/apoptosis events (Lemke & Nelson, 2021; Walck-Shannon & Hardin, 2014).

Meanwhile, multicellular rosettes are formed in wing tissue where apices of 5 to 11 cells meet. The behavior of the rosette structures is similar to that of the four-cell array. Contacts between neighboring cells shorten until they meet to form a pie-shaped structure. Then, contact between neighboring cells in the new

direction increases, causing neighboring cells in the original direction to move away from each other along the resolution axis (Figure 7) (Lemke & Nelson, 2021; Walck-Shannon & Hardin, 2014).

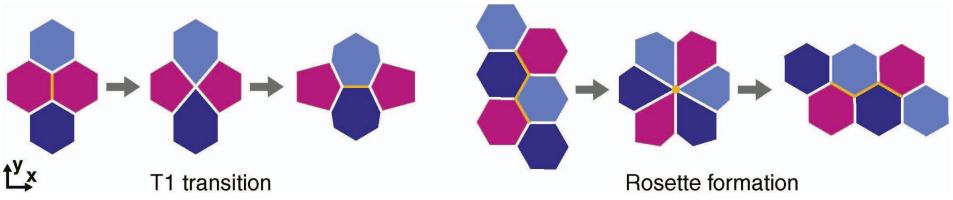

Figure 7. Cell rearrangement motifs in T1 transition (left panel) and Rosette formation (right panel). This figure was originally presented in (Lemke & Nelson, 2021).

1.2.2.3 Cell division

It has been observed that during the transition from the larval to the pupal stage, the process of cell division comes to a halt in the G2 phase. Between 4 and 7 h APF, only a few cells undergo mitosis. This observation confirms that the elongation of the wing occurs through cell shape change and cell rearrangements during the first apposition stage (Etournay et al., 2015).

1.2.3 The inflation stage: The dorsal and ventral communication

1.2.3.1 The dorsal and ventral epithelium is communicated via protrusions

After flattering during the first stage of development, the wing undergoes a process called inflation. This process involves two significant changes. At first, during the separation stage, the basal domains of dorsal and ventral cells of the wing gradually separate due to an increase in the internal pressure of the hemolymph (Brabant et al., 1996). This separation occurs at 8 h to 9 h APF and reaches its maximum at 12 h APF. At this stage, the gas bubble in the abdomen is expelled, and the head everts, marking the transition from pupariation to pupation (Schoborg et al., 2019; Ward et al., 2003).

After this, from 13 h to 20 h APF, the inflation stage of wing development continues. During this phase, the dorsal and ventral layers that form the wings grow in shape and size. Cells previously arrested in G2 until the first apposition stage start proliferating and undergoing mitosis (Etournay et al., 2015). However, it is not yet fully understood how the growth of the two physically separated epithelial layers is coordinated.

Previous research has suggested that transalar cytoskeletal arrays (TCAs) composed of microtubules (MTs) and microfilaments link the dorsal and ventral epithelia (Fristrom et al., 1993), providing a clue to this question. Communication between dorsal and ventral cells has been previously observed (García-Bellido, 1977), and recent studies have emphasized its importance for wing growth and

patterning (Gui et al., 2019). Live imaging in a recent study revealed that long protrusions connect the dorsal and ventral epithelia and attach to the two epithelial layers via a basal integrin-laminin complex. This highlights unique cellular adhesion mechanisms that support the formation of 3D organs during pupal wing development (Singh et al., 2024; T. Sun et al., 2021) (Figure 8). Although TCAs are proposed to hold the dorsal and ventral epithelium together to ensure proper mature wing structure, their role in wing growth remains not yet fully understood.

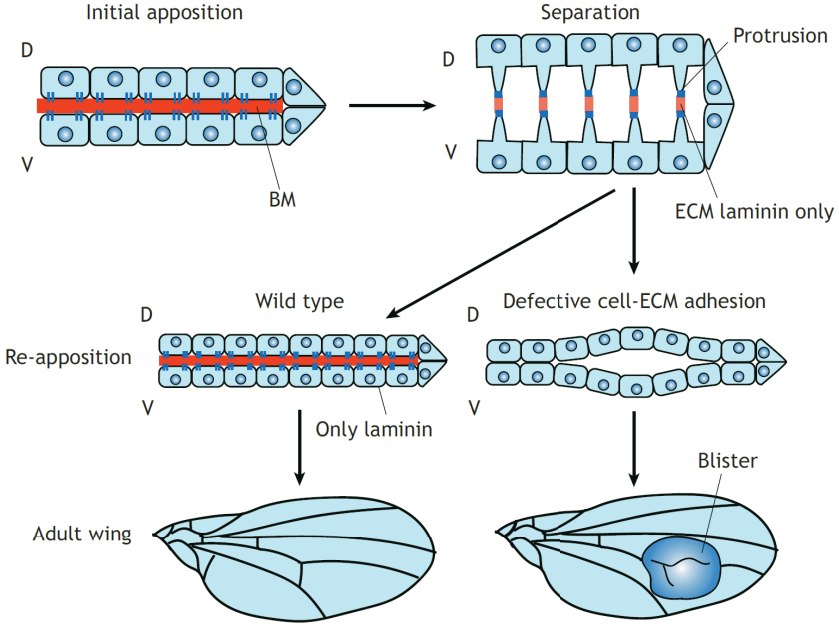

Figure 8. The two epithelial layers (D, dorsal, V, ventral) initially oppose and later separate in the inflation stage, remaining connected by membrane protrusions. Basement membrane/ extracellular matrix (BM/ECM: red) and integrins (blue) are present in the schematic. If there is a defect in the adhesion between the cells and the ECM, it can lead to the formation of blisters during the re-apposition stage, which are visible in adult wings. This figure was originally presented in (Yamaguchi & Knaut, 2022).

1.2.3.2 Dynamic interplay of polarized MT forces drives tissue extension

During the later inflation stage, between 15 h and 20 h APF, the pupal wing continues to undergo remodeling. The wing length increases to three times its width, and it becomes symmetrical in shape relative to the anterior-posterior boundary. The wing has two regions: the proximal wing hinge and the distal wing blade, but their shapes remain indistinct at 15 h APF. Contraction of the hinge is essential during this stage to help the wing achieve its optimal shape by 24 h APF (Figure 9A, B), (Etournay et al., 2015; Matamoro-Vidal et al., 2015). The wings also grow in size due to active mitosis, which means that both hinge contraction and cell division generate tension, leading to changes in tissue shape. This allows

the wing to elongate along the proximal-distal axis and narrow it along the anterior-posterior axis, ultimately changing the overall shape of the wing (Figure 9B), (Aigouy et al., 2010; Xiong et al., 2014).

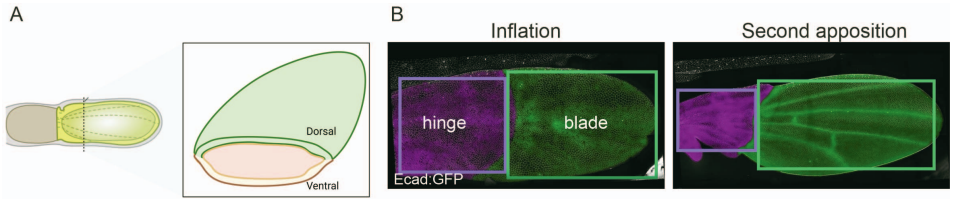

Figure 9. The hinge contraction takes place in the inflation stage. **A.** Schematic of the pupal wing in the inflation stage, dorsal and ventral layers separate in this stage. **B.** The hinge contraction process is tracked by Ecad:GFP. The hinge region is marked in magenta, and the wing blade region is marked in green. Modified from (Diaz de la Loza & Thompson, 2017; Etournay et al., 2015).

Recent research has indicated that MTs are crucial in tissue remodeling from 15 h to 20 h APF. Non-centrosomal MTs form bundles that align with the proximal-distal axis, and they work in conjunction with the Fat-PCP system at adherens junctions to regulate cell behavior and coordinate the collective behavior of cells during tissue remodeling (Figure 10), (Matis, 2020; Röper, 2020; Singh et al., 2018, 2024). However, there is still a lack of understanding regarding cell shape change and tissue dynamics between 15 h – 20 h APF as the dorsal and ventral layers of the wing remain separated. Therefore, further studies are needed to analyze the collaboration between the dorsal and ventral parts and provide a comprehensive understanding of the 3D structural dynamics.

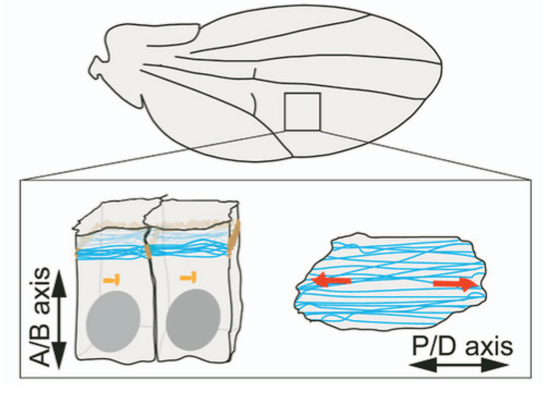

Figure 10. Schematic of *Drosophila* wing epithelium displaying the patterning of the MT array (blue). Left panel: side view, A/B: apical/basal. Right panel: top view, red arrow marks the direction of MT arrays along the P-D axis. This figure was originally presented in (Matis, 2020; Singh et al., 2018, 2024).

1.2.3.3 Non-centrosomal MTs and associated proteins

During the development of a pupal wing, apical junctions arrange non-centrosomal MTs into a polarized network, which is crucial for tissue elongation (Matis, 2020; Singh et al., 2018, 2024). To regulate the non-centrosomal organization of MTs, proteins such as ninein anchor and stabilize MTs by binding to γ-tubulin, which is a tubulin superfamily specialized for microtubule nucleation, and relocate to apical sites outside the centrosome. Additionally, proteins that bind to minus ends independently of γ-tubulin also play a role in this process (Panzade & Matis, 2021; Paz & Lüders, 2018; Sanchez & Feldman, 2017; Wu & Akhmanova, 2017). Several regulatory proteins are required to regulate non-centrosomal MTs in response to external forces. In this session, I will discuss the primary proteins that regulate MT dynamics. Even though some of these regulatory proteins have not been tested in the context of the wing, they will be used to manipulate the dynamics of MTs in my project.

Tau: is a protein found in both humans and *Drosophila* which is important for the proper functioning of the nervous system. It is similar to a protein called microtubule-associated protein 2 (MAP2) which stabilizes MTs in mammals. In *Drosophila*, mutations in the Tau protein can result in neurodegenerative processes and abnormalities in photoreceptor development (Wang & Mandelkow, 2016; K. Zhang et al., 2021).

Katanin: is an enzyme that plays a key role in regulating the length and number of MTs. It does so by breaking existing MTs, which helps to destabilize them. For instance, in the salivary gland, placode cells of *Drosophila*, Katanin, and the minus-end-binding protein Patronin accumulate in an apical-medial position. Katanin severs the single active centrosome, releasing MT minus-ends, which are then anchored by apical-medial Patronin. This promotes the formation of the longitudinal MT array, which is crucial for apical constriction and tube formation (Gillard et al., 2021; Mao et al., 2014).

Patronin: The Calmodulin-regulated Spectrin-Associated Protein (CAMSAP) family in vertebrates and Patronin in invertebrates are responsible for regulating the organization of MTs in a variety of specialized cells. Patronin is capable of identifying and decorating the free minus ends of MTs and offers protection to non-centrosomal MTs from depolymerization (Khanal et al., 2016; Nashchekin et al., 2016; Panzade & Matis, 2021; Takeichi & Toya, 2016).

Spectraplakin Shortstop (Shot): is a linker protein found in all cells of the epithelium of mammals. It acts as a cross-linker between actin, MTs, and the cell membrane. Shot is a large protein that can recruit Patronin to form ncMTOCs, which are a center of growing MTs from stabilized minus-ends. These two proteins' functions have been well studied in *Drosophila* oocytes and microvilli. Shot/Patronin ncMTOCs are independent of γ-tubulin (Khanal et al., 2016; Nashchekin et al., 2016, 2021; Takeichi & Toya, 2016). In *Drosophila* wing cells, on the other hand, Shot is required for stabilizing minus-ends of MTs on the apical compartment and also maintaining cell-cell contacts on the basal compartment (T. Sun et al., 2021).

Spectrin: is a protein that interacts with MTs and actin filaments, but it does not regulate MT dynamics. The cell cortex is a structure located beneath the plasma membrane, which comprises actin filaments and actin-binding proteins. Spectrin plays a critical role in this structure and is responsible for creating the spectrin-actin network by forming tetramers that bind to actin filaments. In polarized epithelial cells, spectrin can be found in either the apical or basolateral domain. Apical spectrin is responsible for bringing Patronin and Shot proteins to the apical membrane. Any mutation in *spectrin* can result in the abnormal placement of Patronin and Shot, which can disrupt the epithelial tissue (Deng et al., 2020; Fletcher et al., 2015; Forest et al., 2018; Khanal et al., 2016).

1.2.4 Second-apposition stage: Core and Fat planar cell polarity dynamics shape the wing

Around 20 h APF, the pupal wing enters the second apposition. During this stage, two layers of epithelial cells re-oppose each other. The rate of cell proliferation significantly reduces and cell shape change facilitates wing elongation and vein patterning. The proximal hinge region of the wing contracts, causing mechanical stress on the blade region (Etournay et al., 2015; Ray et al., 2015).

It is worth noting that during the first apposition, the pupal wing elongates along the P-D axis owing to the planar polarization of MyoII activity, while the second apposition stage regulates the wing's final shape via the global axis of planar cell polarity (Brittle et al., 2022; Merkel et al., 2014; Olofsson et al., 2014).

1.2.4.1 Core planar cell polarity system

Planar cell polarity (PCP) is responsible for aligning cells in epithelial sheets, which is critical for tissue development and maintenance. PCP proteins are distributed asymmetrically based on external cues that create intracellularly polarized domains. In *Drosophila* wing development, there are two PCP systems, Core PCP and Fat PCP, which form asymmetric complexes at cell junctions that help to couple adjacent cells' polarity and develop tissue-wide P-D axis patterns (Butler & Wallingford, 2017; Olguin & Mlodzik, 2010; Strutt & Strutt, 2015).

The Core PCP system comprises six components that form complexes in the cell junctions. Half of the complex consists of a homodimer of the cadherin Flamingo (Fmi), the seven-pass transmembrane protein Frizzled (Fz), the cytoplasmic proteins Dishevelled (Dsh), and Diego (Dgo) on a distal junction. These interact with the four-pass transmembrane protein Strabismus (Stbm, also known as Van Gogh) and a cytoplasmic protein Prickle (Pk) on the proximal junction (Figure 11A).

During the development of wings, Core PCP complexes segregate with opposite polarities to different sides of the cell, generating intracellular polarity coupled between neighboring cells (Figure 11A'). In the tissue scale, global patterns change dynamically during wing morphogenesis. The Core PCP reorients

from a radial to P-D orientation between 16 and 30 h APF, establishing global cues for cellular movements (Figure 11A''). The new PCP axis aligns parallel to the orientation of new cell-cell junctions. In detail, Fz-Dzh localizes towards the wing margin early in development, while later on (24 h – 30 h APF), asymmetric PCP patterns align along the P-D axis of the tissue with Fz-Dsh oriented towards it (Aigouy et al., 2010; Brittle et al., 2022; Butler & Wallingford, 2017; Merkel et al., 2014; Olofsson et al., 2014; Strutt & Strutt, 2015).

