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Dynamics of interplanar bridges and tissue morphogenesis in *Drosophila melanogaster* pupal wing

Master´s thesis

30 EAP

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INFOLEHT

Interplanaarsete sildade dünaamika ning koe morfogenees *Drosophila melanogaster* nuku tiivas

Erinevad rakumembraani väljakasvud on oluliseks intertsellulaarse kommunikatsiooni vahendiks, osaledes rakkude proliferatsioonil, raku saatuse valiku kujunemisel ning migratsioonil. Kuigi membraani väljakasve on täheldatud mitmete mudelorganismide arenevates kudedes, on nende bioloogiline roll siiani ebaselge. Antud töös käsitletud mikrotorukesi sisaldavaid interplanaarseid sildasid, on võimalik vaadelda äädikakärbse *Drosophila melanogaster* areneva tiiva epiteelirakkudes. Selleks, et mõista interplanaarsete sildade dünaamikat ning funktsiooni kasutati 5D-mikroskoopiat ja biokuvamist ning tööriistana α-tubuliin:GFP ja tsentrosomaalse cnn:Cherry floreseeruvaid valke, visualiseerimaks interplanaarseid sildasid ning jagunevaid rakke. Tulemused näitasid, et interplanaarsete sildade dünaamika aitab reguleerida areneva tiiva epiteelirakkude koordineeritud proliferatsiooni.

Märksõnad: *Drosophila melanogaster* nuku tiib, intertsellulaarne kommunikatsioon, mikrotuubulid, MTOCs, -TIPs

CERCS (B350): Biomeditsiin

Dynamics of interplanar bridges and tissue morphogenesis in *Drosophila melanogaster* pupal wing

Different actin and/or tubulin-based membrane protrusions have been proposed to be one of the means for intercellular communication needed for proper cell proliferation, cell fate decision, and migration. Even though existence of membrane protrusions have occasionally been described in developing tissues, their physiological roles are still poorly understood. Microtubule-based protrusions, termed interplanar bridges, have been shown to be present in *Drosophila melanogaster* pupal wing. To study the dynamics and function of these protrusions, 5D time lapse imaging was conducted by using α-Tubulin:GFP and centrosomal cnn:Cherry to visualize the interplanar bridges and mitotic cells. Results gathered from the thesis show, that these structures are present during pupal wing development and appear to play roles in regulating coordinated cell mitosis as the means of intercellular communication.

Keywords: *Drosophila melanogaster* pupal wing, intercellular communication, microtubules, MTOCs, -TIPs

CERCS (B350): Biomedical sciences

1. TABLE OF CONTENT

2. ABBREVIATIONS

A-B – Apico-basal

ACV – Anterior crossvein

AP – After pupariation

A-P – Anterior-posterior

Arf – ADP ribosylation factor

Arl2 – ADP ribosylation factor-like-2

Arm – Armadillo

BMP – Bone Morphogenetic Protein

CAMSAP – Calmodulin-regulated spectrin-associated protein

CH – Calponin homology

CLASPs – CLIP-associated proteins

CLIP – Cytoplasmic linker protein

cMT – Centrosomal microtubule

cMTOC – Centrosomal microtubule-organizing center

Cnn – Centrosomin

Dgl – Discs large

Dpp – Decapentaplegic

Dsh – Dishevelled

DST – Dystonin

D-V – Dorsal-ventral

EB protein – End-binding protein

EGF – Epidermal growth factor

EGFR – Epidermal growth factor receptor

F-actin – Filamentous actin

FGF – Fibroblast growth factor

Gal80^{ts} – Temperature-sensitive Gal80

GAP – GTPase activating protein

GSCs – Germline stem cells

Hh – Hedgehog

Hpo – Hippo

hTau – Human Tau

IBs – Intercellular bridges

IpBs – Interplanar bridges

iTNTs – Individual tunneling nanotubes

Klp10A – Kinesin-like protein at 10A

Lgl – Lethal giant larvae

LVs 1-5 – Longitudinal veins 1-5

MACF1 – Microtubule actin cross-linking factor 1

MAP – Microtubule-associated protein

Msps – Mini spindles

MT – Microtubule

MT-nanotubes – Microtubule-based nanotubes

MTOC – Microtubule-organizing center

Nc-array – Non-centrosomal array

ncMT – Non-centrosomal microtubule

ncMTOC – Non-centrosomal microtubule-organizing center

Nub – Nubbin

PCM – Peri-centriolar material

PCP – Planar cell polarity

PCV – Posterior crossvein

P-D – Proximal-distal

Scrib – Scribbled

Shot – Spectraplakin Shotstop

SRF – Serum Response Factor

TAZ – Transcriptional co-activator with PDZ-binding motif

TBC – Tubulin cofactor

TBC-DEG – TBCD-TBCE-Arl2 GTPase

TGF-β – Transforming growth factor beta

TNTs – Tunneling nanotubes

UAS – Upstream activation sequence

Ubi – Ubiquitin

Wg – Wingless

YAP – Yes-associated protein

Yki – Yorkie

γ-TuRC – γ-tubulin ring complex

+TIPs – Microtubule plus-end tracking proteins

-TIPs – Microtubule minus-end tracking proteins

3. INTRODUCTION

Drosophila melanogaster, also referred to as a fruit fly, has been extensively used as a model organism to investigate various biological processes and to understand the role of genes during the development of embryos (Morgan, 1910). The wing of the fruit fly serves as a powerful tool to study cellular interactions, complex signaling pathways, and mechanisms underlaying the transformation of 2D tissue into 3D organ (Waddington, 1940; Gui *et al*., 2019).

Different membrane protrusions, composing of actin-based filopodia, tubulin-based microtubules, and more, have been identified to function as the means of intercellular communication (Önfelt *et al.*, 2006). This kind of cell-cell communication appears to be needed for proper proliferation, cell fate, and migration during development. These cell protrusions have been occasionally described in tissue development, but the formation and biological functioning of these structures is yet to be understood. That is the reason, why model organisms like *Drosophila melanogaster* provide a powerful tool to uncover the physiological role of membrane protrusions in the context of animal development.

Considering that development requires fine coordination, it was hypothesized, that membrane protrusions might have a role in wing development. In fact, microtubule-based nanotubes, hereby termed interplanar bridges, have been shown to have a role during pupal wing development (unpublished data). These microtubule structures are governed by non-centrosomal microtubule-organizing centers and seem to differ from other known cell protrusions by their structure and dissimilar roles in development. To better understand the role of interplanar bridges in the context of wing development, it was hypothesized that microtubule minus-end proteins that are shown to localize at non-centrosomal microtubule-organizing sites might have an impact in the functioning of interplanar bridges. Hence, one of the goals of the thesis was to screen genes known to be involved in MT minus-end regulation by non-centrosomal organizing centers. Additionally, genes shown to be involved in the stabilization of microtubule minus-ends have an adult wing phenotype, which is characterized by smaller wing size (unpublished data). That would imply, that interplanar bridges might have also a role in regulating cell division during pupal wing development. That is the reason, why another aim of the thesis was to uncover the dynamics of interplanar bridges and their potential role in the intercellular communication that governs the coordinated mitosis in pupal wing.

4. LITERATURE REVIEW

4. 1. *Drosophila melanogaster*

Drosophila melanogaster is a multi-cellular model organism with a rapid and well-defined life cycle divided into four distinguishable stages. They include embryonic, larval, pupal and adult stages (Morgan, 1910; Allocca *et al.*, 2018). The development of the fruit fly takes about 10 days at 25°C. After a day from egg laying, fruit fly embryo is hatched and develops to the larval stage (Fig. 1 A). The larval stages are divided into three separate phases: first, second, and third instar larvae (Fig. 1 B-D). At the end of the third instar larval stage, the animal stops eating and moving. The cuticle hardens and darkens to form the puparium (Fig. 1 E). The term for this process is pupariation. During the pupal stage, metamorphosis occurs for approximately 4 days after which the fly ecloses from the anterior end of puparium (Fig. 1 F). Mature adult flies live about 30 days (Ong *et al.*, 2015; Allocca *et al.*, 2018).