A recent study was conducted to test the hypothesis that Core PCP guides tissue morphogenesis in the *Drosophila* wing. Laser ablation experiments and a rheological model found that while Core PCP mutations affect the fast response to laser ablation, they do not significantly affect overall tissue mechanics. The study suggests that Core PCP is required only as an orientational guiding cue for a short time during *Drosophila* pupal wing morphogenesis (Piscitello-Gómez et al., 2023).

1.2.4.2 Fat planar cell polarity system

On the other hand, the Fat PCP system involves two unique cadherins, Fat (Ft) and Dachsous (Ds), that interact with each other. The kinase Four-joints (Fj) promotes Fat activity. Ft and Ds cadherin complexes are on opposite cell edges (Figure 11B, B'). The expression levels of Ds and Fj vary along the anterior-posterior axis. Four-joint kinase suppresses Ds but promotes Ft, leading to biased activity along the anterior-posterior axis. Ultimately, Ft binds to Ds locally. In the pupal wing, Fat PCP is influenced only near the wing hinge. Between 16 and 30 h APF, Fat PCP reorients from radial to anterior-posterior polarity (Figure 11B'') (Merkel et al., 2014). Recently, one study argued that Fat PCP provides strong gradient cues to shape the P-D axis and establish cellular asymmetries in protein distribution via graded expression of its components. These asymmetries are then potentially amplified across the whole wing (Brittle et al., 2022). In addition, the Fat PCP system has another function to align an apical non-centrosomal MT network at the adherens junction, which patterns MT-based forces across a tissue via polarized transcellular stability (Olofsson et al., 2014; Singh et al., 2018, 2024).

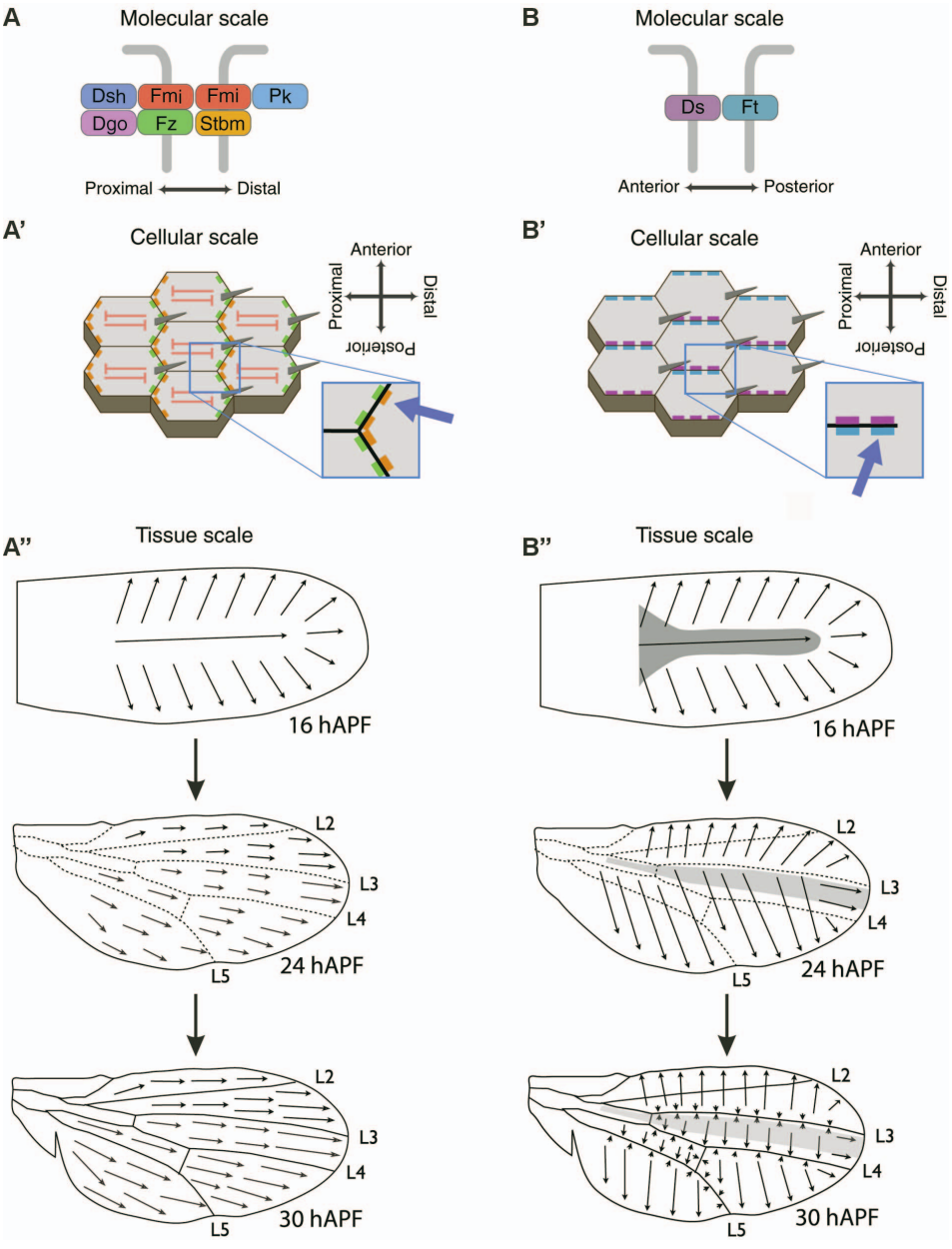

Figure 11. Organization of the Core and Fat PCP systems in the pupal wing. Schematics in **A**, **A**', and **A**'' depict the molecular, cellular, and tissue scales of the Core PCP systems, respectively. Schematics in **B**, **B**'', and **B**'' depict the molecular, cellular, and tissue scales of the Fat PCP systems, respectively. L2, L3, L4, L5 are longitudinal veins in wing. Arrows mean the Core PCP reorients from radial to P-D polarity, whereas Fat PCP reorients from radial to A-P polarity between 16 h and 30 h APF. Modified in (Brittle et al., 2022; Merkel et al., 2014).

1.3 The diversity of cell communication via cellular protrusions

In recent years, various types of cellular protrusions have been reported in different organisms. Despite the confusing description and terminology, these protrusions exhibit unique structural and functional characteristics that have led to the creation of new terms such as thin membrane protrusions, membrane extensions, cellular bridges, lamellipodia, signaling filopodia, cytonemes, microtubule nanotubes, and tunneling nanotubes.

Proper cellular communication is essential for the functioning of biological processes. Cells rely on the ability to accurately receive, process, and respond to signals to perform various biological functions. Recently, cell-to-cell communication modes involving membrane protrusions have been proposed. These types of communication allow for selective signal transmission over varying distances, unlike traditional secretion and diffusion-based intercellular communication (Figure 12A). These protrusions can be distinguished based on their diameter, length, and the cytoskeletal filaments involved in their formation (Figure 12B) (Buszczak et al., 2016; Caviglia & Ober, 2018; Inaba et al., 2022; Sagar et al., 2015; Z. Sun et al., 2017).

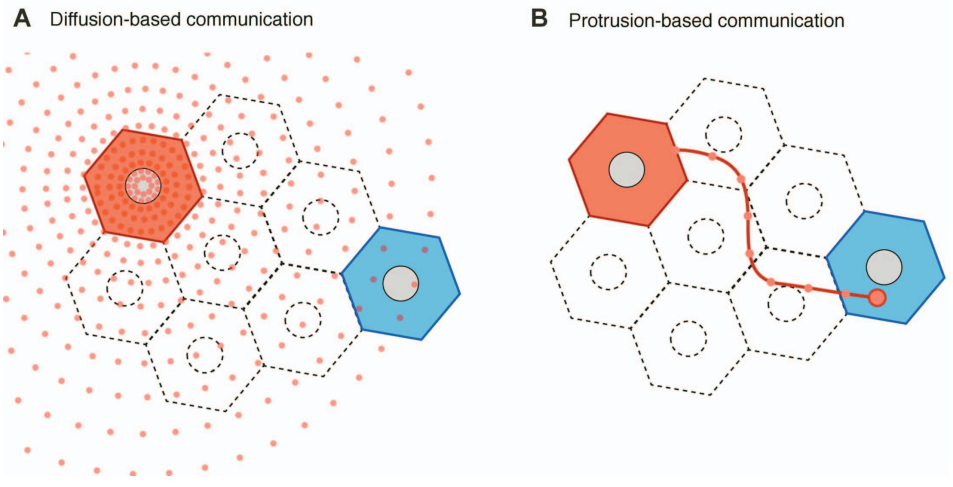

Figure 12. Two modes of cell communication. **A.** Diffusion-based communication. **B.** Protrusion-based communication. The sending cell is red, and the receiving cell is blue. The red dots indicate the signal transmitted by the sending cell. Modified from (Caviglia & Ober, 2018).

In the context of *Drosophila*, three distinct types of cellular protrusions are particularly relevant for cell communication and the transport of different types of molecules. These protrusions are known as cytonemes, MT nanotubes, and tunneling nanotubes (TNTs) (Figure 13). Firstly, cytonemes are membrane protrusions that primarily rely on actin filaments and transport specific ligands and

their receptors to distal cells. They may also contain MTs at their base and are sometimes referred to as signaling filopodia. The second type is signaling MT-nanotubes, closed-ended MT-based membrane protrusions that transport specific receptors by reaching a short range of the signal-sending cell. Finally, tunneling nanotubes (TNTs) are open-ended protrusions that connect the cytoplasm between cells. They are also known as filopodia tubes, bridges, or conduits (Buszczak et al., 2016; González-Méndez et al., 2019).

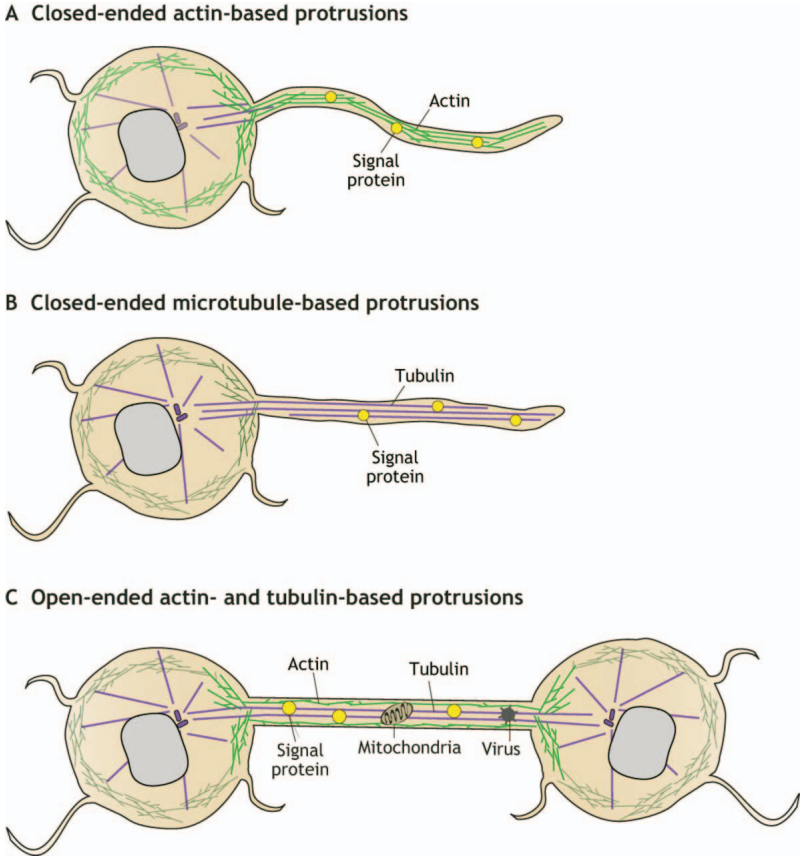

Figure 13. Three types of membrane protrusions are used for cell signaling. **A.** Cytonemes. **B.** MT-nanotubes or closed-ended MT-based protrusions. **C.** Tunneling nanotubes (TNTs) or open-ended actin- and tubulin-based protrusion. This figure was originally presented by (González-Méndez et al., 2019).

1.3.1 A Model for cytoneme-mediated signaling in *Drosophila* wing disc

The first type of cellular protrusions that have been extensively studied for intercellular communication are cytonemes. They are specialized filopodia considered responsible for intercellular signaling and were first discovered in Drosophila's wing imaginal disc cells. These thin fluorescent projections emanate from GFP-

containing cells along the lateral flanks, oriented toward the disc center (Ramírez-Weber & Kornberg, 1999). Cytonemes transmit signals between two cells that are positioned far away from each other through direct contact. They can reach lengths of up to 700 μm and have a diameter of 0.2 μm (Kornberg, 2014; Yamashita et al., 2018; C. Zhang & Scholpp, 2019).

Notably, cytonemes can be seen extending over the apical and basal surfaces of polarized columnar epithelial cells in *Drosophila* third-instar wing discs (Figure 14A, B). Previous studies have shown that these cellular extensions are triggered by localized sources of chemoattractants, depending on their direction. For instance, developing neurons use filopodial extensions to sense guidance cues (Vitriol & Zheng, 2012). Similarly, migratory cells use cellular extensions to navigate gradients of chemoattractants and reach their destination (Swaney et al., 2010).

Recent studies have proposed an alternative method of morphogen BMP-type ligand Dpp diffusion in the *Drosophila* wing disc apical surface. It has been observed that cytonemes contain the BMP type-I receptor called Thickvein (Tkv). These cytonemes extend from the receiving cells on both sides to the Dpp-producer cells at the compartment border. These observations suggest that Dpp might not be diffusing randomly to the target cells, but instead transferring physically at specific sites (Figure 14A) (González-Méndez et al., 2019; Kornberg, 2014).

Similarly, the Hedgehog (Hh) signaling protein plays a crucial role in establishing the A-P organizer in the wing disc. Hh forms distinct particles that remain connected to the signal-sending cell through long, thin cytoplasmic protrusions in the basal compartment. These protrusions often extend across several cell diameters. Live-cell imaging revealed particles moving in a direction along cytoneme-like extensions. Signal-responding cells also form thin filopodia-like structures that contain specific subsets of Hh coreceptors called Patched (Ptc). These extensions reach out to contact those extending from Hh-producing cells, suggesting that these protrusions assist with both sending and receiving particular signals over distances of many cell diameters (Figure 14B) (González-Méndez et al., 2019; Kornberg, 2014).

Cytonemes have also been found in cells from different *Drosophila* larval tissues, such as eye-antenna imaginal discs, air sac primordium in tracheal systems, and cells of the abdominal epidermis. These structures can be visualized using soluble and membrane-bound GFP in live tissue (Buszczak et al., 2016; González-Méndez et al., 2019; C. Zhang & Scholpp, 2019).

In addition to existing research on cytonemes in *Drosophila*, a recent study highlighted the significance of cytonemes in the morphogenesis patterning of the dorsal neural tube in mice. According to the imaging datasets, cytonemes play a crucial role in the deployment of sonic hedgehog during neurodevelopment. The study also identified motor myosin 10 (MYO10) as a regulator of cytoneme function in this model (Hall et al., 2024).