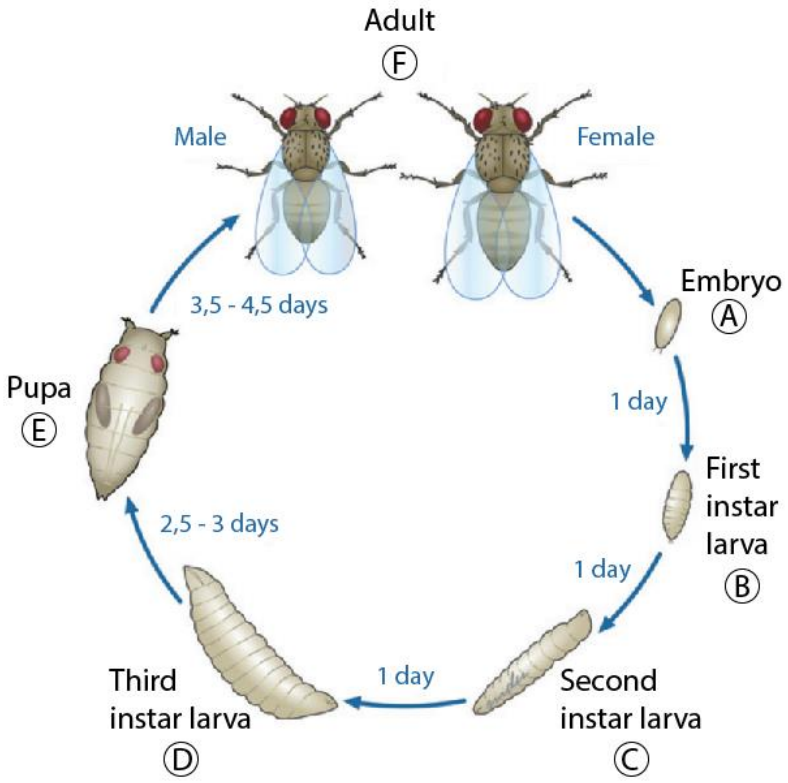

Figure 1. Life cycle of *Drosophila melanogaster*. (A-F) Fruit fly´s life cycle is divided into four stages: embryonic, larval (1st, 2nd and 3rd instar), pupal and adult stages. Development of *Drosophila melanogaster* takes about 10 days at 25°C and mature adult flies live approximately 30 days. Modified from Ong *et al.*, 2015.

4. 2. Wing morphogenesis

4. 2. 1. Larval stage

The wing of *Drosophila melanogaster* originates by invaginating from the embryonic ectoderm. The cells that establish the wing imaginal disc are determined early in embryogenesis where within the first hour of development, 12 primordial cells are given rise to. The imaginal disc is first detectable in newly hatched larvae as a "small oval group of cells", which possess a high rate of proliferation. During the first instar larval stage up to the pupariation, the wing imaginal disc cells divide at an exponential rate reaching from about 50 to about 50.000 cells, which are the total number of adult cells (Garcia-Bellido and Merriam, 1971; Diaz de la Loza and Thompson, 2017).

Wing imaginal disc, made up of a single sheet of epithelial cells, takes on a series of characteristic folds and establishes a sac-like structure as the wing disc grows in size (Fig. 2 A). It contains two territories that will give rise to the adult wing blade, or the hinge and part of the notum (Johnston and Gallant, 2002; Aegerter-Wilmsen *et al.*, 2007; Diaz-Garcia *et al.*, 2016; Diaz de la Loza and Thompson, 2017).

The interactions with neighboring cells play crucial role in defining tissue growth rates. The coordination of cell proliferation within the wing imaginal disc is mediated by local signals that control cell-cell interactions. WNT/Wingless (Wg), Bone Morphogenetic Protein (BMP)/Decapentaplegic (Dpp), Hedgehog, Notch, and other signaling pathways have been shown to influence tissue growth. Genes involved in growth can be divided into two classes. The first class contains the genes affecting the rate of growth and the final size of the organ by controlling the synthesis of proteins and other metabolic processes. The second class of the genes determines the identity, pattern, structure, and final size of wing imaginal discs (Burke *et al.*, 1999; Wang *et al.*, 2000; Johnston and Gallant, 2002; Aegerter-Wilmsen *et al.*, 2007; Schweisguth and Corson, 2019).

The developing wing imaginal disc cells progressively establish precise positional information. Determination events taking place during larval stage divide wing imaginal discs into anterior-posterior (A-P) and dorsal-ventral (D-V) compartments (Fig. 2 B). WNT protein Wg and BMP protein Dpp are the primary pattern organizers expressed in two perpendicular stripes. Dpp expression is observed at the anterior cells of A-P boundary. Wg expression is detectable at the D-V boundary. They are defined by cell-linage restrictions and the cells cannot cross from one side to other. Together, Wg and Dpp specify proximal-distal (P-D) axis as well as vein and

bristle patterning (Fig. 2 B) (Waddington, 1940; Garcia-Bellido and Merriam, 1971; Diaz de la Loza and Thompson, 2017). Boundaries between compartments form organizing centers, becoming the source of long- and short-range diffusible signals known as morphogens. Short-range signals are generated by planar polarity myosins that promote tissue elongation during larval stage along P-D axis. Long-range signals are crucial in shaping the wing and patterning during pupal stage (Olguin and Mlodzik, 2010; Raftery and Umulis, 2012). Boundaries can control the expression of transcriptional activators and repressors. Each region of the wing disc will have a distinct combination of prepattern genes, which will determine different cell fates and presages the final differentiated adult wing structure (Waddington, 1940; Garcia-Bellido and Merriam, 1971; Raftery and Umulis, 2012).

Coordination among neighboring cells is important for development of imaginal discs. Apico-basal (A-B) polarity of epithelial cells allows them to share information by connecting neighboring cells with structures such as adherens junctions, septate junctions, and gap junctions (Johnston and Gallant, 2002). They provide the tools for anchoring the actin cytoskeleton of one cell to another. In addition, they are responsible for clustering receptors and their ligands into signaling foci within the plasma membrane. The junctions are crucial for the maintenance of the wing imaginal disc, and are needed for flexibility to accommodate cell division and folding of the wing disc as it grows (Johnston and Gallant, 2002). Several genes, classified as tumor suppressors, are needed for growth regulation. They have been divided into two groups based on their characteristics, neoplastic and hyperplastic. The "neoplastic" class of genes are responsible for A-B polarity to organize A-B axis and to maintain the architecture of an epithelial cell. They are located at the basal-lateral plasma membrane and depend on each other for this position. Neoplastic tumor suppressor genes include *lethal giant larvae* (*lgl*), *discs large* (*dgl*) and *scribbled* (*scrib*). They are required for the formation of the apically located adherens junctions. The "hyperplastic" class of gene are needed to retain the polarized structure (Johnston and Gallant, 2002). Cadherins and their cytoplastic partners catenins are adhesion proteins belonging to the hyperplastic class and they form the core of adherens junctions. They arrange the membrane actin network by linking transmembrane proteins to the cytoskeleton and are responsible for the control of wing disc growth. Both neoplastic and hyperplastic tumor suppressor mutants lead to massive disc overgrowth (Johnston and Gallant, 2002).

4. 2. 2. Pupal stage

At the end of the larval stage, growth of the wing disc decreases to start a dramatic morphogenesis into a fully formed adult wing. The wing pouch region expands, elongates, and continues the final differentiation of the external wing structure of the adult (Fig. 2 C) (Garcia-Bellido and Merriam, 1971; Diaz de la Loza and Thompson, 2017).

During pupal stage, imaginal wing disc everts and moves from the inside of the larvae through the larval wall to obtain a two-layered three-dimensional wing containing two epithelial layers (Garcia-Bellido and Merriam, 1971). Wing development during the pupal stage can be divided into three phases (Fig. 2 D-F). During first apposition, from the start of pupariation till 10 hours after pupariation (AP), single-layered wing epithelium forms dorsal and ventral epithelia giving rise to two-layered wing. From 10 to 20 hours AP, the wing swells by the accumulation of fluid between the dorsal and ventral epithelial layers. This phase is termed as inflation. Second apposition starts from 20 hours AP and continues until the end of the pupal stage. During this stage, wing returns to a flat form except for the veins, which retain a narrow channel for fluid (Fig. 2 G) (Waddington, 1940; Etournay *et al.*, 2016; Gui *et al.*, 2019). Throughout this stage, mitotic waves are detectable and contraction of the wing hinge and anchorage of the wing blade to the pupal cuticle defines the final shape of the wing. This results in the wing blade growing in size, while hinge part will becomes smaller (Garcia-Bellido and Merriam, 1971; Etournay *et al.*, 2016). Serum response factor (SRF)/Blistered is a transcription factor, which is expressed in intervein regions. It is responsible in determining the spatial pattern of the wing. It has been shown that loss of *srf/blistered* leads to blisters and ballooned wings, where the direction between vein and intervein is lost. Blistering implies to the condition, where dorsal and ventral wing epithelia are separated (Fristrom *et al.*, 1994). Epidermal growth factor receptor (EGFR), BMP/Dpp, Hedgehog, and Notch signaling determines the expression of SRF/Blistered, which themselves are ultimately determined by compartment boundary organizers (Artavanis-Tsakonas *et al.*, 1999; Shimmi *et al.*, 2014; Diaz de la Loza and Thompson, 2017).

4. 2. 3. Adult stage

In the end of the pupal stage, adult flies eclose from their pupal cases and the folded wings spread out. Female wings are generally larger than the wings of male flies. The shape, size, and patterning of the wing is by then already predetermined. Adult fly wing contains five longitudinal veins (LVs) 1, 2, 3, 4, and 5 that are on the P-D axis. Two crossveins termed

anterior crossvein (ACV) and posterior crossvein (PCV) are on the A-P axis (Fig. 2 H) (Diaz de la Loza and Thompson, 2017).

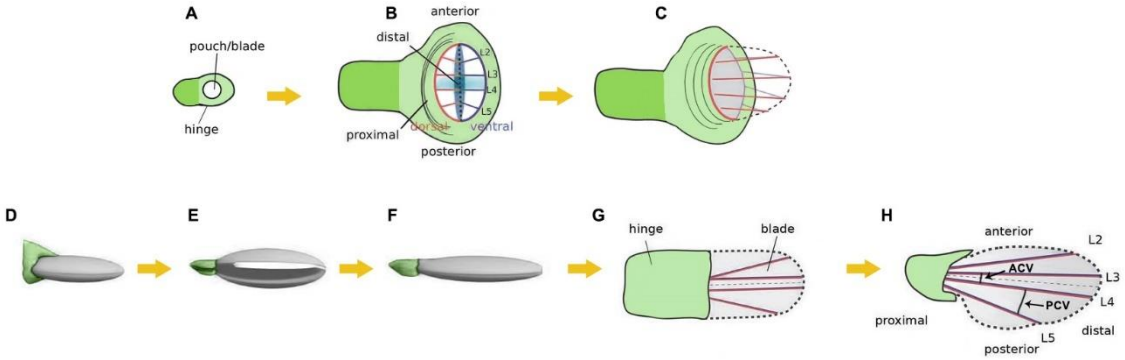

Figure 2. Overview of the wing morphogenesis in *Drosophila melanogaster*. (A) During early larval stage, two regions will give rise to wing blade, hinge, and notum. (B) During wing disc development, A-P and D-V polarity is determined by Dpp and Wnt signals, respectively. Together, A-P and D-V boundaries give rise to P-D axis. (C) From the late larval stage, wing pouch starts to elongate. (D, E, F) During pupal stage, three detectable stages, I apposition, inflation and II apposition, can be identified. (G) Wing size, shape, and patterning are already predetermined in late pupal stage. (H) Adult wing contains 5 LVs (LV1 – 5), ACV, and PCV. Regions between LVs are termed intervein region. (A-C, G-H) Dorsal view of the wing. (D-F) Lateral view of the wing. White – wing pouch, gray – wing blade, light green – hinge, dark green – notum, light blue – Dpp signal, dark blue – Wnt signal. Axes are as follows: proximal left, distal right, anterior up, posterior down. Modified from Matamoro-Vidal *et al.*, 2018 and Gui *et al.*, 2019.

4. 3. Signaling pathways

The proliferation and differentiation of fruit fly wing is regulated by several conserved signaling pathways, such as Hippo, Hedgehog, BMP/Dpp, EGFR, Notch, Wnt/Wg and more, which all have roles on their own and are in coordination with each.

4. 3. 1. Hippo signaling pathway

The Hippo signaling pathway, initially identified in *Drosophila melanogaster*, is evolutionarily conserved pathway. The main function of this pathway is to restrict tissue growth in adults and modulate cell proliferation, differentiation, migration, and apoptosis in developing organ. Hippo pathway limits the organ size through phosphorylation and inhibition of transcription co-activators Yes-associated protein (YAP) and its paralog transcriptional co-activator with PDZ-

binding motif (TAZ) in mammals (Yorkie (Yki) in *Drosophila melanogaster*) (Liu *et al.*, 2010; Zhao *et al.*, 2011; Koontz *et al.*, 2013; Meng *et al.*, 2016; Nashchekin *et al.*, 2016; Plouffe *et al.*, 2016; Zheng and Pan, 2019).

In fruit fly wing, Hippo signaling pathway is a key regulator of imaginal disc growth influencing the rate of proliferation and cell survival (Diaz de la Loza and Thompson, 2017). Disruptions in the Hippo pathway result in the loss of tissue homeostasis and mutants exhibit uncontrolled tissue growth and reduced apoptosis resulting in the imaginal disc overgrowth. Using wing imaginal disc as a model, it was found that knockdown of *yki* or *hippo* (*hpo)* result in the formation of smaller or bigger adult wings, respectively (Halder and Johnson, 2011; Plouffe *et al.*, 2016; Diaz de la Loza and Thompson, 2017; Hevia *et al.*, 2017). Thus, Hippo signaling pathway regulates organ growth and tissue size in the wing of *Drosophila melanogaster*. Studies have also shown that the Hippo signaling pathway is regulated by actin cytoskeleton as well as cell polarity, cell adhesion, and cell junction proteins indicating a crosstalk with Hippo pathway to regulate organ size (Zheng and Pan, 2019).

The prime mediator, YAP/TAZ/Yki plays an important role in the control of cell fate and tissue regeneration as a link and integrator of multiple prominent pathways such as WNT/Wg, Epidermal growth factor (EGF)/Egf, BMP/Dpp, and Notch pathways (Varelas *et al.*, 2010; Halder and Johnson, 2011).

4. 3. 2. WNT/Wingless signaling pathway

WNT proteins are a family of secreted lipoproteins that activate different intracellular signaling pathways through binding to several receptors and co-receptors locating at the cell surface (Palomer *et al.*, 2019). The canonical WNT/Wg signaling pathway is involved in proliferation and growth, cell fate specification and differentiation by controlling the expression of WNT/Wg target genes by stabilizing cytoplasmic β-catenin (β-CAT)/Armadillo (Arm) (Wang *et al.*, 2000; Nusse, 2008; Gattinoni *et al.*, 2010; Varelas *et al.*, 2010; Yang, 2012; Cervello *et al.*, 2017; Palomer *et al.*, 2019).

The non-canonical WNT/Wg signaling pathway plays a role in proper cell migration, the establishment of body axes through reorganizing the cellular cytoskeleton, cell fate specification and differentiation, apoptosis, and more (Wang *et al.*, 2000; Nusse and Clevers, 2017; Palomer *et al.*, 2019). Planar cell polarity (PCP) pathway is a non-canonic pathway, that does not require β-CAT/Arm (Olguin and Mlodzik, 2010; Palomer *et al.*, 2019). This signaling pathway is important in organ morphogenesis and in polarized cell movement by regulating

cytoskeletal rearrangement (Nusse and Clevers, 2017; Palomer *et al.*. 2019). It has been shown, that Wg signaling pathway is involved in the control of cell polarity and MT cytoskeleton development through the interactions between Dishevelled (Dsh) and Par-complex proteins in *Drosophila melanogaster* wing (Yang, 2012).

During imaginal disc development, Wg together with Dpp signaling pathways are shown to be "patterning" signal transduction pathways in fruit fly wing development (Johnston and Gallant, 2002). In early larval stage, Wg is responsible for the formation of D-V compartment to establish body A-P and D-V axes of the wing. Together with Egf's and others, concentration gradients are formed in the imaginal disc. Areas with higher concentration establish posterior region, areas with lower concentration become the anterior region (Waddington, 1940; Garcia-Bellido and Merriam, 1971; Diaz de la Loza and Thompson, 2017). Wg has also shown to play a role in development of the wing and notum during later stages. For example, *Wg* over-expression mutants show tissue overgrowth while knock-down of the gene leads to the loss of wings (Wang *et al.*, 2000).

4. 3. 3. BMP/Dpp signaling pathway

The transforming growth factor-β (TGF-β) signaling pathway is involved in many aspects of development by controlling tissue and organ development, extracellular matrix production, cellular proliferation, differentiation, motility and apoptosis (Cohen, 2003). The activity of this pathway in *Drosophila melanogaster* is mainly required to promote the proliferation of neuronal and epithelial tissues (Hevia *et al.*, 2017). The TGF-β superfamily of ligands consist of about 40 secreted polypeptide growth factors that comprise of TGFs, activins, nodals, and BMPs (Cervello *et al.*, 2017).

BMPs are members of the TGF-β superfamily shown to be an important group of signaling molecules conserved in both vertebrates and invertebrates (Padgett *et al.*, 1993; Carreira *et al.*, 2014). They play a crucial role in regulating cell lineage, differentiation, proliferation, apoptosis, and morphogenesis, making BMPs essential for conserved embryonic developmental and adult homeostatic processes (Padgett *et al.*, 1993; Derynck *et al.*, 1998; Katagiri and Watabe, 2016).