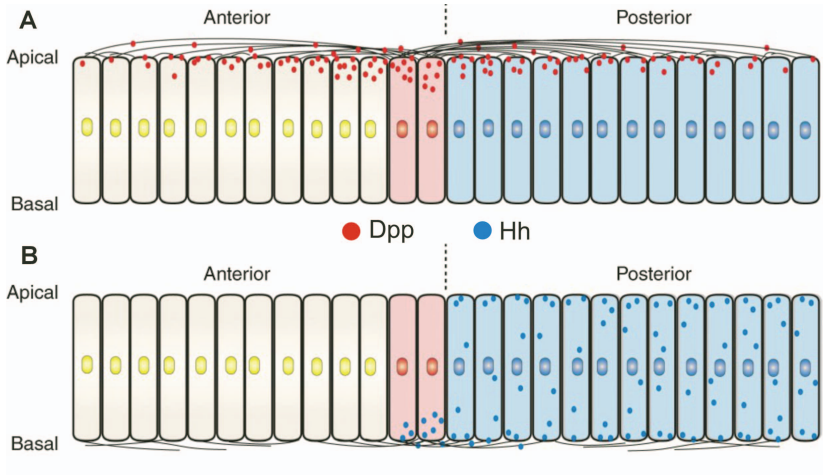

Figure 14. Cytoneme-mediated signaling in *Drosophila* wing disc. **A.** The columnar epithelium of the wing disc can be illustrated in transverse sections indicating that Dpp (red dots) moves from the anterior/posterior signaling center via apical cytonemes. **B**. Basal cytonemes transfer Hh (blue dots) from the cells that produce it in the posterior compartment to the cells in the anterior compartment, crossing the compartment border (dashed line). Modified from (Kornberg, 2014).

1.3.2 MT Nanotubes provide for short-range signaling in male germline stem cells of *Drosophila*

The second type of cellular membrane protrusion is MT nanotubes. These were first discovered in male germline stem cells (GSCs) of *Drosophila*. Unlike cytonemes, MT nanotubes consist primarily of MT, facilitating short-range signaling rather than long-range intercellular communication (Figure 15), (Inaba et al., 2015; Kornberg & Gilboa, 2015).

Live-cell imaging experiments have revealed that thin MT nanotubes extend into the hub cell cluster and transmit Dpp signals back to GSCs from their niche, similar to cytonemes. The Tkv receptor for Dpp is localized to specific puncta that move along the MT nanotubes to receive the Dpp ligand expressed by the niche cells. Colocalization analysis suggests that Dpp produced by the hub cells binds to the Tkv receptor expressed by GSCs on the surface of MT nanotubes. Additionally, research has shown that increasing the thickness of MT nanotubes by reducing the activity of the kinesin Klp10A enhances Dpp signaling while reducing the frequency of MT nanotubes by using an intraflagellar transport-B (IFT-B) mutant, which disrupts the formation of MT nanotubes, reduces Dpp signal transduction. These findings suggest that the components involved in Dpp/BMP signaling may promote the formation and stabilization of MT nanotubes (Amoyel & Bach, 2015; Buszczak et al., 2016; Inaba et al., 2015; Yamashita et al., 2018).

Further research is needed to understand the role of MT nanotubes in organisms other than the GSCs of *Drosophila*, as well as how the movement in and

out of these extensions is controlled. The discovery of MT nanotubes provides a new way to study communication between stem cells and specialized cells in an *in vivo* model.

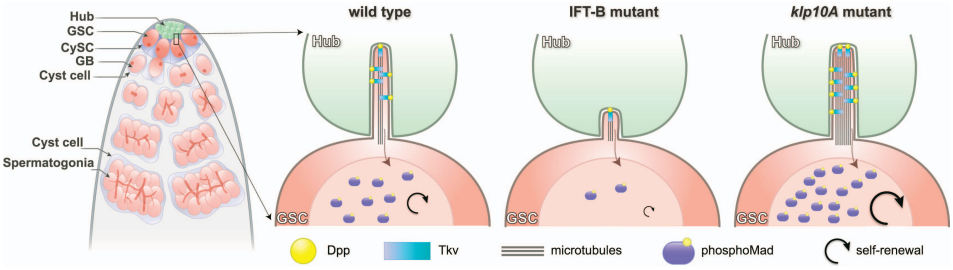

Figure 15. Membrane protrusions known as MT-nanotubes extend from GSCs towards hub cells. The BMP receptor, Tkv, travels along these structures and gets activated by Dpp on the MT nanotubes. Three different conditions of wild type, IFT-B mutant, and *klp10A* mutant, are presented in the schematic. GSC, germline stem cells; CySC, cyst stem cells; GB, gonialblast. This figure was originally presented in (Amoyel & Bach, 2015).

1.3.3 Tunneling nanotubes in cell – cell communication

Tunneling Nanotubes (TNTs) were discovered in cultured cells back in 2004. These tiny tubes connect the plasma membranes of cells, creating a continuity between them (Figure 16) (Rustom et al., 2004). TNTs were initially characterized by their tunneling ability, which means they connect the cytoplasm of two cells and are open-ended. TNTs can transfer organelles such as mitochondria and lysosomes, endosome vesicles, pathogens, prions, and bacteria. They can also propagate cell death signals, including the transfer of active caspases, and affect membrane potential and calcium signals (Buszczak et al., 2016; González-Méndez et al., 2019; Yamashita et al., 2018).

Recent studies have confirmed the existence of TNTs *in vivo* models. Scientists have discovered membrane nanotubes that display TNT characteristics in the corneal stroma of mice using mosaic/chimeric or transgenic conditions (Chinnery et al., 2008). A critical study was conducted on thin cellular protrusions crucial in transporting active Wnt5b-Ror2 complexes in zebrafish (*Danio rerio*) embryos (C. Zhang et al., 2024). Despite initial confusion, the study showed that these protrusions, named “cytonemes” can transport both signal and receptor, exhibiting TNT and not just cytoneme characteristics. This was the first time protein complex transport had been observed through live imaging outside of *in vitro* cell cultures. The discovery has significant implications for developmental biology.

To date, no structures resembling TNT have been observed in *Drosophila*. However, current studies are exploring the existence of an open-ended tube in the *Drosophila* pupal wing model and investigating dynamic trafficking through the protrusion network of cells (Shimmi O, unpublished data).

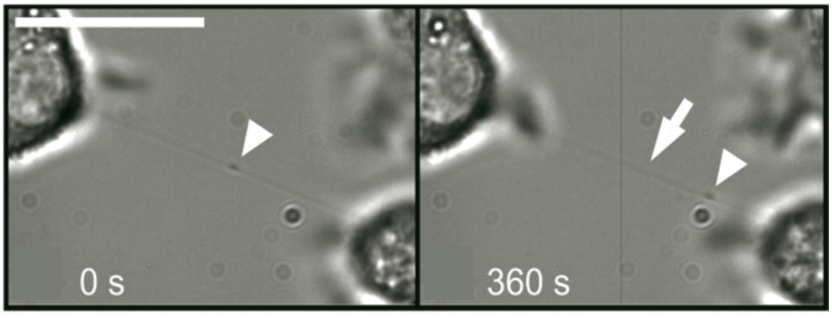

Figure 16. *De novo* formation of TNTs of PC12 cells, which are an immortalized cell line derived from rat pheochromocytoma that resembles primary cultures of fetal neurons. An arrowhead indicates an object traveling along TNTs, and an arrow indicates the starting position. The images show the object at 0s and 360s. Scale bar: 20 μm. This figure was originally presented in (Rustom et al., 2004).

1.4 Coupling between Bone morphogenetic protein signaling and morphogenesis in *Drosophila* pupal wing

1.4.1 Overview of the canonical BMP pathway in *Drosophila*

Bone morphogenic protein (BMP) signal transduction pathway plays a critical role in morphogenesis, growth, and pattern formation during the development of wing veins at the pupal stage of the *Drosophila* life cycle (Matsuda & Shimmi, 2012; Shimmi et al., 2014). The BMP ligand Decapentaplegic (Dpp) can form a homodimer or, alternatively, heterodimers with two other *Drosophila* BMP ligands (Gbb or Scw). The dimeric ligand binds to tetrameric receptor complexes comprising Type I receptors Tkv/Sax and Type II receptors Wit/Punt at the plasma membrane. Once activated, Tkv phosphorylates the transcription factor Mad, a reliable indicator of the BMP/Dpp pathway activity. pMad combines with the co-SMAD Medea and moves into the nucleus to transcribe many target transcription factor genes, including *spalt* (*sal*), *optomotor blind* (*omb*), and the anti-Smad, *Daughters against Dpp* (*dad*) (Figure 17) (Affolter et al., 2001; Montanari & Tran et al., 2022).

Dad serves as a negative feedback regulator of the pathway by inhibiting Type-I receptor activation and subsequent Mad recruitment. While the expression of some genes is upregulated by BMP/Dpp signals, others, like Brinker (Brk), get suppressed. Therefore, the expression patterns of Dad and Brk are frequently utilized to assess BMP signaling activity positively and negatively in developing the *Drosophila* wing (Figure 17), (Montanari & Tran et al., 2022).

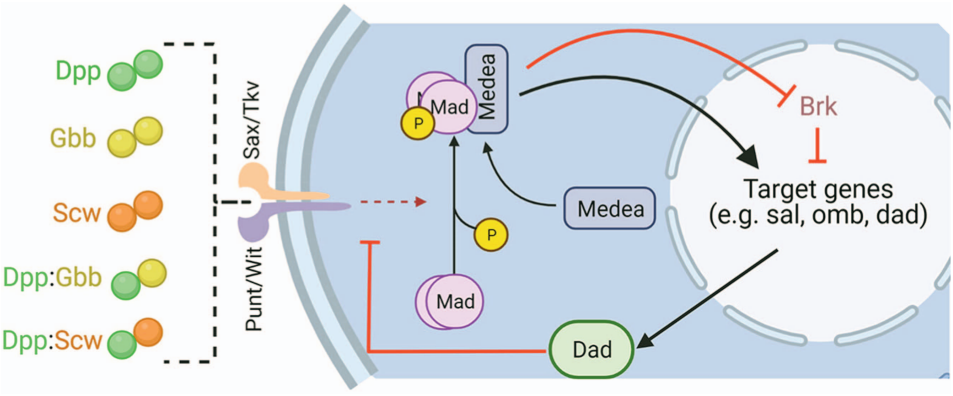

Figure 17. BMP signal transduction in *Drosophila*. BMP signal transduction in *Drosophila* begins with the association of Dpp homodimers with tetrameric receptor complexes consisting of Tkv/Sax and Wit/Punt. Activated receptors phosphorylate Mad, accumulating in the nucleus to modulate gene expression. This figure was originally presented in (Montanari & Tran et al., 2022).

1.4.2 BMP signaling and morphogenesis in *Drosophila* pupal wing

The pupal wing of the *Drosophila* is an excellent model for studying BMP signaling and morphogenesis. Although BMP is known to determine wing veins in the pupal wing, recent studies suggest that BMP signaling coordinates both growth and patterning/differentiation during this stage. When *dpp* is conditionally knocked out in the pupal wing, tissue size is reduced and vein formation is impaired. It has been found that BMP signaling plays a complex role in coordinating growth, which requires broader tissue-level control, as well as vein development that is specific to wing vein progenitors. However, it has been observed that Dpp forms a long-range signal during the early pupal stage (proliferation stage), and later operates over a short-range during differentiation (Gui et al., 2019; Montanari & Tran, 2022).

Interestingly, the change in spatial distribution of BMP signaling coincides with changes in 3D tissue architecture. When the two epithelial layers separate during inflation, BMP helps sustain cell proliferation by lateral long-range trafficking. When the two epithelial layers come together, the BMP signaling range becomes more refined laterally, with active vertical Dpp trafficking between the two epithelial sheets for wing vein patterning/differentiation. The idea that 3D tissue architecture itself plays an instructive role in signal distribution is further supported by experiments that artificially change 3D tissue architecture, which sufficiently changes the spatial distribution of the BMP signal (Figure 18) (Gui et al., 2019; Montanari & Tran, 2022).

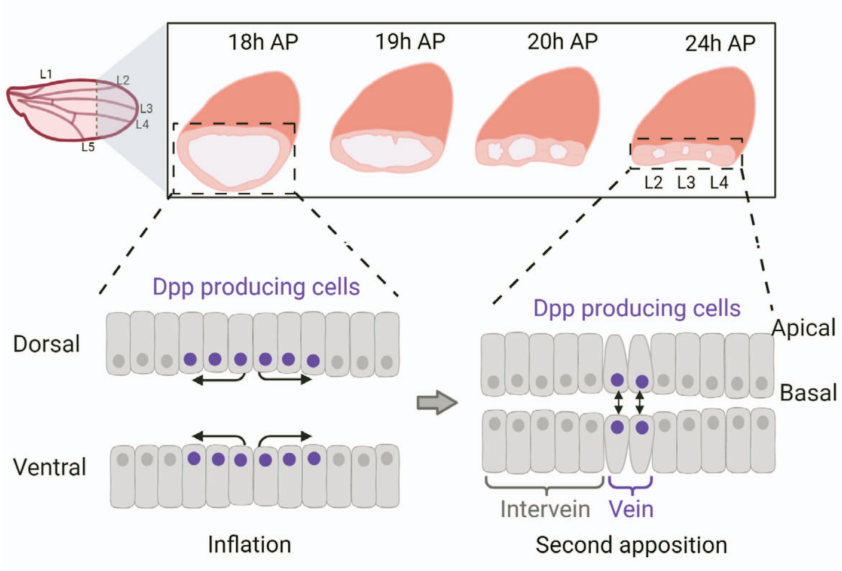

Figure 18. Coupling between 3D tissue architecture and BMP signaling. The diagram illustrates the coupling between 3D tissue dynamics and BMP signal. Dpp controls tissue growth and vein differentiation by diffusing laterally during inflation and later signaling between dorsal and ventral cells to refine vein patterning and differentiation. This figure was originally presented in (Montanari & Tran et al., 2022).

1.4.3 Posterior crossvein development and BMP signaling

Drosophila wings have five longitudinal veins (LVs) and two cross veins (CVs), which are important features of the normal wing (Gui et al., 2019; Matsuda & Shimmi, 2012; Shimmi et al., 2014).

The posterior crossvein (PCV) development provides a unique model to address how spatiotemporal regulation of BMP signaling leads to wing vein patterning in *Drosophila* pupal wing. During the early stages of pupal development, the *dpp* gene is expressed in the longitudinal veins (LVs), and a positive feedback mechanism involving Tkv is used to keep Dpp ligands specifically in the LVs. This mechanism is important for maintaining vein width. However, between 18 h – 26 h APF, Dpp ligands are transported to the PCV region to show long-range signaling, and Dpp signaling is observed in all veins. This is achieved through facilitated transport, whereby Dpp ligands are carried from the LVs to the posterior crossvein region (Antson et al., 2022; Montanari & Tran et al., 2022).

The cells in the longitudinal veins produce Dpp or Dpp: Gbb and hold it in a complex with BMP-binding proteins like Sog and Tsg-related *Crossveinless* complex. This complex is then transported through a facilitated mechanism. Once it reaches the posterior crossvein, Sog is cleaved by an extracellular protease called Tolloid-related (Tlr), releasing the Dpp:Gbb heterodimer from the ligand transporter complex. After this, the released ligands bind to receptors and transduce the signal (Figure 19), (Antson et al., 2022; Montanari & Tran et al., 2022).

The activity of Dpp signaling increases and becomes refined for future PCV cells. However, since most Dpp ligand is immobilized in the LVs, PCV cells receive only limited amounts of Dpp ligands. This limited amount is translated into a solid and robust signal activity, ensuring cell differentiation. It suggests that future PCV cells have evolved specific programs to boost and optimize the Dpp signal.