The biological activities of BMPs are highly conserved between flies and mammals. That is the reason why fruit fly has provided a powerful platform to study the biological mechanisms and biochemical properties of BMPs (Padgett *et al.*, 1993). Dpp is a long-range morphogen responsible for the formation of A-P boundary during early larval stage (Garcia-Bellido and

Merriam, 1971; Aegerter-Wilmsen *et al.*, 2007). In later stages, it has a role in inducing more distant patterning along the A-P axis and plays a role in the growth of the wing imaginal disc (Burke *et al.*, 1999; Haines and Van den Heuvel, 2000; Aegerter-Wilmsen *et al.*, 2007; Gui *et al.*, 2019). In the development of fruit fly pupal wing, loss of Dpp signal has shown to lead to adult wing phenotypes, which are characterized by reduced wing size and loss of vein formation, leading to the conclusion that Dpp signal regulates both the proliferation and patterning during the pupal wing development (Gui *et al.*, 2019).

4. 3. 4. Epidermal growth factor receptor signaling pathway

EGFR signaling pathway is responsible for regulating cell remodeling during development. It is needed for cell proliferation, differentiation and survival, inhibition of apoptosis, migration, cell adhesion, and more (Wang *et al.*, 2000; Buchon *et al.*, 2010; Malartre, 2016). In *Drosophila melanogaster*, the pathway´s overexpression results in uncontrolled cell division and migration, loss of Egfr signaling leads to widespread apoptosis. That is a reason why the inhibition of EGFR/Egfr pathway has been a target for cancer therapy. This pathway is also been shown to have an impact on cell polarity during development (Lusk *et al.*, 2017).

Egfr mediated signaling controls the growth and patterning of the wing imaginal disc. During early wing imaginal disc development, Egfr signaling is required for the formation of D-V compartment and wing-notum differentiation (Wang *et al.*, 2000). *Egfr* mutants exhibit abnormal wing phenotype, where the wing blades are present, but they have patterning abnormalities, and the notum is lost. This leads to the conclusion, that Egfr signaling is required for normal wing-notum development (Wang *et al.*, 2000; Zecca and Struhl, 2002).

4. 3. 5. Notch signaling pathway

Notch signaling pathway is a highly conserved pathway involved in a variety of essential cellular processes, including cell fate determination, cell polarity and tissue homeostasis (Artavanis-Tsakonas *et al.*, 1999; Lai, 2004). Notch signaling directly affects cell proliferation and patterning during fruit fly wing morphogenesis. The ectopic expression of Notch or its ligand Delta/Serrate, has been shown to cause adult wing phenotypes, which are characterized by outgrowths (Baonza and Garcia-Bellido, 2000).

4. 3. 6. Hedgehog signaling pathway

The Hedgehog signaling pathway is evolutionarily conserved pathway implicated to have a role in regulating development and differentiation of tissues and organs during embryogenesis (Cervello *et al.*, 2017; Li *et al.*, 2017). It has been documented that short-range inducer Hedgehog (Hh) controls the expression of long-range morphogens Wg and Dpp in *Drosophila melanogaster* wing. During imaginal disc development, wing primordium is subdivided into A-P compartments. Cells in the posterior compartment are programmed to secrete Hh (Haines and Van den Heuvel, 2000). In comparison, cells in the anterior compartment transduce the Hh signal, which is essential for correct wing patterning. Hh signaling has shown to directly determine the position of the future wing vein LV3 (Burke *et al.*, 1999). It is also implemented in inducing a narrow band of cells to express Dpp. Alterations in the *dpp* expression leads to dramatically affected patterning, characterized by loss of veins, and wing size reduction (Aegerter-Wilmsen *et al.*, 2007; Shimmi *et al.*, 2014; Gui *et al.*, 2019) .

4. 4. Intercellular communication through membrane protrusions

Cell cytoskeleton is a complex and dynamic network of interlinking protein filaments in the cytoplasm. It performs multiple functions, like establishing and preserving cell shape, allowing cell migration, and being involved in many cell signaling pathways (Huber *et al.*, 2013). Three main components, actin-based microfilaments, intermediate filaments, and tubulin-based microtubules (MTs), build up the cytoskeleton in eukaryotic cells (Ridley, 2006; Cohen *et al.*, 2010; Huber *et al.*, 2013; Caswell and Zech, 2018).

Intercellular communication is a vital property of multicellular organisms during development and tissue homeostasis (Buszczak, *et al.*, 2016). It is needed for the adequate responses for proliferation, cell fate determination, and coordinated migration. Different structures such as tunneling nanotubes (TNTs), cytonemes, intercellular bridges (IBs), and MT-based nanotubes (MT-nanotubes) have been shown to be a part of the cell-to-cell communication allowing signals to be transmitted in a selective manner over a wide range of distances (Rustom *et al.*, 2004; Önfelt *et al.*, 2006; Sartori-Rupp *et al.*, 2019). These protrusions can be distinguished based on their diameter, length, and cytoskeletal elements involved in their formation filaments (Sherer and Mothes, 2008; Kornberg and Roy, 2014; Buszczak *et al.* 2016).

4. 4. 1. Tunneling nanotubes

TNTs are long cell-to-cell conduits representing a novel means for intercellular communication (Rustom *et al.*, 2004). They are straight, filamentous actin (F-actin) based bridge-like structures that are actively formed under a broad range of stresses and are proposed to exist under physiological conditions (Korenkova *et al.*, 2020). They establish cytoplastic continuity and can traffic diverse cargos, such as mitochondria, endosomal vesicles, viruses, and Ca^{2+} (Rustom *et al.*, 2004; Vignais *et al.*, 2017). TNTs are able to couple cells over long distances and the length of the TNTs varies as the connected cells move apart or migrate (Abounit and Zurzolo, 2012). Importantly, even though majority of TNTs are shown to contain only F-actin, they have also been shown to contain MTs (MT-containing TNTs) (Wang and Gerdes, 2015).

There has been a proposed categorization of TNTs according to their diameter (Önfelt *et al.*, 2006). Thin TNTs are mainly composed of F-actin and allow the exchange of smaller cargo. Thick TNTs contain both F-actin and MTs. They are shown to be more stable and to have an ability to transfer larger cargo such as mitochondria (Önfelt *et al.*, 2006). But it has also been anticipated that structures that appear to be "thick" may in fact be made from several individual TNTs (iTNTs). Thin TNTs appear to connect iTNTs between each other, conceivably holding them in a bundle and conferring higher stability (Sartori-Rupp *et al.*, 2019).

TNTs are thought to be involved in development by helping to arrange morphogenetic gradients, that allow cells to reach their specific niche in different tissues or cell types (Korenkova *et al.*, 2020). Additionally, they may have a role in immune cell activation and immune response and are thought to contribute to various pathological conditions, like cancer, AIDS, and neurodegenerative diseases (Venugopal *et al.*, 2014).

Several pathways have been shown to be involved in actin remodeling. Ras GTPases, Rab proteins, and EGFR have been shown to promote TNT formation in different cellular contexts (Hase *et al.*, 2009; Delage *et al.*, 2016; Zhu *et al.*, 2018). Still, the molecular details of the mechanisms of TNT formation are largely unclear (Korenkova *et al.*, 2020).

TNTs differ from other protrusions, like filopodia, and intercellular structures, such as IBs and cytonemes, both from the structural and functional point of view (Buszczak *et al.*, 2016).

4. 4. 2. Filopodia

Filopodia is a needle-like protrusion consisting of bundled and polymerized linear F-actin filaments. It can align with focal adhesions and have a role in cell migration by sensing the

chemical and physical environment, facilitating cell-cell adhesion, and forming protrusions (Caswell and Zech, 2018). Compared to TNTs, filopodia have short-range filaments and do not appear to contain vesicles. Another difference comes from the fact, that compared to TNTs, filopodia displays various actin arrangements, namely parallel bundles (Sartori-Rupp *et al.*, 2019). Even though the difference between filopodia ja TNTs exists, it is important to note, that the possibility that TNTs could arise from filopodia should not be excluded (Korenkova *et al.*, 2020). During migration, cells actively form filopodia not only for movement, but also for signaling purpose. It has been speculated, that cells could form numerous protrusions in different directions when seeking information about migration direction. Later, cells could form fewer protrusions towards the direction of migration to synchronize their migration with other cells (Korenkova *et al.*, 2020). Protrusion formation has also been associated with cell division. It is important to note, that different types of protrusions could be formed by the same cell at the same time (Danilchik *et al.*, 2013).