Previous studies have identified several crucial factors for optimal BMP signaling activity and PCV morphogenesis. These factors include *Crossveinless*-2 (Cv-2), *Crossveinless*-d (Cv-d), and *Crossveinless*-C (Cv-C). Cv-2 is a protein that binds to BMP and interacts with Tkv. Depending on its concentration, it can either enhance or inhibit BMP signaling. The positive feedback mechanism between BMP and Cv-2 ensures that the BMP signal is finely tuned during PCV morphogenesis. Cv-d, on the other hand, is a co-factor for BMP signaling during PCV morphogenesis. It affects the range of BMP movement in the pupal wing and acts as part of a lipid-BMP-lipoprotein complex. Finally, *Cv-C* encodes the RhoGAP88C protein, which inactivates Rho-type small GTPases (including Cdc42, Rac1, Rac2, and Rho1) and downregulates integrins in the PCV field (Antson et al., 2022).

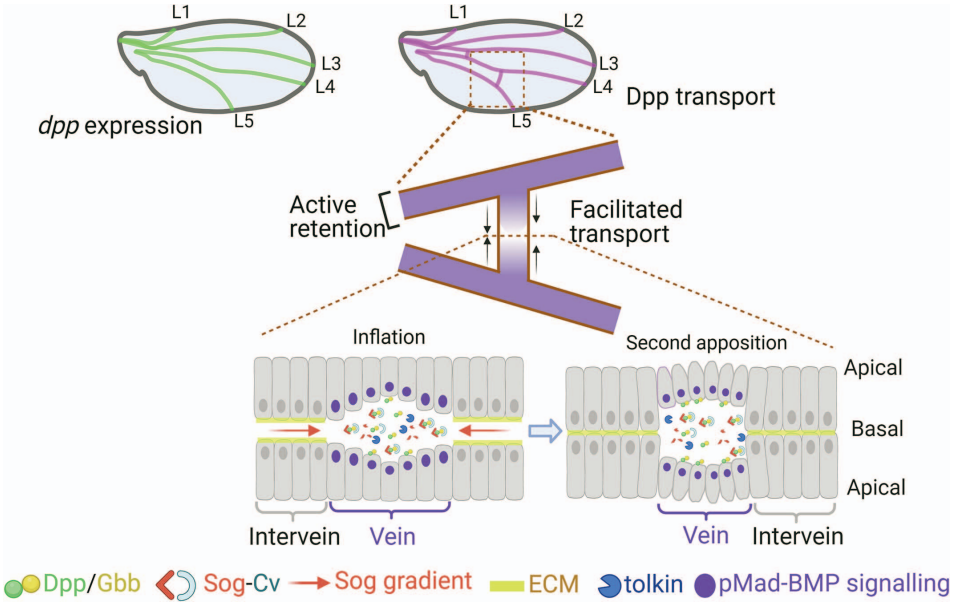

Figure 19. BMP signaling in the posterior crossvein (PCV) morphogenesis. Top: *dpp* mRNA is expressed in the longitudinal veins (L1 – L5) and not in the crossveins during early pupal stages. Whereas, Dpp signals are detected in all wing vein primordia, including crossveins. Bottom: Dpp ligands move from longitudinal veins to posterior crossvein via facilitated transport. Tolkin cleaves Sog, releasing Dpp:Gbb, enabling Dpp:Gbb signaling in the PCV. This figure was originally presented in (Montanari & Tran et al., 2022).

2. AIMS OF THE STUDY

Epithelial morphogenesis plays a crucial role in the formation of 3D tissue structures and organs during animal development. However, our current understanding of how successive cell shape changes are coupled with dynamic tissue morphogenesis is far from complete. To better understand this, there is a need for a system that can observe real-time changes in cell shape.

By employing the *Drosophila* pupal wing as an *in vivo* model, this thesis aims to study the correlation between cell shape changes and 3D morphogenesis during *Drosophila* wing development.

1. To elucidate the programmed disassembly of the microtubule-based membrane protrusion network that coordinates 3D epithelial morphogenesis.
2. To examine the role of α-Spectrin in the regulation of cell shape changes during the disassembly of microtubule protrusions in *Drosophila* pupal wings.
3. To investigate how cell shape changes form a mechanical feedback loop with BMP signaling for posterior cross-vein development in *Drosophila* pupal wings.

3. MATERIALS AND METHODS

The thesis is based on three publications that utilized advanced *in vivo* live imaging techniques and 5D dataset analysis protocols for quantification purposes (Table 1). A brief summary of the methods used is included, but for more detailed information about the experiments and materials, please refer to the original publications (Ref I, II, III) and the corresponding supplemental materials. The schematics in the dissertation were created with Biorender.com.

Table 1. The methods

Fly husbandry	Ref I, II, III
Fly genotypes	Ref I, II, III
Immunofluorochemistry	Ref III
Live imaging	Ref I, II, III
Confocal microscopy	Ref I, II, III
Data analysis with ImageJ	Ref III
Data analysis with Imaris 9.7	Ref I, II, III

3.1 Reference I and II

3.1.1 A non-invasive live imaging protocol to observe pupal wing development

In references I and II, a non-invasive method was developed to directly observe the structure inside the pupal wing during the inflation stage.

To conduct the experiment, crosses were established and maintained at room temperature to produce pupae for imaging. After 13.5 h APF at 22 °C (or equivalent 10.5 h APF at 29 °C), head eversion was used as a developmental marker to determine the suitability of pupae for live imaging.

After inverting the head of the pupa, a micro-scalpel was used to carefully cut open a small window on the pupal case, specifically over the wing area. This pupa was then put under the confocal microscope for live imaging. The protocol allowed the pupal flies to continue to develop normally during the imaging period (Figure 20A, B). The Result and Discussion section will provide detailed information regarding the duration of each experiment and the time intervals of every stack. All time-lapse images were acquired using a Leica TCS SP8 STED 3X CW 3D confocal microscope.

The original confocal data was saved as .lif files and then converted to .ims files using Imaris File Converter from Oxford Instruments. All subsequent analysis was performed using Imaris software from Oxford Instruments.

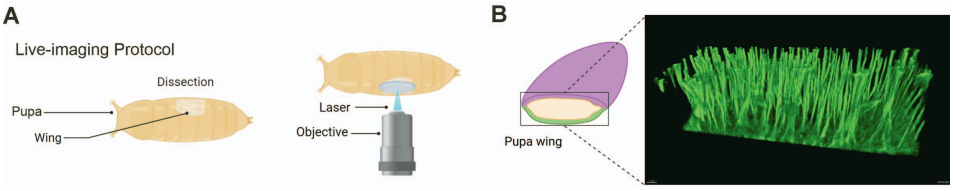

Figure 20. A non-invasive live imaging protocol to observe pupal wing development. **A.** Schematic of dissection procedure: open the wing window and place the sample under the confocal microscope. **B.** Example of the 3D dataset under confocal microscope. Left: schematic for the pupal wing in inflation. Right: 3D view of MT protrusions visualized by αTubulin:GFP. The dorsal epithelium is towards the top of the view.

3.1.2 Quantitative analysis

– Quantifying Mitoses
  During the four-hour imaging period, mitotic cells were manually identified and marked as surfaces in each 3D dataset timeframe. This process was carried out for both dorsal and ventral layers. The total number of surfaces in the final frame represented the total number of mitoses that occurred in the 510×1025 pixel area.

– Quantifying MT Distribution
  To analyze the spatial distribution of GFP-tagged MTs, we extracted the z-profile of GFP intensity for each time point of the dorsal and ventral epithelial layers. Then we calculated the average intensity values of the inter-epithelial space and determined the relative expression level of MT in the interepithelial space by computing the ratio of the interepithelial value to the average of the dorsal and ventral values for each time point.

– Quantifying Cell Distribution Across Stages
  Stage I, II, III, and IV classification of 25 random cells per wing was performed using αTubulin:GFP data. The experiment was repeated four times.

– Quantifying Apical Cell Area
  CAAX:mCherry was expressed and live-imaged for two hours (10.5 h – 12.5 h APF at 29 °C) to observe the apical cell compartment. The apical surfaces of individual cells within the constant region were measured during stages I and II to analyze the apical cell areas.

3.2 Reference III

3.2.1 Immunofluorescence

To conduct immunofluorescence experiments, the pupa was fixed with 3.7% formaldehyde for two overnights. The pupal wing was then dissected for the next staining step. Overnight blocking of the pupal wings was done using normal goat serum (10%). Both primary and secondary antibody incubations were also carried out overnight at 4 °C. The primary antibodies used were mouse anti-DLG1 (1:40; Developmental Studies Hybridoma Bank (DSHB), University of Iowa) and rabbit anti-phospho-SMAD1/5 (1:200; Cell Signaling Technologies). The secondary antibodies used were anti-rabbit IgG Alexa 568 (1:200, Invitrogen), anti-mouse IgG Alexa 647 (1:200; Life Technologies), and anti-rabbit IgG Alexa 647 (1:200; Life Technologies). F-actin was stained with Alexa 488-conjugated phalloidin (1:200; Life Technologies).

3.2.2 Time-Lapse imaging and quantification analysis

The live imaging protocol has been described previously and focuses on the PCV region.

To measure BMP signaling during the BMP refinement period in cells, a specific protocol was used. The first step is to exclude the nearby LV nuclei to determine the number of pMad-positive cells in the PCV field. This can be achieved by marking the predicted path of the LV-PCV boundary and drawing a line across from the edge of L4 and L5 on either side of the PCV. The pMad-positive nuclei that fall between these lines can then be counted.

4. RESULTS AND DISCUSSION

4.1 *Drosophila* pupal wing during inflation as a model for microtubule-based protrusion drive 3D epithelial morphogenesis (Ref I and II)

Although many studies indicate that the pupal wing is one of the good 2D models for epithelial morphogenesis and has been studied for many years, research has mainly focused on the first and second apposition stages (Etournay et al., 2015; Gui et al., 2019; Piscitello-Gómez et al., 2023; Salis et al., 2017). The reason is that during these stages, the two epithelial layers are opposed, allowing researchers to dissect and fix the sample with formaldehyde. However, fixed tissue cannot resolve the structure during the inflation stage when two wing epithelia separate because all structures within the wing are damaged. To address this issue, a non-invasive method was developed to visualize the pupal wing *in vivo* and observe the structure inside the wing during the inflation stage.

4.1.1 The *Drosophila* pupal wing forms a cellular network between dorsal and ventral epithelia during the inflation stage

To begin, we conducted a study using live imaging techniques through a confocal microscope to explore how dorsal and ventral epithelium communicate with each other in a pupal wing during the inflation stage. Previous research has revealed physical links between the two epithelial layers (Fristrom et al., 1993; García-Bellido, 1977; Gui et al., 2019; T. Sun et al., 2021). Our study confirmed the presence of a unique long basal protrusion by imaging GFP-tagged Tubulin (αTubulin:GFP) at 13 h APF. This protrusion consists of MT projections that originate in the apical compartment of the cell and extend basally. MT foci are distributed medioapically on both dorsal and ventral cells (Figure 21 A, B, C, D).

Next, we investigated the MT structures inside cells to determine whether they were nucleated by centrosomal or non-centrosomal MT organizing centers (cMTOCs or ncMTOCs, respectively). To answer this question, we conducted a co-expression experiment using GFP-tagged Patronin. Patronin is an ortholog of human CAMSAP2 and a tubulin-binding factor at the minus end of MTs. It has been identified as one of the key components of ncMTOCs (Nashchekin et al., 2016, 2021). Our data showed that when GFP-tagged Patronin and mCherry-tagged αTubulin were co-expressed, Patronin localized at the minus ends of MT protrusions. Therefore, we concluded that MT protrusions were nucleated by ncMTOCs located in the apical compartment (Figure 21E).

Additionally, we observed pupal wings expressing α-Tubulin:GFP and Life-Act:Ruby (Ref I, Figure 1). We found that MT-based protrusions occur longitudinally, while actin filaments extend both longitudinally and laterally in a thread-like manner. These filaments form a lateral network between the longitudinal protrusions, creating filopodia-like structures. By integrating data from α-Tubulin:

GFP, LifeAct:Ruby, and CAAX:mCherry (Ref I, Figure S1C), we clarify that the pupal wing contains a 3D structure comprising longitudinal MT-based protrusions and lateral extensions formed by actin filaments. Our high-resolution images provide a detailed description of the cell protrusions within the pupal wing and confirm the organizing centers of these MT structures, which have not been mentioned in previous studies. Therefore, we have named this structure the Interplanar Amida Network (IPAN) (Ref I, Figure 1F).

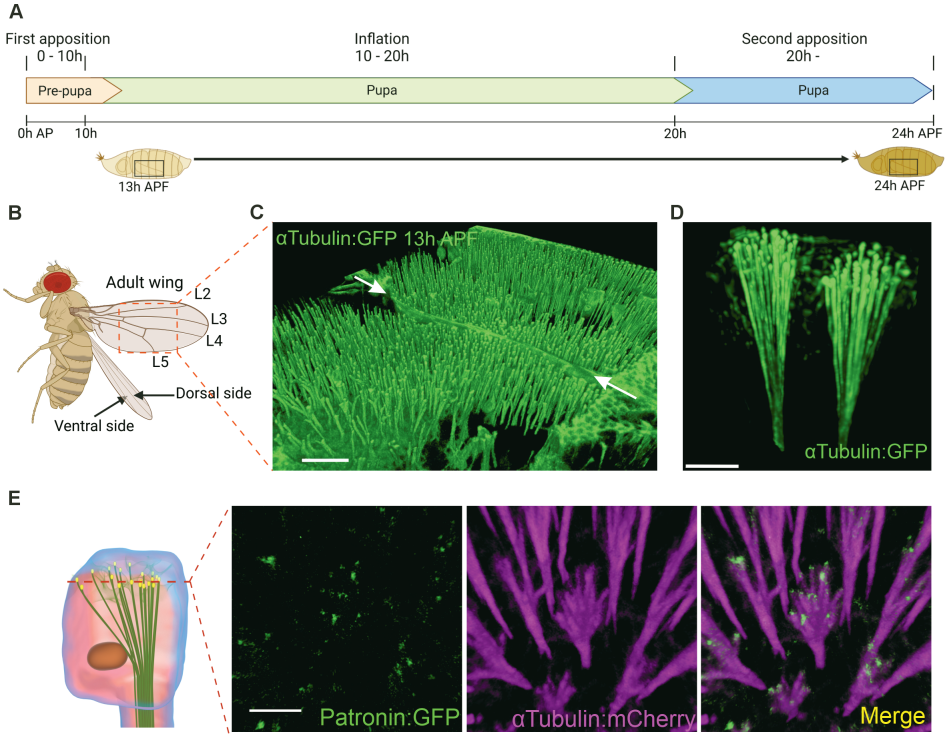

Figure 21. A network of MT projections nucleated by ncMTOCs within the *Drosophila* pupal wing during the inflation stage. **A.** Timing of wing development during the first 24 h after pupariation at ~22 °C. **B.** The red box in the schematic of the adult fly wing shows the targeted pupal wing region during live imaging. L2-L5: longitudinal veins. **C.** MT projections in the pupal wing expressing αTubulin:GFP. White arrows indicate the trachea. **D.** A high-resolution image of αTubulin:GFP at 13 h APF. We see MT protrusions composed of individual MTs, bundling as they extend basally from the apical surface. **E.** Left: A schematic illustration including minus-end foci (yellow dots) and MT fibers (green) in a single cell. Right: Live-imaging of pupal wing express αTubulin:mCherry and Patronin:GFP were taken at 14 h APF (~22 °C). Patronin:GFP localized to minus-ends of MT protrusions in a single cell. Scale bar: 40 μm (**C**), 5 μm (**D**, **E**). Parts of the results of this dissertation have been published in Ref I.