4. 4. 3. Intercellular bridges

IBs are type of protrusions that allow the cytoplastic continuity and cargo transfer of organelles and/or macromolecules similar to TNTs (Greenbaum *et al.*, 2011). The common components of IBs are actin and anillin (Robinson and Cooley, 1997). Two IBs, germline and somatic IBs, have been shown. Germline IBs are relatively short and are shown to be responsible for germ cell communication, allowance of nutrient transport, and synchronizing mitotic cell division and entry into meiosis (Robinson and Cooley, 1997; Greenbaum *et al.*, 2011; Haglund *et al.*, 2011; Korenkova *et al.*, 2020). Somatic IBs have much shorter length compared to TNTs. They promote exchange of cytoplasm, synchronization of cell division or differentiation, and coordination of cell behavior during development (Haglund *et al.*, 2011). IBs differ from TNTs by having generally shorter and thicker dimensions. They interconnect neighboring cells and even though IBs are thought to connect cells only in short distance, study in developing zebrafish showed, that they are also able to connect cells over long distances (Caneparo *et al*., 2011). Another difference between TNTs and IBs comes from the fact, that IBs are formed between dividing cells while TNTs are formed *de novo* between two distinct cells (Rustom *et al.*, 2004). Thereby even though the proposed functions of IBs and TNTs could overlap, they represent distinct structures and their role should also be different during development (Korenkova *et al.*, 2020).

4. 4. 4. Cytonemes

Cytonemes are another type of cell protrusions that resemble TNTs morphologically, but have a distinct function. While TNTs allow transfer of cargos through establishing cytoplasmatic continuity, cytonemes allow signal transduction through protein-protein interactions (Rustom *et al.*, 2004; Kornberg and Roy, 2014). Cytonemes are actin-based structures, that are found in diverse tissues and are shown to promote signaling between specific cells over variety of distances (Buszczak *et al.*, 2016). They were first noted in *Drosophila melanogaster* wing disc cells as long cellular protrusions (Ramírez-Weber and Kornberg, 1999). They were shown to be extended between wing disc morphogen-receiving cells and the morphogen-expressing cells suggesting an alternative possibility to diffusion-based models of morphogen dispersion (Ramírez-Weber and Kornberg, 1999). While the conventional view is that concentration gradient is formed by simple diffusion, cytonemes appear to be a likely mechanism for establishing morphogen gradients both in *Drosophila melanogaster* and vertebrate (Stanganello *et al.*, 2015). Cytonemes are shown to promote BMP/Dpp, fibroblast growth factor (FGF), and Hh signaling, that are vital for tissue pattern formation during wing disc development (Cohen *et al.*, 2010; Gradilla *et al.*, 2014). For example, Dpp and Wnt gradients may be dependent on cytoneme length and the frequency of contact with recipient cells (Stanganello and Scholpp, 2016). In addition, they are also shown to contribute signal transduction of Wg, EGF, and Delta/Notch pathways (Timothy *et al.*, 2013; Buszczak *et al.*, 2016). Cytoneme formation appears to be specific for precise signaling pathways (Roy *et al.*, 2011). For example, in wing disc, over-expression of *dpp* or disruption of EGF signaling has shown to result in the formation of short cytonemes suggesting their role in growth and/or stabilization of cytonemes (Buszczak *et al.*, 2016). Thereby cytonemes could represent specific means of direct intercellular communication during development (Korenkova *et al.*, 2020).

4. 4. 5. Microtubule-based nanotubes

MT-nanotubes are thin tubulin-based protrusions (Inaba *et al.*, 2015). They are shown to provide an exclusive surface for productive signaling between niche and stem cells in *Drosophila melanogaster* germline stem cells (GSCs) (Buszczak *et al.*, 2016). It has been indicated, that manipulations of the size and frequency of MT-nanotubes impact the Dpp signal transduction within GSCs (Buszczak *et al.*, 2016). For example, increasing the thickness of MT-nanotubes increases the Dpp signaling. In comparison, decreasing the frequency of MT-nanotubes leads to the reduction of Dpp signal transduction (Buszczak *et al.*, 2016). Thereby it

could be concluded that Dpp signaling components may promote the formation and/or stabilization of MT-nanotubes.

Much of the formation and functioning of MT-nanotubes is yet to be understood. Like with cytonemes, it remains unclear how MT-nanotubes are made specific for different signaling pathways and how trafficking through these structures is regulated.

4. 5. Microtubules

MTs are hollow cylinder-like structures present in all eukaryotic cells. They play together with intermediate filaments and F-actin an important role in cell cytoskeleton formation. MTs possess an enormous flexibility making them a vital player in different cellular functions like cell division, cell shape regulation, intracellular transport, cell polarization, and migration (De Forges *et al.*, 2012; Muroyama and Lechler, 2017).

4. 5. 1. Microtubule dynamics

αβ-tubulin heterodimers are the building blocks of MTs giving them their distinguishable structure, dynamics, and polarity. Tubulin superfamily is highly conserved family of proteins, composed of alpha-(α), beta-(β), gamma-(γ), delta-(δ), epsilon-(ε), zeta-(ζ) and eta- (η) tubulin members (Dutcher, 2001; Lopez-Fanarraga *et al.*, 2001). While α-, β, and γ-tubulins are found in all eukaryotic cells and are shown to be essential for MT polymerization, then δ-, ε-, ζ-, and η-tubulins do not appear to have a ubiquitous distribution in eukaryotic cells (Dutcher, 2001).

In order for the MTs to form, αβ-tubulin heterodimers have to interact among themselves and bind to those already at the MT lattice (Hirata *et al.*, 1998; Lopez-Fanarraga *et al.*, 2001). In comparison to actin and γ-tubulin, where folding concludes after polypeptides are released from the chaperonin, α- and β-tubulin folding can be achieved only after a second GTP-hydrolysis-dependent step, where coordinated interactions with other tubulin cofactors are required (Fontalba *et al.*, 1993).

A group of proteins called tubulin cofactors (TBCs) are crucial in the folding of tubulin dimers. They control the availability of tubulin subunits and regulate tubulin subunit synthesis, dimer formation, and MT stability in all eukaryotic cells (Hirata *et al.*, 1998; Lopez-Fanarraga *et al.*, 2001; Szymanski, 2002). The conserved group consists of TBC-A, -B, -C, -D, and -E proteins (Tian *et al.*, 1997). In addition, another crucial player in tubulin biogenesis and/or degradation is an enzyme called ADP ribosylation factor-like-2 (Arl2) (Bhamidipati *et al.*, 2000; Nithianantham *et al.*, 2015). This protein belongs to the Arl family, which represents a group

of ADP ribosylation factor (Arf) related proteins with little or no Arf activity (Tian *et al.*, 1997). Together these cofactors and Arl2 GTPase regulate the assembly of α- and β-tubulin into heterodimers and are needed for the synthesis of polymerization-competent tubulin subunits (Tian *et al.*, 1997; Bhamidipati *et al.*, 2000; Al-Bassam and Bement, 2017).

TBCB and TBCA were the first cofactors shown to be involved in α- and β-tubulin folding, respectively (Bhamidipati *et al.*, 2000; Lopez-Fanarraga *et al.*, 2001). In the process of tubulin folding, newly synthesized α- and β-tubulin polypeptides bind to the cytosolic chaperonin complex. α- and β-tubulin bind to TBCB and TBCA, respectively (Fig. 3 1-2). α-tubulin is then handed off to TBCE, β-tubulin to TBCD, respectively (Fig. 3 3-4). This results in the formation of TBCE-α/TBCD-β complex (Fig. 3 5a). TBCC is then bound to the complex (Fig. 3 5b). Arl2 is next requited to the complex resulting in the formation of a stable heterotrimeric chaperone, termed TBCD-TBCE-Arl2 GTPase (TBC-DEG). Following the establishment of the complex, Arl2 is stimulated to hydrolyze GTP through the GTPase activating protein (GAP) function of TBCC (Fig. 3 6). This leads to the activation of Arl2 activity, which releases energy needed to alter the shape of αβ-tubulin and results in the release of the dimer from the complex, which is the foundation of MT formation (Fig 3. 7) (Tian *et al.*, 1997; Bhamidipati *et al.*, 2000; Szymanski, 2002; Nithianantham *et al.*, 2015).