Next, we conducted time-lapse imaging experiments using the αTubulin:GFP marker to better understand the behavior of MT protrusions in the pupal wing. The experiments were carried out at a temperature of 29 °C for 10.5–14.5 h APF,

equivalent to 13–17 h APF at room temperature (~22 °C). Our findings revealed that the MT structure undergoes dynamic changes over time. The dorsal and ventral MT projections collaborate to form a bundle structure in both layers, which then disassembles (Figure 22). These findings hint to us that the dynamic changes of MT projections play an important role in remodeling the tissue shape of the pupal wing during the inflation stage. Earlier research has highlighted that ncMTOC-regulated MTs are used in differentiated cells, such as neurons and epithelial organs (Booth et al., 2014; Gillard et al., 2021; Muroyama & Lechler, 2017; Toya & Takeichi, 2016; Wu & Akhmanova, 2017). Our data emphasizes that the IPAN, which is a transient cellular structure facilitated by ncMTOC-nucleated MTs, is used to remodel the structure of the pupal wing in the early inflation stage.

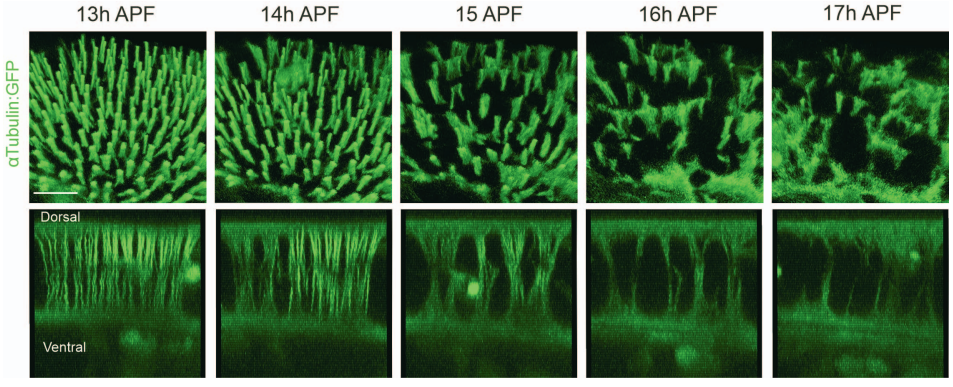

Figure 22. Dynamics of MT projections. Apical (top panel) and lateral (bottom panel) view of pupal wing express αTubulin:GFP at 10.5 h, 11.5 h, 12,5 h, 13,5 h and 14,5 h APF at 29 °C (equivalent to 13 h, 14 h, 15 h, 16 h and 17 h APF at room temperature). Scale bar: 10 μm.

4.1.2 The IPAN is dynamic and transient

In the next step, we analyzed the structural changes in IPAN from the inflation to the re-apposition stage, spanning from 13 h to 24 h APF, using pupal wings expressing membrane-bound CAAX:mCherry to capture IPAN structures. Our results showed that most protrusions were temporary, unstable, and had three evident characteristics. Firstly, dorsal and ventral protrusions were disassembled time-dependently, similar to the dynamics of MTs. Secondly, the remaining protrusions joined with neighboring protrusions locally and left the bundle to either disassemble or extend proximally on the bundle fronts throughout the wing. This led to the formation of an inter-epithelial cytoskeleton that mediates the attachment of the two layers of epithelial cells. Thirdly, the dynamics of protrusions seemed to be similarly coordinated in the future wing hinge and wing blade until 18 h APF, although the hinge contraction started prior to the re-apposition stage (Ref I, Figure 2, Figure 23A).

To examine the relationship between the cell membrane and MT projections, we used a wing expressing αTubulin:GFP and CAAX:mCherry. Our findings indicated that the cell membrane and MT structures are similarly regulated. Rigid MT projections inside the cell appear to coordinate membrane protrusion, indicating that structural changes in MTs affect the membrane and the overall shape of the cell (Figure 23B).

The key events of the IPAN dynamics, from the wing inflation phase to the re-apposition stage, are summarized in Figure 23C.

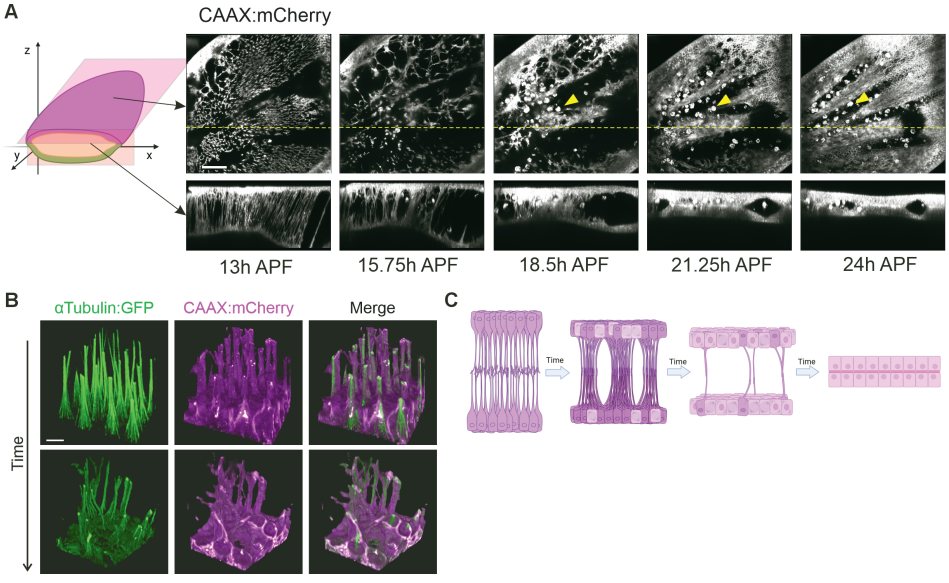

Figure 23. The IPAN is dynamic and transient. **A.** Time-lapse images show the pupal wing expression driven by CAAX:mCherry (white) between 13 h – 24 h APF. The upper panels display interepithelial transverse (XY) views of the IPAN at different time points, with corresponding lower panels showing optical cross-section views (yellow dot line). Bright, amorphous objects (yellow arrowheads) are macrophage-like hemocytes. **B.** Basal views of the dorsal layer of αTubulin:GFP (green, left), CAAX:mCherry (magenta, middle), and merged images (right). In the early stage at ~13 h APF (top panel), there are robust MT and membranous protrusions, while thinner protrusions form bundles in later phases at ~16 h APF (bottom panel). **C.** Main events of IPAN in the pupal wing from inflation to second apposition stage. Scale bar: 50 μm (**A**), 5 μm (**B**). Parts of the results of this dissertation have been published in Ref I.

4.1.3 The formation of cell-cell contacts is necessary to maintain IPAN from dorsal and ventral cells

According to research conducted by Sun et al. (T. Sun et al., 2021), extracellular matrix (ECM) components are crucial in the focal adhesion between two layers of epithelial cells. During the inflation stage, the adhesion between the two opposing wing epithelial layers is maintained by integrin-mediated adhesion.

However, the formation and interaction of the dorsal and ventral protrusions are still unclear. Therefore, we conducted further investigations to understand how the two epithelial layers interact to form cell-cell contacts in IPAN.

To study cell-cell interaction, we focused on the interepithelial space during the inflation stage. We used a two-color system and high-resolution imaging to distinguish between cell membranes derived from the dorsal or ventral epithelium. rCD2:RFP and mCD8:GFP were utilized for visualizing dorsal and ventral cells, respectively (Ref I, Figure 3, Figure 24A). Our data revealed that each cell has a protrusion basally, branching out before coming into contact with the protrusions of the opposite epithelial sheet. Interestingly, interepithelial contacts are not established by single pairs of dorsal and ventral cells but through multiple cells (Ref I, Figure 3 A, B).

We also monitored the bundling process of IPAN using a two-color system. Our observations suggest that the dorsal and ventral layers form a complex network in the interepithelial space that can withstand dynamic movement or disassembly of the bundles without disrupting the overall connectivity of the IPAN (Figure 24A, B).

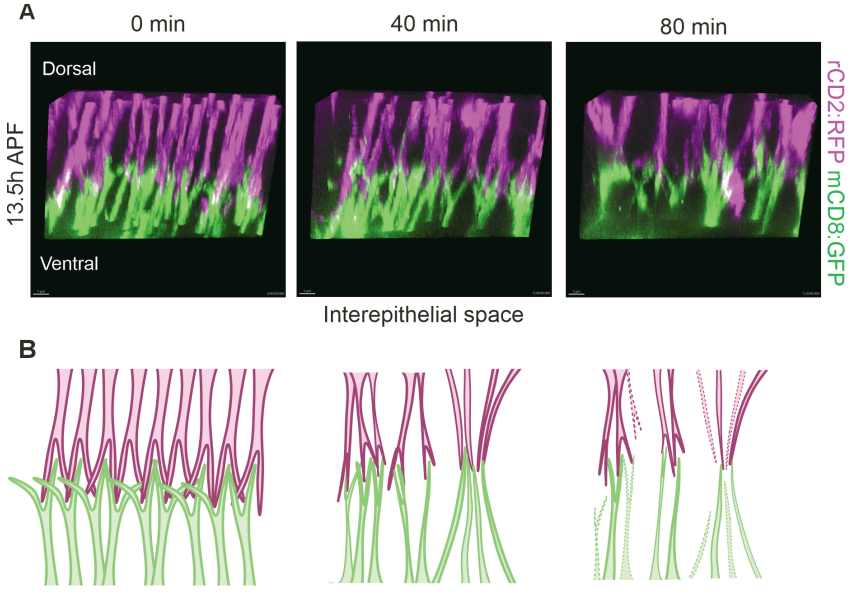

Figure 24. Cell-cell contacts are necessary to maintain IPAN from the dorsal and ventral epithelial layers. **A.** Time-lapse images of pupal wing expression driven by membrane-bound rCD2:RFP (dorsal) and mCD8:GFP (ventral) from 13.5 h APF, showing interepithelial space position. **B.** Schematics of dorsal and ventral protrusions forming cell-cell contacts in interepithelial space. Scale bar: 10 μm (**A**). Parts of the results of this dissertation have been published in Ref I.

4.1.4 Reorganization of MTs from ncMTOC to cMTOC during morphogenesis

We noticed changes in the distribution of MTs within the cell during the IPAN disassembly process. As the process progressed, the number of MT foci visibly decreased, and the apical lateral distribution of MTs became more pronounced. A significant amount of the αTubulin:GFP utilized for MT projections gradually reorganized from ncMTOC to cMTOC, forming the mitotic spindle (Ref I, Figure 4, Figure 25A). Under certain physiological and pathological conditions, such as the embryonic epithelial tubules of the salivary gland, *Drosophila*, epithelial-mesenchymal transition (EMT), and epithelial tissue neoplasia, nucleation of MTs is regulated between the cMTOC and ncMTOC (Burute et al., 2017; Gillard et al., 2021; Schnerch & Nigg, 2016). This study provides evidence for the first time of MT reorganization from ncMTOC to cMTOC in epithelial tissue.

To better understand MT protrusion dynamics, we analyzed several independent cells and identified four stages of MT change over time with distinct characteristics. The stages range from the αTubulin being used for protrusions to the formation of the mitotic spindle in the cell undergoing mitosis. Stage I is characterized by the majority of αTubulin used for membrane protrusions. Around 30 MT foci can also be found in the apical compartment of the cell. In stage II, the vertical MT projections degenerate partially, leading to a decrease in MT foci to 10 or fewer per cell. During stage III, the vertical MT projections continue to degenerate, ultimately resulting in the loss of basal protrusions. In this stage, a lateral MT fiber can be observed in the apical-medial and cortical parts of the cell. Finally, in stage IV, αTubulin forms the mitotic spindle, and the cell undergoes mitosis (Figure 25A, B). After observing that mitosis occurs after the loss of MT-based protrusion, we wondered if the disassembling of MT-based protrusion is tightly coupled with mitosis.

To elucidate the hypothesis, the current protocol using only αTubulin:GFP, is insufficient to effectively capture mitotic cells. Therefore, the mitotic spindle and the pericentriolar material (PCM) marker Cnn:RFP were utilized for live imaging. The αTubulin protein captured MT protrusions of the IPAN, while Cnn accumulated at the centrosome only during mitosis and served as a tool to observe mitotic cells. The time-lapse images captured from a basal view display a sequence of events, beginning with the loss of MT spikes, followed by the appearance of mitotic cells. These findings indicate that the IPAN disassembly takes place in a programmed manner. These data hint to us that the dynamics of the MT disassembly process include changes in MT organization and subsequent mitosis (Figure 25C).

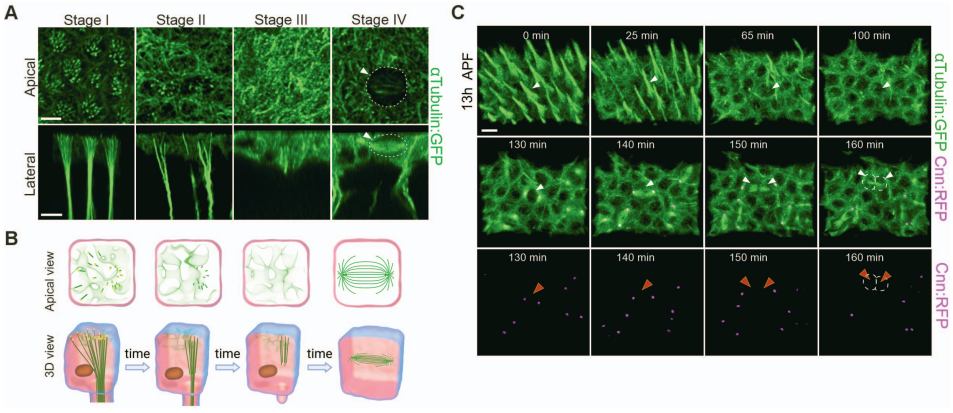

Figure 25. The process of MT disassembly involves alterations in the organization of MTs followed by mitosis. **A.** There are four stages during which MT protrusions regress, followed by the process of mitosis. In stage I, MTs join to form a protrusion extending basally. In stage II, fewer MT foci are found apically. In stage III, MTs no longer form apical foci and the protrusion disassembles. In stage IV, a spindle and mitotic cell rounding occur during mitosis. The pupal wing expression is driven by αTubulin:GFP. The arrow and dotted circle indicate the mitotic cell in stage IV. **B.** Schematic of MT structural changes in which MT protrusions regress, with subsequent mitosis. **C.** These images show the dorsal epithelium at the basal view expression driven by αTubulin:GFP and Cnn:RFP, starting at 13 h APF at ~22 °C. The top and middle images show αTubulin:GFP in green and Cnn:RFP in magenta, while the bottom ones only show Cnn:RFP in magenta, taken between 130–160 minutes after 13 h APF. The arrowheads indicate tracked cells, and the dashed lines show two cells post-division. Scale bar: 5 μm (**A**, **C**). Parts of the results of this dissertation have been published in Ref I.

4.1.5 Quantitative analysis of the dynamic IPAN during pupal wing development

For our next research phase, we needed to quantitatively analyze the IPAN dynamics and mitosis in both dorsal and ventral epithelial layers. For this, we used a 5D imaging protocol that included the x, y, and z dimensions and the t (time) and λ (wavelength) dimensions for the pupal wings. Live imaging was carried out on a fixed position in the wing expressing αTubulin:GFP and the PCM marker Cnn:mCherry (Figure 26A). This allowed us to quantify mitosis in both dorsal and ventral epithelium and the intensity of GFP-tagged MTs in the interepithelial space every 5 minutes for 4 hours in both the dorsal and ventral epithelium (Figure 26B).