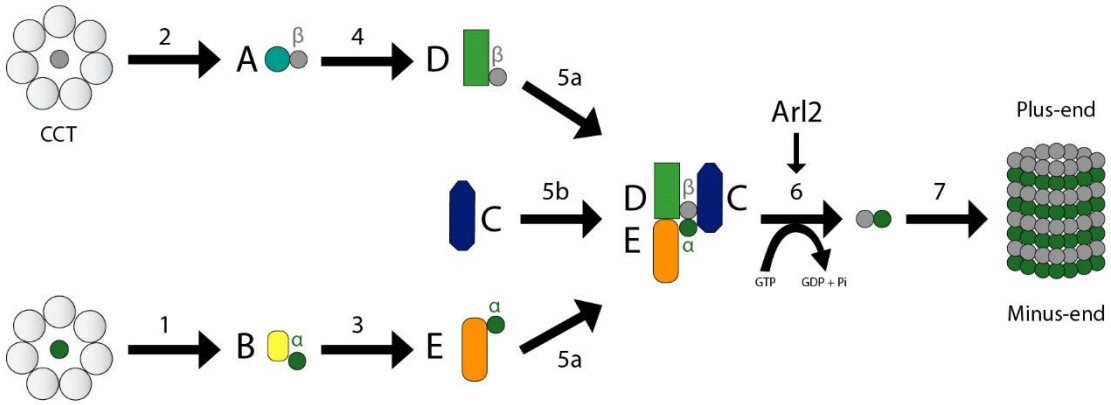

Figure 3. The formation of α- and β-tubulin heterodimers to form MTs. During MT tubulin folding, α- and β- tubulin are bound to TBCB and TBCA, respectively (1, 2). They are then handed off to TBCE and TBCD, respectively (3, 4), which forms a complex with additional cofactor, TBCC (5a, 5b). Arl2 is then bound to the complex and it hydrolyzes GTP into GDP, which is needed for α- and β-tubulin heterodimer release (6). They are assembled to MTs in a manner that α-tubulin is exposed at minus-ends, and β-tubulin at plus-ends (7). According to Tian *et al.*, 1997.

It has been shown, that overexpression of TBCE, similar to its β-tubulin counterpart TBCD, can destroy the complete MT network in the cell (Bhamidipati *et al.*, 2000). More recent experiments with yeast have demonstrated that TBC-DEG can form a stable complex. It was found that mutant form of *arl2* that lacked enzyme activity inhibited ternary complex dissociation in vitro and caused several defects in MT dynamics in vivo due to problems in forming MTs (Nithianantham *et al.*, 2015). From these results it was concluded that TBC-DEG chaperone has a role in regulating MT functioning. They are essential for proper MT dynamics in eukaryotic cells and their loss or overexpression are both lethal in most eukaryotes, stemming from the loss of the MT cytoskeleton (Nithianantham *et al.*, 2015).

Two MT ends are intrinsically distinct as α-tubulin is exposed at the minus-end and β-tubulin is at the plus-end. The dynamics and polarity of MTs stems from the polymerization and depolymerization at their ends.

MTs undertake phases of growth and shrinking, which are separated by events of catastrophe (transition from growing to shrinking) and rescue (transition from shrinking to growing) (Fig. 4). This dynamic behavior of assemble and disassemble from MT ends was termed “dynamic instability” (Desai and Mitchison, 1997). Positively charged plus-ends can undergo transition from a rapidly growing state to one in which the MT undergoes complete depolymerization. The negatively charged minus-ends have a slower rate of dimer addition and/or loss compared to the plus-ends (Desai and Mitchison, 1997). They are often capped *in vivo* making them relatively stable compared to the plus-ends (Hirata *et al.*, 1998). The fact, that individual tubulin units are either added to or removed from the filament ends gives MTs an enormous plasticity capable of rapid change in their length.

The behavior of MT instability is mainly explained by the GTP-cap model (Desai and Mitchison, 1997; Akhmanova and Hoogenraad, 2015). Both α- and β-tubulin can bind to GTP but only GTP bound to the β-tubulin can be hydrolyzed. Thereby MT lattice is built from GDP-tubulin, where the GTP-cap binds to β-tubulin and stimulates MT growth. During polymerization, GTP-bound tubulin heterodimers are added at the plus-end of the MTs, resulting in their assembly. A slight delay between polymerization and hydrolysis of the GTP by β-tubulin creates a GTP-tubulin cap. This protects the plus-ends by stabilizing them. The loss of GTP-cap induces quick depolymerization which results in rapid disassembly of the MT (Desai and Mitchison, 1997; De Forges *et al.*, 2012).

The switch from MT polymerization to depolymerization is sensitive to the intrinsic rate of hydrolysis of the GTP molecule bound to the β-tubulin subunit. An inactive non-exchangeable

site, termed N-site, on α-tubulin, is thought to stabilize αβ-tubulin dimers during their biogenesis. An active exchangeable site, termed E-site, on β-tubulin, is stimulated to hydrolyze GTP upon αβ-tubulin incorporation into MT lattice at the plus ends. GTP hydrolysis at the E-site leads to dynamic instability at the MT plus ends. GTPase helps to build tubulin units (αβ-tubulin) capable of attaching to the MTs (Tian *et al.*, 1997; Alushin *et al.*, 2014).

In cell behavior, MT plus-ends are mostly extended toward the cell periphery. In contrast, the minus-ends are attached to different cellular structures, such as mitotic kinetochores or the cell cortex (Mitchison, 1993; De Forges *et al.*, 2012; Muroyama and Lechler, 2017).

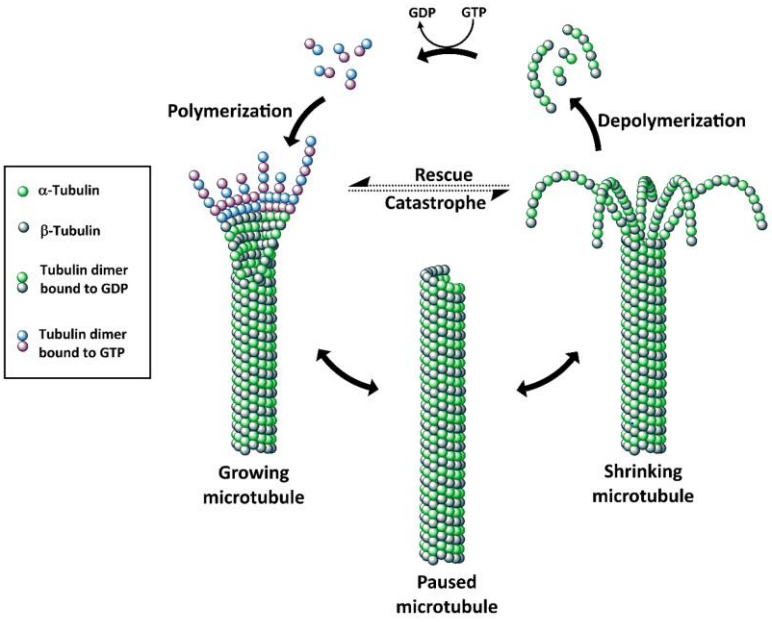

Figure 4. MT dynamics. MTs undergo phases of growth and shrinking, which are separated by events of catastrophe and rescue. The polymerization and depolymerization of MTs takes mainly place at β-tubulin exposed plus-ends. In comparison, α-tubulin exposed MT minus-ends are relatively stable and exhibit growth and shrinkage in a smaller rate. Modified from Chen *et al.*, 2017.

4. 5. 2. Microtubule organizing centers

Microtubule-organizing centers (MTOCs) are responsible for regulating inherent MT dynamic instability. MTs are anchored to the MTOCs by their minus-ends. Considering this fact, MTOCs can be divided into two, centrosomal- and non-centrosomal-MTOCs (cMTOCs and ncMTOCs) depending on the cell type, cell cycle, and differentiation stage (Bornens, 2012; De Forges *et al.*, 2012; Muroyama and Lechler, 2017).

During cell division, centrosomes are the main MTOCs in all eukaryotic cells. They acts as cMTOCs to regulate centrosomal-MTs (cMTs) through nucleation and anchoring (Bornens, 2012; De Forges *et al.*, 2012).

Centrosomes are comprised of two centrioles, each having nine-triplet MTs, surrounded by dense mass of proteins, termed peri-centriolar material (PCM). It encompasses components like microtubule associated proteins (MAPs), destabilizing factors, and severing proteins, that help additional MTs to form (De Forges *et al.*, 2012; Akhmanova and Hoogenraad, 2015). Spindle apparatus organizes and separates chromosomes into two daughter cells during cell division. The minus-end dynamics is important for controlling the structure of mitotic spindle during this process (Bornens, 2012; De Forges *et al.*, 2012). Even though centrosomes are regarded as major MTOCs, there are cells, where not all MT minus-ends are attached to the centrosome. This is an indicator, that these minus-ends are instead associated with other structures or just lie free in the cytoplasm (Akhmanova and Hoogenraad, 2015).