Moreover, we can clearly understand the mechanism of this entire process by combining quantitative analysis with conditional gene expression manipulation in a specific manner in the wing compartment with *in vivo* live imaging. Specifically, to obtain gene expression manipulation in pupal wings, white pupae (0 h APF) were collected at room temperature for ectopically expressed transgenes, then transferred to 29 °C until 10.5 h APF (a condition I or UAS condition). For pupae

carrying the RNAi transgene, fly crosses were transferred to 29 °C 16 hours before collecting white pupae and kept at that temperature until imaging at 10.5 h APF to ensure the RNAi phenotype (condition II or RNAi condition) (Figure 26C).

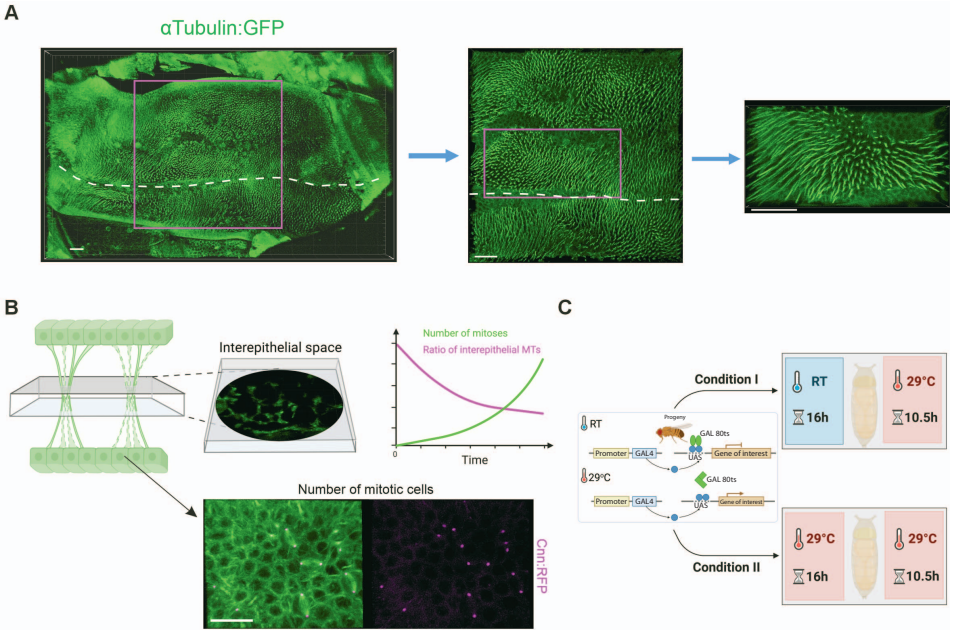

Figure 26. Protocol for quantification programmed disassembly IPAN lead to mitosis. **A.** To count the loss of MT protrusion and mitoses in dorsal and ventral epithelia, we need to limit the region of interest (ROI). The ROI is represented by a magenta box located near the future hinge of the wing adjacent to the trachea, represented by a white dashed line. The pupal wing expression is driven by αTubulin:GFP. **B**. Predicted position for analysis of interepithelial space and label mitosis. **C.** Schematics of the Gal4-Gal80^{ts}/UAS system were used to allow spatiotemporally controlled expression of transgenes in the pupal wing. Condition I was used to overexpress proteins that impact IPAN, while condition II was used to express RNAis that knock down the expression of IPAN-impacting proteins. RT: room temperature. Scale bar: 30 µm (**A**), 10 µm (**B**). Parts of the results of this dissertation have been published in Ref I.

4.1.6 Coordinated mitosis takes place between dorsal and ventral epithelia following the programmed disassembly of the IPAN

We conducted a study to examine the relationship between disassembled MT projections and mitoses using the above quantification protocol. We examined a control experiment by observing *in vivo* 5D imaging over 4 hours at 29 °C. The results showed that at 10.5 h APF, most cells contained MT projections. By 12.5 h APF, most MT projections degenerated or formed bundles. During the 4-hour observation period, approximately 30–35% of the observed cells underwent mitosis. Notably, dorsal and ventral mitotic cells cooperated with each other, initiating and accelerating mitosis as MT protrusions bundle and disassemble (Figure 27A, A'). Our results show evidence that the disassembly of MT protrusions and the release of cell-cell contacts can trigger mitosis in both layers.

Assuming that MT disassembly is responsible for mitosis, we hypothesized that a delayed MT disassembly process affects mitosis. To test this hypothesis, we conducted an experiment to ectopically stabilize the MT structure. We used the MT stabilizing factor Tau (human Tau, hTau) (Wang & Mandelkow, 2016) and found that the bundling and disassembly of MT projections were significantly delayed when hTau was ectopically expressed in both the dorsal and ventral epithelium using the *nubbin-Gal4* (*nub-Gal4*) driver. Our data showed that delaying programmed disassembly of MT projections significantly affects mitoses in both layers, resulting in smaller adult wings (Figure 27B, B' and Ref I, Fig EV3A, C). These findings suggest that the timing of MT disassembly is critical and that delaying it has a significant impact on tissue development.

In another experiment, we tested whether disrupting MT projections could enhance mitosis. To achieve this, we used Katanin-60 (Kat60), an MT-severing factor (Mao et al., 2014), to disrupt MT projections only in the dorsal compartment by using the *apterous-Gal4* (*ap-Gal4*) driver. This led to the disruption of most cell-cell contacts. The results showed that the number of mitotic cells remained low in both the dorsal and ventral epithelial layers, which resulted in smaller, blistered adult wings (Figure 27C, C', Ref I, Fig 6C). These findings underscore establishing cell-cell contact before disassembly leads to mitosis is significant. Both formation and disassembly of the IPAN are required for coordinated mitosis between two-layered epithelia.

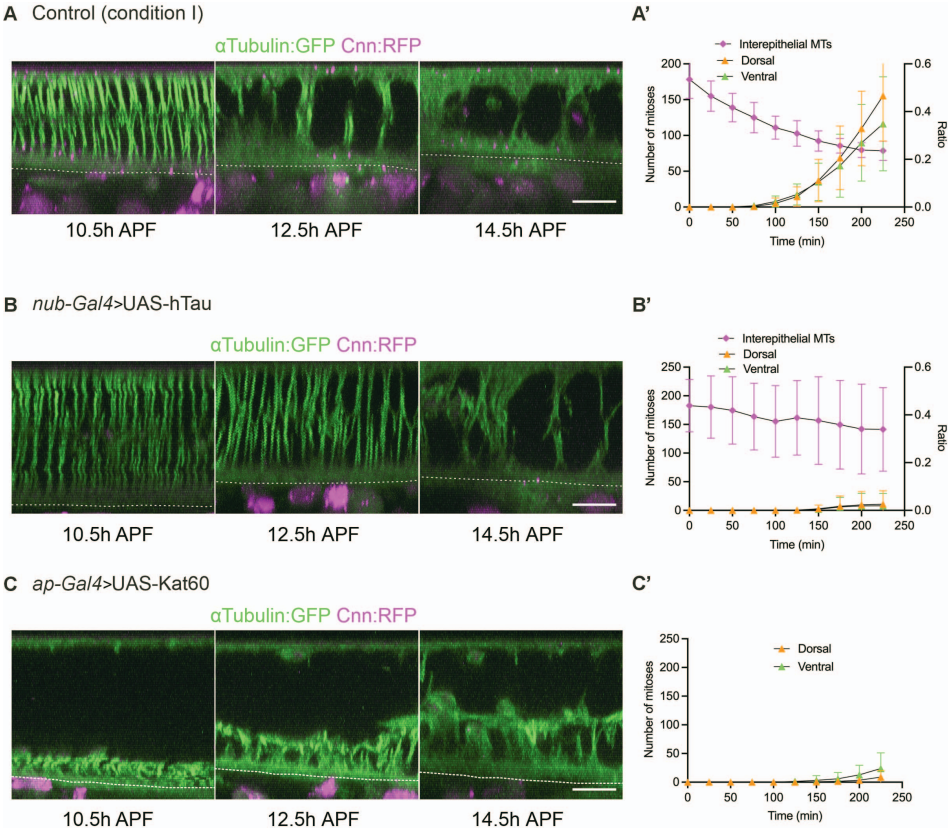

Figure 27. Modulation of MT stability affects coordinated mitosis. Time-lapse images of αTubulin:GFP (green) and Cnn:RFP (magenta) in optical cross sections of MT protrusions in control (condition I) (**A**), overexpressing hTau in both dorsal and ventral cells (*nub-Gal4*>UAS-hTau) (**B**), and Kat60 overexpression in only dorsal cells (*ap-Gal4*>UAS-Kat60) (**C**) at 10.5 h, 12.5 h, and 14.5 h APF (29 °C). Dashed lines represent the boundary between the ventral wing cells and inner tissue (pupal leg). Dorsal cells are at the top, and ventral cells are at the bottom. Note that disassembly of MT protrusions is significantly delayed in wings expressing hTau, and cell-cell contacts through the IPAN are largely disrupted in the wing expressing Kat60. **A'–C'**. Number of mitotic cells (dorsal: orange triangle, ventral: green triangle) in wing epithelium and ratio of interepithelial MTs (magenta) at different time points in control pupal wings (**A'**), hTau overexpression (both dorsal and ventral) pupal wings (**B'**), and Kat60-overexpressing pupal wings (**C'**). Time 0 corresponds to 10.5 h APF. N = 5. Data are means ±95% confidence intervals (CIs). Scale bars: 20 µm (**A, B, C**). Parts of the results of this dissertation have been published in Ref I.

4.1.7 Patronin and Short stop sustain MT-based protrusions and coordinated mitosis

As mentioned previously, MT-based protrusions are nucleated by ncMTOCs. Therefore, we investigated whether the absence of co-factors that work with ncMTOCs has any impact on mitosis.

Before proceeding with further time-lapse experiments, we first evaluated the effectiveness of the knockdown protocol on Patronin, a protein that stabilizes the minus end of MTs (Khanal et al., 2016; Nashchekin et al., 2016). We confirmed that the knockdown of *patronin* significantly reduced the levels of Patronin. Our results showed that the Patronin knockdown significantly decreased the apical foci of the MT projections as compared to the control (Figure 28A). Additionally, at 14 h APF, Patronin:GFP signal was specifically lost at the apical end of MT projections (Figure 28B).

Next, we conducted experiments to test the effects of conditional knockdown of Patronin in both the dorsal and ventral wings (*nub-Gal4>patronin*^{RNAi}). The results showed a significant decrease in the number of mitosis in the dorsal and ventral epithelial layers. Our data reveal that the dorsal and ventral projections were less bundled, resulting in more protrusions over the 4-hour imaging period. Moreover, the MT distribution of the interepithelial space was more prolonged than that of the control, indicating that the loss of cell-cell contact was facilitated less effectively, leading to a lower number of mitotic cells in both dorsal and ventral tissue (Figure 28C, C'). This outcome is similar to the hTau phenotype when MT projection stabilizes and reduces the MT programmed disassembles process, as discussed above (Figure 27B, B').

In another experiment, we knocked down Patronin only in the dorsal compartment using the *ap-Gal4* driver (*ap-Gal4>patronin*^{RNAi}). Interestingly, we observed a time-dependent disassembly of MTs in the interepithelial space. As a result, the number of mitotic cells remained unaffected, and coordinated mitoses were regulated similarly to the control (Figure 28D, D').

Our analysis has once again verified that MT facilities lose cell-cell contact over time in both the dorsal and ventral epithelium, a process that is coordinated with mitosis. Moreover, if the IPAN structure in one layer is compromised, it can be functionally restored by the IPAN of the opposite layer, ensuring coordinated proliferation. However, we still lack sufficient knowledge about how Patronin enables the loss of cell-cell contact.

As part of our investigation into the role of ncMTOCs in interepithelial mitotic coordination, we looked into other factors associated with ncMTOCs. One of the proteins we tested was Short stop (Shot). Shot interacts with ncMTOCs and has multiple functions (Khanal et al., 2016; Voelzmann et al., 2017). We found that in the pupal wing when Shot is knocked down conditionally, the minus-ends of MT projections in the medioapical region are severely compromised. Instead of vertical protrusions, curled structures are formed, which leads to the loss of dorsal-ventral contacts and largely abolishes the IPAN (Figure 29A). This is consistent with a previous report (T. Sun et al., 2021).

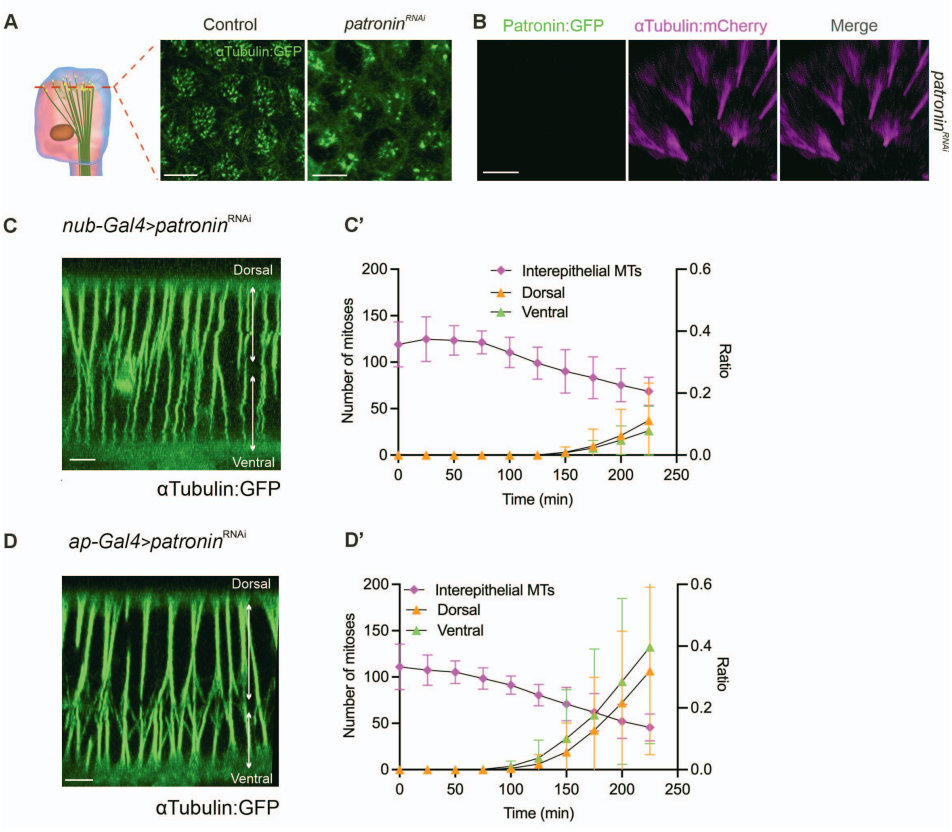

Figure 28 Patronin sustains MT-based protrusions and coordinated mitosis. **A.** Live-imaging in the apical compartment of αTubulin:GFP at 10.5 h APF (29 °C) in control (left) and conditional RNAi knockdown of *patronin* (right). Upon patronin knockdown, the number of apically oriented MT foci is reduced. A schematic showing the apical compartment, Patronin (yellow dots), and MT protrusions (green) in a single cell is also provided. **B.** Live-imaging of αTubulin:mCherry and Patronin:GFP in a conditional RNAi knockdown of patronin at 10.5 h APF (29 °C) shows that Patronin is lost in minus-ends of MT protrusions. The images consist of Patronin:GFP (left), αTubulin:mCherry (center), and a merged image (right). (**C–D**) Lateral view of αTubulin:GFP during conditional *patronin*^{RNAi} in both dorsal and ventral layers (*nub-Gal4> patronin*^{RNAi}, **C**) or only in the dorsal layer (*ap-Gal4> patronin*^{RNAi}, **D**) at 10.5 h APF. **C**'. A number of mitotic cells (dorsal: orange triangle, ventral: green triangle) in wing epithelium and the ratio of interepithelial MTs (magenta) at different time points in *patronin*^{RNAi} (both dorsal and ventral) pupal wings (right). Time 0 corresponds to 10.5 h APF. Data are from five individual replicates (N = 5). Data are means ±95% CIs. **D"** Number of mitotic cells (dorsal: orange triangle, ventral: green triangle) in wing epithelium and the ratio of interepithelial MTs (magenta) at different time points in *patronin*^{RNAi} (dorsal) pupal wings (right). Time 0 corresponds to 10.5 h APF. Data are from five individual replicates (N = 5). Data are means ±95% CIs. Note that in *nub-Gal4>patronin*^{RNAi}, protrusions are thin and long (**C**). Additionally, dorsal protrusions are longer and thinner than ventral protrusions in *ap-Gal4 > patronin*^{RNAi} (**D**). Scale bar: 5 µm (**A, B**), 10 µm (**C, D**). Parts of the results of this dissertation have been published in Ref I.