Cell differentiation is coupled with MT reorganization from centrosomal- to non-centrosomal-MT (cMT, ncMT) arrays (Bartolini and Gundersen, 2006). For this kind of reorganization to occur, centrosomes must lose or reduce their cMTOC activity. New non-centrosomal sites (nc-sites) have to be specified and activated to acquire ncMTOC function (Sanchez and Feldman, 2017).

Different models have been postulated to explain possible differentiation-induced centrosome inactivation. Transcriptional downregulation of centrosomal proteins coupled with decreased pro-proliferative signals may lead to MT reorganization. A possible mechanism may be loss of specific MT anchoring proteins, like Ninein, or centrosomal inactivation which can be mediated through increased MT severing by MT severing proteins(Muroyama and Lechler, 2017). It is also plausible that centrosomal inactivation is created by general centrosome disassembly and dispersal of PCM (Muroyama and Lechler, 2017).

Another necessity for ncMTOCs to form is the activation of ncMTOC sites. Activation may occur through relocation of centrosomal proteins to new nc-sites. Another possibility is that distinct set of non-centrosomal proteins may generate ncMTOCs themselves. Lastly, nc-arrays may be generated by re-purposing previously generated MTs by MT severing proteins (Muroyama and Lechler, 2017).

ncMTOCs regulate MT dynamics in differentiating cells and are essential for cell polarity, organization and functioning (Bartolini and Gundersen, 2006).

During cell division MT polarization emanates from the centrosome, which acts as cMTOC. The minus-ends are anchored to the centrosome, which is usually in the middle of the cell and the plus-ends extend outward toward periphery. This gives them their distinguishable radial MT system (Fig. 5) (Bornens, 2012; Akhmanova and Hoogenraad, 2015; Sanchez and Feldman, 2017). Many differentiated cells lack centrosomes but still possess highly polarized microtubule networks. These ncMTOCs are found in differentiating cells, where depending on the cell type MT minus-ends are anchored in different locations in the cell (Nashchekin *et al.*, 2016). ncMTOCs are predominant in cell types such as neurons and epithelial cells, where MTs can be arranged into parallel or anti-parallel arrays. This strongly contributes to the cell polarity crucial in differentiating cells (Fig. 5) (Bornens, 2012; Muroyama and Lechler, 2017; Sanchez and Feldman, 2017).

In neurons, nc-arrays play a very prominent role (Akhmanova and Hoogenraad, 2015). Most differentiated neurons have one long axonal process and several branched dendrites, strongly dependent on MTs. Mammalian, *Drosophila melanogaster* and *C. elegan*s neurons organize axonal MTs in a way that their plus-ends are facing away from the cell body (Fig. 5). Dendrites have a mixed MT polarity in mammalian neurons and predominantly minus-end-out MT orientation in invertebrate neurons (Fig. 5) (Baas and Lin, 2011). In mammalian neurons, the centrosome acts as an active MTOC in the start of differentiation. Over time, the centrosome-dependent MT organization is lost. It is strongly believed, that differentiated neurons have many ncMTs and that the minus-ends are distributed throughout the whole cell (Baas and Lin, 2011).

Many simple epithelial cells form A-B MTs. Their minus-ends are anchored to the apical surface of the cell and plus-ends are pointed towards the basal side (Fig. 5) (Bellett *et al.*, 2009; Khanal *et al.*, 2017; Sanchez and Feldman, 2017). When cells differentiate and polarize, the proportion of ncMTs increase, and MTs can become tied to the apical cortex or to cell-cell junctions (Akhmanova and Hoogenraad, 2015). Centrosome inactivation leads to their relocation to the apical surface which is linked to γ-tubulin (Sanchez and Feldman, 2017). For example, during fruit fly trachea morphogenesis γ-tubulin is released and repositioned to apical surface where it is stabilized by transmembrane protein PioPio (Brodu *et al.*, 2010). Calmodulin-regulated spectrin-associated protein (CAMSAP)/Patronin is another protein responsible in MT minus-end dynamics at ncMTOC sites (Akhmanova and Hoogenraad, 2015; Sanchez and Feldman, 2017). Interestingly, it has been shown that radial MT arrays and MT minus-ends attached to membrane organelles, and not to the centrosome, may be present in the cell at the same time (Bornens, 2012).

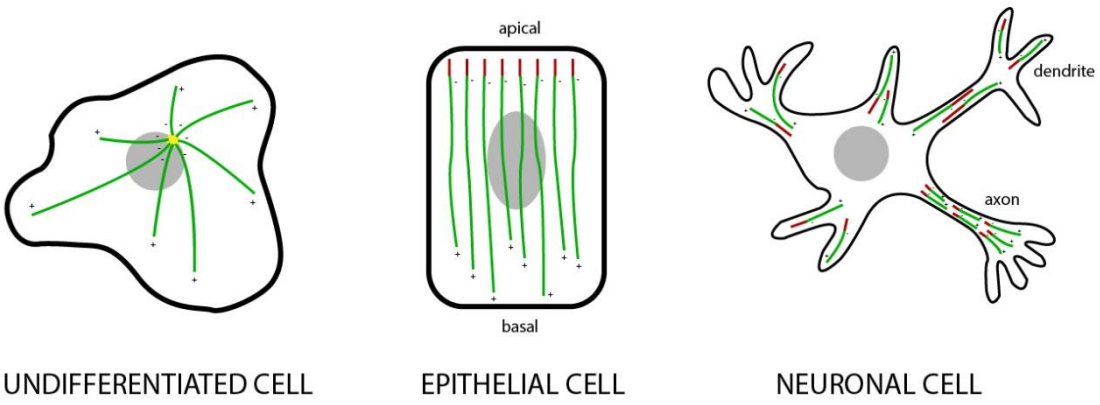

Figure 5. Location of MTOCs and organization of MTs in different cell types. MTs (green) are organized by MTOCs. In undifferentiated cells, MT minus-ends are anchored to centrosomes (yellow). In epithelial cells, MT minus-ends are anchored to ncMTOCs (red) at the apical side of the cell, plus-ends growing toward the basal side. In neurons, axonal MTs are usually arranged in the manner that MT plus-ends are facing away from the cell body. Dendrites have mixed MT polarity, with mostly minus-end-out MT orientation (according to Sanchez and Feldman, 2017).

4. 5. 3. Microtubule associated proteins

Even though the formation and accurate functioning of MTs is linked to TBCs and intrinsic processes such as the presence of GTP-cap, additional factors play a role in the correct MT dynamics. MTs are also regulated extrinsically through post-translational modifications. They influence polymer dynamics by tuning the activity and affinity of MTs. Numerous modifications are often correlated with MT stability (Muroyama and Lechler, 2017; Sanchez and Feldman, 2017). Additionally, many MAPs have been shown to play a role in correct MT functioning (De Forges *et al.*, 2012).

MAPs are responsible for the nucleation, stabilization, anchoring, depolymerization, and clustering of MTs (De Forges *et al.*, 2012; Akhmanova and Hoogenraad, 2015; Muroyama and Lechler, 2017). They influence MT behavior by binding to MTs and regulating their dynamics. These proteins have been categorized as MT-plus-end tracking proteins (+TIPs), MT-minus-end targeting proteins (-TIPs), MT motor proteins, and MT severing proteins. Numerous studies have identified many +TIPs that play an important role in regulating the MT plus-ends. In contrast, the players controlling the behavior and organization of MT minus-ends remain largely still to be uncovered (Akhmanova and Hoogenraad, 2015; Sanchez and Feldman, 2017).

4. 5. 3. 1. MT-plus-end tracking proteins

The polymerization and depolymerization takes mainly place at MT plus-ends. An important family of MAPs are the +TIPs. They play a role in tracking plus-ends of MTs, regulating their dynamics, and participating in the interactions of MTs during proliferation (De Forges *et al.*, 2012).

Cytoplasmic linker protein (CLIP) 170/190 was first identified as a +TIP that plays an important role in rescue events (Perez *et al.*, 1999; Komarova *et al.*, 2002). +TIPs form an interacting network at the core of which are the proteins of End-binding (EB) family. They bind to the growing ends of MTs by sensing the nucleotide-bound state of β-tubulin. EBs bind dynamically to the tips of plus-ends and promote continual MT growth through suppressing catastrophe events (Komarova *et al.*, 2009). EB-proteins are able to bind other +TIPs to the MT plus-ends. CLIP-associated proteins (CLASPs)/Multiple asters (Mast)/Orbit and XMAP215/Mini spindles (Msps) are partners of EBs that recruit tubulin dimers to the MT plus-ends thereby promoting rescue events (De Forges *et al.*, 2012). It is important to note that only a few +TIPs are known to bind to MT minus-ends (Muroyama and Lechler, 2017).