Moreover, we found that when we conditionally knocked down Shot only in the dorsal layer (*ap-Gal4>shot*^{RNAi}), it caused the loss of dorsal-ventral contacts and essentially abolished IPAN (Figure 29B). As a result, there was a significant reduction in the number of mitoses in both the dorsal and ventral epithelium, which led to smaller and inflated adult wings (Figure 29C, Ref I, Figure EV4D).

Our findings further confirm that the formation and loss of cell-cell contacts are necessary for coordinated mitoses between the two epithelia. This was evident from the fact that even in a ventral epithelium which was a wild-type background, the loss of cell-cell contacts led to a significant reduction in the number of mitoses (Figure 29B').

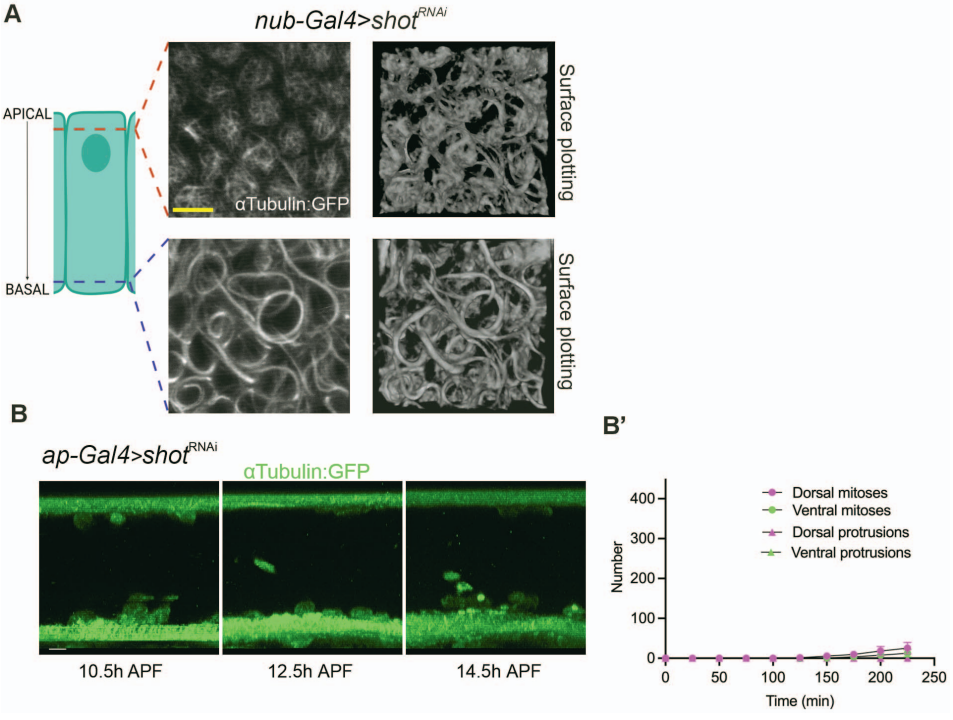

Figure 29. The role of Shot in sustaining structure of MT-based protrusions and co-ordinated mitosis. **A.** Conditional RNAi knockdown of *shot* abolishes apical MT foci, leading to MT protrusions that form curls in the basal cell body and fail to extend into the interepithelial space. Left: schematic showing approximate apical and basal regions for greyscale images of the pupal wing at 10.5 h APF (29 °C) at right. Middle: apical (upper) and basal (bottom) view of αTubulin:GFP. Right: surface plots of apical (upper) and basal (bottom) view of αTubulin:GFP. **B.** Time-lapse images of αTubulin:GFP (green) and Cnn:RFP (magenta) in optical cross sections of MT protrusions at 10.5 h, 12.5 h, and 14.5 h APF of wings in which *shot* is knocked down in dorsal epithelium only (*ap-Gal4>shot*^{RNAi}). Dorsal cells face up, and ventral cells down. **B'.** The number of mitotic cells (dorsal: orange triangle, ventral: green triangle) in wing epithelium at different time points in *shot*^{RNAi} pupal wings (right). Time 0 corresponds to 10.5 h APF. Data are from five individual replicates (N = 5). Data are means ±95% Cis. Scale bar: 5 μm (**A**), 10 μm (**B**). Parts of the results of this dissertation have been published in Ref I.

4.1.8 The G2/M transition in dorsal and ventral epithelia takes place autonomously

During the prepupal stage, wing cells are arrested in G2 (Etournay et al., 2015; Milán et al., 1996). We hypothesize that MT projections disassembly aids in the G2/M transition. To test the role of G2/M transition in IPAN-mediated tissue proliferation, we conducted a knockdown of String (Stg), a key cell cycle regulator that activates CDK1 (Glover, 2012).

After inducing conditional RNAi of *stg* in the dorsal epithelium (*ap-Gal4> stg*^{RNAi}), we noticed that mitotic cells persisted ventrally while the suppression of mitosis was evident dorsally. We noted that dorsal and ventral MT projections formed bundled structures, and the distribution of MTs in the interepithelial space decreased, albeit slightly delayed, compared to the control. These findings suggest that the IPAN-mediated MT dynamics-induced G2/M transition is autonomously regulated in the two epithelia (Ref I, Figure 8).

To confirm these findings, we conditionally knocked down POLO kinase, a cell cycle regulator controlling the G2/M transition and mitotic events (Glover, 2012), in the dorsal epithelium (*ap-Gal4>polo*^{RNAi}). The results showed the loss of mitosis in the dorsal layer, while the ventral layer showed mitotic activity (Ref I, Figure EV5).

These results suggest that the loss of cell-cell contacts provides a proliferative signal in both dorsal and ventral epithelia, and executors of the G2/M transition thus function autonomously.

Taking all the data together, we conclude that IPAN plays a pivotal role in regulating the proliferation of 3D epithelial tissues. IPAN sustains the connections between two-layered epithelial cells in the inflated pupal wing. When IPAN disassembles, it leads to the loss of cell-to-cell contacts, resulting in coordinated cell proliferation between two-wing epithelia.

4.1.9 α-Spectrin regulates cell shape changes during disassembly of MT-based protrusions in *Drosophila* wings

To better understand the molecular mechanisms involved in pupal wing development, we performed RNAi screening in pupal wings to identify essential genes involved in wing development. We found that when we conditionally knocked down α-Spectrin (*nub-Gal4>α-spectrin*^{RNAi}), the size of the adult wing increased (Figure 30A, B). Since α-Spectrin functions with multiple interacting partners, including MTs and actin filament (Deng et al., 2020; Fletcher et al., 2015; Forest et al., 2018), we wonder whether α-Spectrin may regulate IPAN-mediated cellular mechanisms.

To investigate this, we followed individual cells between stage I and stage II in the control (staging of IPAN have been clarified in the previous section, Figure 25), the data showed that the apical area of the cells was smaller in Stage I but increased in stage II, suggesting that apical relaxation takes place. However, the apical compartments in the group where *α-spectrin* was conditionally knocked

down (*nub-Gal4>α-spectrin*^{RNAi}) had no significant expansion from stage I to stage II (Ref II, Figure 1E, F). This data suggests that fluctuations in apical cell size occur during the disassembly of MT-based protrusions and that α-Spectrin may regulate apical compartment changes during this process.

To further understand this phenomenon, we imaged live membrane-bound mCD8:GFP and Spaghetti squash (Sqh):mCherry (myosin II light chain)-expressing control wing in stages I and II. During stage I, we observed significant Sqh:mCherry in the apicomedial and cortical regions. However, in stage II, most of the Sqh:mCherry was detected only in the cortical region, indicating that the cells relaxed during stage II (Ref II, Figure 1G). In the *α-spectrin* knockdown wing, Sqh:mCherry was not spatially distributed in the apicomedial region during stage I (Ref II, Figure 1H). This suggests that α-Spectrin is necessary for apical relaxation between stage I and stage II, which is mediated by regulating MyoII localization.

Next, we analyzed the stages of IPAN-mediated mitosis. We found that α-Spectrin plays a role in regulating the temporal sequence of MT structure, leading to the loss of cell-cell contacts and subsequently coordinated mitosis. Accordingly, *α-spectrin*^{RNAi} wings experienced faster degeneration of stage I and shorter stages II and III durations than control wings (Figure 30C).

Finally, the cell membrane becomes more relaxed in *α-spectrin*^{RNAi} wings, which directly affects the dynamic of MTs within cells. During time-lapse imaging of αTubulin:GFP and CAAX:mCherry in α-spectrin RNAi wings, it was observed that changes in the cell's shape did not follow the movement of the MTs. The results showed that there were membrane protrusions occasionally in *α-spectrin*^{RNAi} wings, even after the MT protrusion disassembly and the mitotic phase. Interestingly, MTs were frequently regenerated after mitosis if these protrusions remained intact, which was not observed in the control wing (Figure 30D, E).

Taken together, our data indicate that α-Spectrin is crucial in supporting tissue homeostasis during IPAN-mediated morphogenesis. It mediates cell shape changes related to the temporal sequence of the gradual changes of MT structures and the robustness of IPAN structure dynamics.

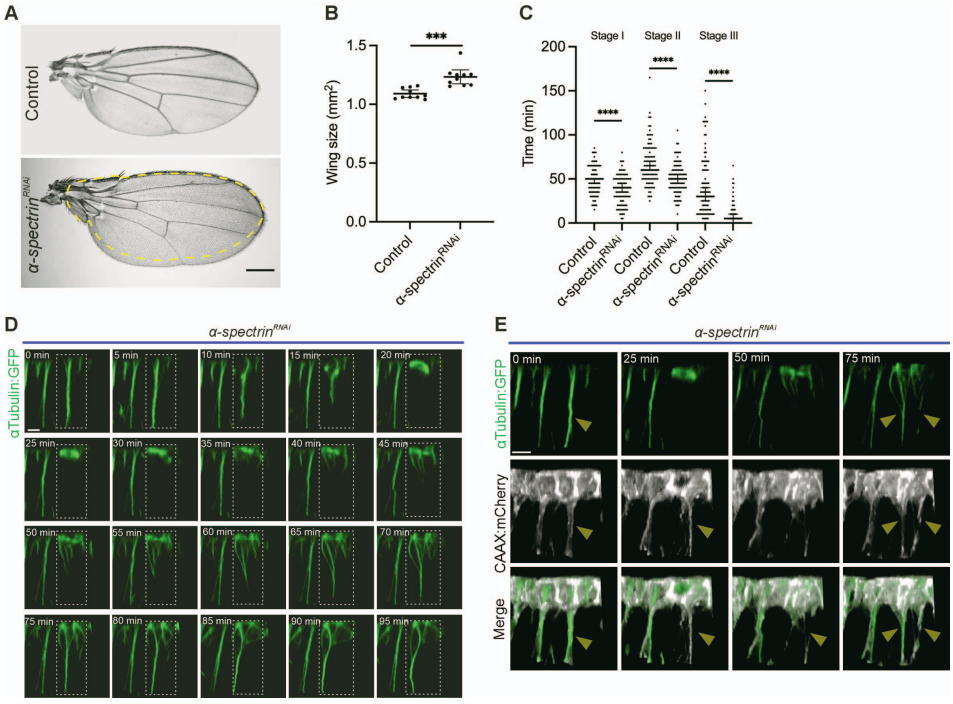

Figure 30. α-Spectrin regulates cell shape changes during disassembly of MT-based protrusions. **A**. Adult male wings in control (upper panel) and conditional RNAi knockdown of *α-spectrin* (lower panel). The yellow dotted line represents the size of the control wing. **B**. Adult male wing size in control and conditional RNAi knockdown of *α-spectrin*. ***P = 0.0001. Data are means ±95% CIs. Statistical significance was calculated by a two-tailed t-test. **C**. Graphs showing the distribution of cells in Stages I, II, and III within the region of interest (ROI) in control and conditional RNAi knockdown of *α-spectrin*. Data are means ±95% CIs. Each data point represents one cell. ****P < 0.0001. Data were analyzed by two-sided Mann-Whitney U test. **D**. A lateral view of αTubulin:GFP (green) is captured every 5 minutes, illustrating the reform MT after cell division in *α-spectrin*^{RNAi} knockdown. **E**. A lateral view of αTubulin:GFP (green) and CAAX:Cherry (white) in the pupa wing epithelium is captured every 25 minutes. The yellow arrows indicate a cell undergoing mitosis, then reforming its protrusion after division. Scale bar: 250 μm (**A**), 5 μm (**D**, **E**). Parts of the results of this dissertation have been published in Ref II.

4.2 The posterior crossvein morphogenesis as a model for apical constriction determines vein fate (Ref III)

Our next project focuses on how changes in cell shape refine BMP signaling. *Drosophila*'s posterior crossvein (PCV) development is an excellent system for understanding BMP signaling's regulation of epithelial patterning and differentiation. Vein morphogenesis and BMP signaling start simultaneously at 20 h – 24 h APF, refining the BMP signaling pattern during vein morphogenesis in the longitudinal veins and intensifying in the PCV. However, it is unclear how BMP pattern refinement occurs in PCV.

4.2.1 BMP signaling induces cell shape changes during pattern refinement

In the development of *Drosophila* wings, the posterior crossvein (PCV) cells are defined by the presence of BMP signaling, which can be identified by the pMad signal. The refinement of the PCV field takes place during a certain period of time known as the "refinement period", which occurs between 20 h to 28 h APF (Ref III, Figure 1D,E).

During the refinement period, wing veins exhibit apical constriction, which is responsible for shaping the PCV cells. BMP signaling initiates PCV development (Matsuda & Shimmi, 2012; Shimmi et al., 2005); does it affect cell shape? By analyzing *crossveinless* mutant wings with inactive BMP signaling in PCV, it has been observed that apical constriction does not occur in the region where PCV normally forms (Ref III, Figure 1F). This implies that BMP signaling is essential for the cell shape changes that take place during the development of PCV.

Furthermore, our data also highlighted BMP signaling affects cell shape change by promoting the apical localization of MyoII, which induces apical constriction of PCV cells. MyoII is concentrated in the apical compartment of PCV cells in wild-type pupal wings, whereas in *crossveinless* pupal wings, apical MyoII enrichment is not observed in the PCV region (Ref III, Figure 1G). Additionally, ectopic expression of the constitutively active form of BMP type I receptor Thickveins (Tkv^{QD}) in the pupal wing induces apical enrichment of MyoII and apical constriction (Ref III, Supplemental Figure 1B).

Taken together, these findings suggest that BMP signaling facilitates the apical localization of MyoII to promote the apical constriction of PCV cells.

4.2.2 Time lapse imaging during PCV morphogenesis

In the next step, we used live imaging of E-cadherin tagged with GFP (E-cad: GFP), a marker for apical cell shape changes, to investigate how BMP signaling coordinates the development and refinement of wing veins. Our observations revealed that during refinement, cells forming the posterior crossvein (PCV) constrict apically and maintain vein-like morphology, while some cells flanking

them also constrict apically but fail to keep the same morphology (Ref III, Figure 2A–C).

We then observed cell shapes inside and outside the PCV field were linked to their BMP signaling state during refinement. Cells positive for BMP signaling in the field remained constricted, whereas cells outside did not show this trait. As the field was refined, cells on its edges lost their BMP signaling state and fate competence, which caused their cell shape changes to become reversible and transient (Ref III, Figure 2D–F).

Therefore, the BMP signaling state is coupled with transient and reversible cell shape changes, and the fate competence of cells is also transient and reversible.

4.2.3 Cell shape change and pattern refinement are coupled

We proposed that changes in cell shape could affect the refinement of signaling patterns, ultimately determining the fate of cells in the Posterior Crossvein (PCV) region. To test this idea, we inhibited Myosin Heavy Chain activity (MyoII) using a dominant negative form (MyoII-DN) to modulate cell shape changes in the developing wing during PCV morphogenesis. Our findings showed that inhibiting MyoII activity disrupted apical constriction in the PCV region and LV cells of 23 h APF pupal wings (Ref III, Figure 3A-E). Disrupting MyoII activity throughout the posterior wing resulted in a broader range of BMP signaling in the PCV region, indicating that cell shape changes refine the BMP signaling pattern, but not the BMP signaling itself. We observed disrupted PCV patterning in adult wings, indicating that the organization of cell fates had been disrupted (Ref III, Figure 3F).

Our findings suggest that a self-organizing mechano-chemical loop occurs during crossvein patterning, as the BMP signal induces apical Myosin II localization to instruct cell shape changes, the activity of which is necessary for the proper patterning of the BMP signal.

4.2.4 Competition for BMP signaling takes place at the PCV region

What is the role of cell shape changes in signaling pattern refinement? The hypothesis is that cell morphology, compared to other cells in the field, is more important than cell shape change, which can cause less signaling and subsequent fate loss in cells surrounded by those more readily able to change shape.

To test the hypothesis, we generated clones that attenuate cell shape changes amongst neighboring wild-type cells to test this hypothesis. Interestingly, the loss of BMP signaling can be often observed context-dependently when small MyoII attenuated clones are produced within the PCV field (Ref III, Figure 3G). Larger clones expressing Myo-DN lacked refinement, similar to when expressed throughout the posterior wing blade (Ref III, Supplemental Figure 2D). This suggests that cell shape changes based on MyoII play a crucial role in whether a cell retains vein fate during refinement. When neighboring cells cannot form vein-

like shapes, refinement does not happen. Only cells that can change shape retain the signal and acquire vein fate. Cells of the PCV field may compete for BMP signaling through their changes in cell shape. The self-organizing MyoII-BMP feedback loop is how pattern refinement occurs at the PCV. BMP signaling induces cell shape changes, influencing the cells' ability to retain that signal.

As BMP-signaling-induced cellular changes appear needed to refine the signal, we decided to test whether increasing BMP signaling levels beyond the normal range could result in supercompetitive cells that are better suited to receive the signal, as BMP-signaling-induced cellular changes appear to be necessary to refine the signal.

We created clones of the constitutively active form of BMP type I receptor Thickveins (Tkv^{QD}) that showed strong BMP signaling. When we induced Tkv^{QD} clones in wings, cells expressing Tkv^{QD} became supercompetitive for BMP signaling, disrupting signaling in other cells at the PCV region (Ref III, Figure 3I). This supports the idea that cells compete for BMP signaling in the PCV field, and high signaling levels in some cells can disrupt signaling in others.

4.2.5 Basal cell shape is a potential mechanism of competition between cells

We next consider how cells compete for the BMP signal and how cell shape affects this. When cells undergo apical constriction, the basal domain tends to expand. This is important in vein morphogenesis, where a lumen needs to form. Recent research found that Dpp ligands are mainly localized on the basal side of the wing epithelia (Matsuda & Shimmi, 2012). Therefore, basal expansion could increase a cell's competency for the basally localized ligand.

During the refinement period, we investigated whether BMP-positive cells in the PCV field expand basally. We found that as these cells apically constrict, they basally expand. Since the volume of cells remains mostly unchanged during the refinement period, it appears that BMP-induced apical Myosin II causes basal expansion without affecting cell volume.

We also measured the basal sizes of cells in areas of low and high competence for the BMP signal. Peripheral cells had smaller basal surfaces than central cells, suggesting that basal expansion enhances the cells' competence for capturing the basally localized ligand, and thus contributes to the mechanism of competition (Ref III, Figure 4).

When MyoII activity is disrupted, there are fewer differences in basal cell size between central and peripheral cells, suggesting that basal cell size dynamics play a role in the mechanism of refinement (Ref III, Supplemental Figure 3).

In summary, during the morphogenesis of PCV, changes in cell shape induced by myosin are linked to BMP signaling. A mechanical-chemical feedback loop leads to competition for the BMP signal. Cells with higher levels of BMP signaling outcompete their neighbors for the role of vein-forming cells, while cells with lower levels acquire an intervein fate. When BMP signaling is induced

ectopically, it causes super-competence for the signal, leading to the accumulation of myosin, apical constriction, and loss of BMP signaling in neighboring cells.

Our study highlights the occurrence of competition for BMP signaling in the PCV. When there are many cells with low competence, BMP-positive cells retain their signaling state. Smaller clones of less competent cells lose the signal autonomously, while clones of super-competent cells outcompete neighboring wild-type cells non-autonomously. Therefore, we propose a new mechanism, known as “mechano-chemical feedback mediated competition for the developmental signal.” This mechanism may commonly be utilized during pattern formation.

5. FINAL REMARKS AND FUTURE PERSPECTIVES

This study utilizes genetic tools to investigate cellular dynamics in epithelial morphogenesis, focusing on the *Drosophila* pupal wing. Our findings show significant cell shape changes during pupal development, making it an excellent model for studying real-time cell-cell communication and tissue morphogenesis.

In the inflation stage, the study highlights the importance of the microtubule-based membrane protrusion network and provides valuable insights into the field. Although membrane protrusions have been occasionally described in developing tissues (Korenkova et al., 2020; Sartori-Rupp et al., 2019; T. Sun et al., 2021), genetic manipulation focused on these protrusions is often challenging. Therefore, current knowledge of the physiological significance of such structures is limited. Our *in vivo* live imaging protocol can be conducted using confocal microscopy and quantification methods, providing valuable insights into the shapes of epithelial cells and the dynamics of microtubule-based protrusions with high resolution and precision. Combined with *Drosophila* genetic tools, such as the Gal4/Gal80^{ts} system of spatiotemporally controlled gene expression, it can be utilized alongside multi-colored fluorescent live imaging to establish a comprehensive system for understanding cell shape changes affecting tissue morphogenesis. Thus, our study underscores the significance of the Interplanar Amida Network (IPAN) structure in developing wing tissues and suggests that the loss of cell-cell contacts and apical relaxation plays a crucial role in coordinating mitoses (Figure 31A). The convenient and comprehensive system of the IPAN in the *Drosophila* pupal wing holds promise for addressing many questions about cell shape changes impacting tissue morphogenesis that were previously challenging to answer.

Furthermore, in the second apposition, vein cells undergo differentiation using an apical constriction motif. Our results indicate that apical constriction regulated by Myosin II is coupled with the refinement of the signaling pattern by allowing cells to compete to activate signaling pathways. Accordingly, Myosin II-induced apical constriction allows cells to compete for BMP signaling through a mechano-chemical feedback loop between cell shape changes and BMP signaling. This leads to the self-organizing refinement of the developmental field during cross-vein pattern formation (Figure 31B).

This research enhances our comprehension of the dynamic changes in different cell shape motifs to coordinate 3D morphogenesis, especially during the inflation stage. However, it has also led to further questions. Our recent data indicates that the IPAN not only coordinates mitoses but also acts as a tunneling nanotube (TNT)-like structure to maintain a cellular network. It is interesting to note that TNTs facilitate intercellular trafficking of vesicles, organelles, and pathogens (Cordero Cervantes & Zurzolo, 2021; Korenkova et al., 2020). However, the exact physiological roles of these structures are still not well understood due to limitations in studying them *in vivo*. Therefore, future studies can help

clarify how the TNT-like structure in IPAN facilitates the trafficking of organelles in both physiological and pathological conditions.

Moreover, if IPAN allows for intercellular trafficking through protrusions, it may facilitate the exchange of signaling information and molecules between the two layers during 3D pupal wing formation when the two epithelial layers are at a distance. In order to comprehend how signal proteins or RNA can traverse cell membranes in the interepithelial space, we will need to further develop new tools for imaging and tissue manipulation. This will aid us in discovering new mechanisms of signaling and cellular communication in IPAN, contributing to a better understanding of human diseases such as cancer and neurodegenerative diseases.

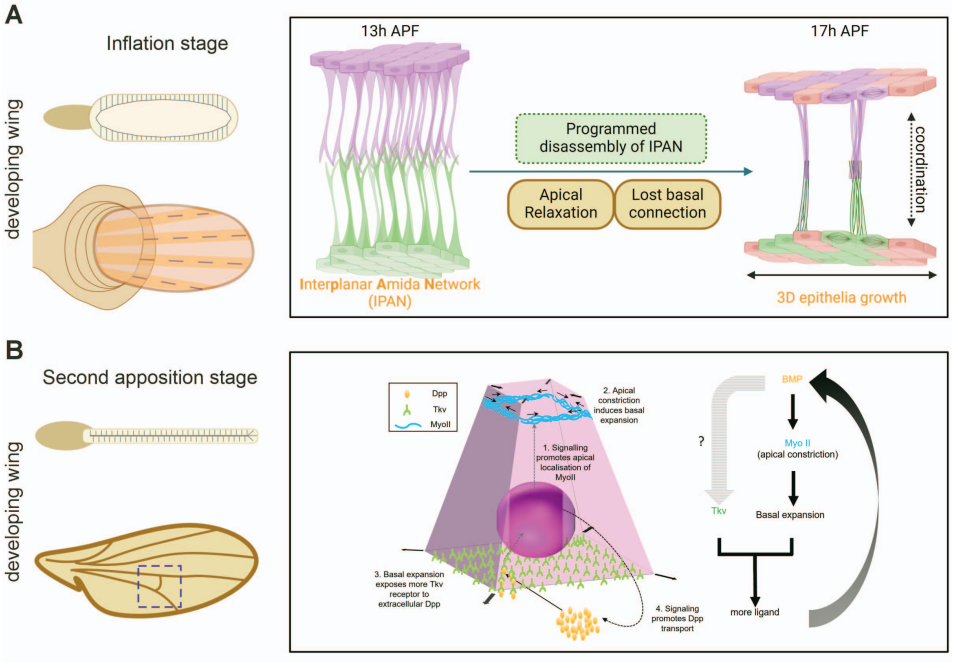

Figure 31. The cellular dynamics and epithelial morphogenesis in *Drosophila* wing development. **A.** Schematics of IPAN-mediated 3D morphogenesis during inflation stage. **B.** Schematic depicting the model of mechano-chemical feedback mediated competition for BMP signaling leads to pattern PCV formation in the second apposition stage. The dotted box in the left-wing schematic indicates the PCV region.

SUMMARY IN ESTONIAN

Rakkude dünaamika ja epiteeli morfogenees *Drosophila* tiiva arengus

Epiteelirakud mängivad üluolulist rolli keerukate 3D organite ja kudede kujunemisel loomade arengus. Vaatamata üha täienevatele teadmistele kudede morfogeneesi (kuju tekke) tekkemehhanismide kohta, on endiselt ebaselge, kuidas rakustruktuuride kuju muutused aitavad reguleerida morfogeneesi. Kuna need muutused toimuvad arengus dünaamiliselt, siis on vaja nende uurimiseks mudelsüsteemi, mis võimaldaks jälgida ajaliselt-ruumiliselt arenguprotsesse eluskudedes. Üheks paindlikuks mudelsüsteemiks, millega koe-morfogeneesi uurida, on täismoonet läbivad äädikakärbse (*Drosophila melanogaster*) nukud. Antud doktoritöö keskendubki teadusküsimusele, kuidas epiteliaalsete rakkude kuju muutused koordineerivad 3D koe morfogeneesi äädikakärbse nuku-staadiumi tiiva arengus. Töö fookus on suunatud spetsiifilisele arenguaknale, mil tiiva epiteelid (selgmine ja kõhtmine) liiguvad teineteisest eemale ja seejärel uuesti kokku. See on hetk, mil tiiva epiteelirakkudes toimub koordineeritud rakujagunemine, mis on omakorda aluseks tiiva morfogeneesi kujunemisele.

Doktoritöös käistletud töödes avastati dünaamiline rakkudevaheline membraansete väljakasvude võrgustik, mis kujuneb tiiva selgmiste (dorsaalsete) ja kõhtmiste (ventraalsete) epiteelirakkude mikrotorukesi sisaldavate väljakasvude ja planaarsete aktiinifilamente sisaldavate filopoodi-laadsetest väljakasvudest, millele pandi nimeks interplanaarne Amida võrgustik (IPAN). IPAN on ülioluline struktuur koordineerimaks epiteelirakkude jagunemist selgmises ja kõhtmises tiivaepiteelis ja läbi selle tiiva morfogeneesi. Olulise ja uudse leiuna avastati, et rakukuju muutused aitavad reguleerida üleminekut mittetsentrosomaalsetelt mikrotorukesi organiseerivatelt keskustelt tsentrosomaalsetele organiseerimiskeskustele, ehk siis suunates mitoosi masinavärki. Seega IPAN pakkub unikaalset raamistikku koe-morfogeneesi uurimiseks.

Teiseks keskenduti doktoritöös ka rakukuju muutustele mikrotorukesi sisaldavate väljakasvude dünaamilisel taandumisel. Leiti, et valk α-spektriin mängib olulist rolli tiivaepiteeli rakkude tipmise ehk apikaalse osa kuju reguleerimisel läbi kontraktiilse aktomüosiini võrgustiku, mis on vajalik 3D-koe morfogeneesil.

Kolmanda uuringu eesmärk oli välja selgitada kuidas raku kuju muutus ja luu morfogeneetilise valgu (BMP) signalisatsioon on seotud äädikakärbse nuku-staadiumi areneva tiiva tagumise (posterioorse) ristsoone morfogeneesiga. Tulemused näitasid, et tiiva rakkude apikaalne ahenemine aitab reguleerida BMP signalisatsiooni, mis omakorda suunab rakkude diferentseerumist tiivasoonte rakkudeks või tiivasoonte vahelisteks rakkudeks.

Kokkuvõtvalt, doktoritöös käsitletud artiklid toovad selgust küsimustele, kuidas rakukuju muutused koordineerivad 3D koe-morfogeneesi, kuidas IPAN aitab sünkroniseerida selgmise ja kõhtmise tiivaeipiteeli rakkude jagunemist läbi rakk-rakk kontaktide kaotamise, kuidas on seotud α-spektriin ja tiiva epiteeliraku apikaalse osa „lõdvestus" ja kuidas läbi raku apikaalse osa ahenemise ja raku kuju muutuse on vahendatud konkurents BMP arengusignaali pärast, mis suunab tagumise tiivasoone morfogeneesi.

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