4. 5. 3. 2. MT-minus-end tracking proteins (-TIPs)

While there are many proteins shown to interact with MT plus-ends, only few proteins associated with MT minus-ends have been identified. -TIPs regulate MT minus-end dynamics. For the appropriate regulation, the nucleation, stabilization, and anchoring of MT minus-ends must occur (Akhmanova and Hoogenraad, 2015; Sanchez and Feldman, 2017).

γ-tubulin is a primary MT nucleator found in MTOCs (Dutcher, 2001; Kollman *et al.*, 2011; Nashchekin *et al.*, 2016; Sanchez and Feldman, 2017). It is incorporated into a larger complex called γ-tubulin ring complex (γ-TuRC), which is known to associate with MT minus-ends (Gunawardane *et al.*, 2003). It nucleates MTs and can cap their minus-ends (Muroyama and Lechler, 2017). γ-TuRC is responsible for initiating nucleation by forming rings that act as templates for new MT growth. This model of γ-TuRC-mediated MT nucleation was termed as "template model" (Kollman *et al.*, 2011). After nucleation, MT plus-ends start to grow into the cytoplasm while γ-TuRC caps MT minus-ends to prevent their depolymerization (Zhang *et al.*, 2007; De Forges *et al.*, 2012; Petry and Vale, 2015). However, it has been shown that not all MT minus-ends are capped with γ-TuRCs (Akhmanova and Hoogenraad, 2015). This has led to raising questions about the function of other -TIPs that influence the behavior of MT minus-ends.

The CAMSAP/Patronin/Nezha family members were the first proteins shown to interact with MT minus-ends directly and are thereby classified as -TIPs. The family has a signature amino-terminal calponin homology (CH) domain and carboxy-terminal CKK domain, involved in MT minus-end binding (Goodwin and Vale, 2010; Jiang *et al.*, 2014; Yau *et al.*, 2014; Akhmanova and Hoogenraad, 2015). The identification of this protein family has given us new insight into the mechanisms governing the MT minus-end dynamics. It has helped us to uncover the importance of MT minus-ends in the control of mitotic spindle length, epithelial cell migration, proper organelle distribution, and in neuronal development and regeneration (De Forges *et al.*, 2012; Akhmanova and Hoogenraad, 2015; Adikes *et al.*, 2018).

Vertebrates possess three CAMSAPs 1-3. A whole-genome screen in *Drosophila melanogaster* has identified one member of this family, named Patronin (Rubin and Lewis, 2000; Goodwin and Vale, 2010; Tanaka *et al.*, 2012; Cao *et al.*, 2020).

CAMSAP3 was the first family member shown to recognize and bind to MT minus-ends. The main function of CAMSAP2/3 is to stabilize MT lattice for the MT outgrowth. They cooperate to organize epithelia-specific organization of ncMTs. In addition, the proteins have a strong effect on MT dynamics by inhibiting MT minus-end polymerization and thereby the catastrophe event (Khanal *et al.*, 2017).

CAMSAPs require MT minus-end polymerization to form stable MTs. Experiments with laser-induced severing have shown that CAMSAPs are promptly recruited to free MT minus-ends (Jiang *et al.*, 2014). Importantly, studies revealed that the main function of CAMSAPs is to stabilize the minus-ends that are not embedded in the centrosome (Jiang *et al.*, 2014). This function was shown, where in cultured mammalian epithelial cells, the depletion of CAMSAP2/3 reduced the number of ncMTs significantly, which led to the conclusion that a significant proportion of free MT minus-ends are dependent on CAMSAPs (Tanaka *et al.*, 2012). The depletion also inhibited cell migration, which shows that ncMTs are required for efficient cell migration (Jiang *et al.*, 2014). CAMSAP family members are important determinants of MT density and stability in neurons (Akhmanova and Hoogenraad, 2015). Loss of CAMSAP2 has a strong effect on axon formation and in the extension and branching of dendrites. It is important during axon specification, neuronal polymerization, and neuronal remodeling (Yau *et al.*, 2014). Experiments with epithelial cells and neurons have demonstrated, that γ-TuRC and CAMSAPs have independent roles in MT formation and maintenance although they may also act sequentially to nucleate and stabilize MTs (Tanaka *et al.*, 2012).

It has been demonstrated, that Patronin can associate with free MT minus-ends. It acts as a cap by binding to minus-ends protecting them from depolymerization and stabilizes them in vivo. Patronin inhibits the MT disassembly by binding to MT minus-ends through its C-terminal CKK domain and protects them from kinesin-13 family depolymerase Kinesin-like protein at 10A (Klp10A) mediated degradation (Goodwin and Vale, 2010; Akhmanova and Hoogenraad, 2015). Klp10A has been shown to induce MT minus-end depolymerization to regulate MT length and dynamics (Rogers *et al.*, 2004).

The antagonism between the CAMSAP/Patronin and the MT depolymerase manifests itself in interphase and mitosis. It has been uncovered, that depletion of Patronin leads to shorter bipolar spindles. Whether CAMSAPs have similar functions in mammalian cells remain to be uncovered, but so far CAMSAP1 may be a likely candidate as a mitotic regulator (Akhmanova and Hoogenraad, 2015). Another antagonist to CAMSAPs/Patronin is the MT-severing enzyme Katanin. It may contribute to disassembly of CAMSAP-decorated MT stretches or limit their growth by cutting or depolymerizing them from the minus-ends (Jiang *et al.*, 2014).

Thereby, CAMSAP/Patronin are crucial regulators that determine the behavior of free ncMT minus-ends by possessing their microtubule-stabilizing.

Spectraplakin Shotstop (Shot), Microtubule actin cross-linking factor 1 (MACF1) and Dystonin (DST) in mammals, is an actin binding protein, known to crosslink MTs to the actin cytoskeleton. It can bind F-actin through its N-terminal actin-binding domain and MTs through its C-terminal domain (Nashchekin *et al.*, 2016). Shot has been shown to be responsible for the recruitment of Patronin to form ncMTOCs. Together they act as a source of growing MTs from stabilized minus-ends. Shot/Patronin ncMTOCs are shown to be independent of γ-tubulin. This kind of function of these two proteins has been demonstrated for example in fruit fly oocytes and microvilli (Nashchekin *et al.*, 2016; Khanal *et al.*, 2017).

In *Drosophila melanogaster* microvilli, Patronin together with Shot act in parallel at the apical domain of epithelial cells to polarize MTs. Polarization of these two proteins is dependent on the apical spectrin cytoskeleton, which in turn is dependent of determinants of cell polarity, like Par-complex (Nashchekin *et al.*, 2016; Khanal *et al.*, 2017).

Patronin and Shot localize apically in fruit fly follicle cells and have a potential role in polarizing the MT cytoskeleton along the apical-basal axis (Khanal *et al.*, 2017). Both Patronin and Shot mutants exhibit disordered MT cytoskeleton phenotype, where Shot was shown to affect MT polarization (Khanal *et al.*, 2017). The results with fruit fly follicle cells made it

possible to speculate that Patronin and Shot work in parallel to polarize MTs(Khanal *et al.*, 2017). But by which means do Patronin and Shot become polarized at the apical domain?

Cell polarity is crucial for epithelial organ function and morphogenesis, in cell shape change and proper MT polarization (Gilmour *et al.*, 2017). In epithelial cells, key determinants of A-B cell polarity are responsible for polarizing all other proteins in the cell. Par-complex proteins have a role in the A-B regulation (Suzuki and Ohno, 2006). Two key determinants, Cdc42 and Lgl, are shown to be important in organizing the polarization of apical spectrins. In polarized epithelial cells, $\alpha_2\beta_{H2}$-spectrin is localized in the apical domain, whereas $\alpha_2\beta_2$-spectrin localizes at the basolateral domain. Apical spectrins are shown to interact with Patronin and Shot to recruit them to the apical membrane (Khanal *et al.*, 2017). *cdc42* and *lgl* mutants exhibit mis-location of apical spectrins, thereby also Patronin, and Shot, causing a dramatic disruption of the epithelial tissue (Fletcher *et al.*, 2015). *α-spectrin* mutants are shown to cause Shot mis-localization at the apical side. Thereby A-B polarity determinants, like Par-complex proteins, act upstream of Spectrin polarization to control the location of Shot and Patronin (Fletcher *et al.*, 2015; Khanal *et al.*, 2017).

Tau is a MT-binding protein found in different *Drosophila melanogaster* cell types and tissues as well as in human Hela cells. It is known to be phosphorylated during mitosis to generate MTs with lower affinity (Gustke *et al.* 1994; Bouge and Parmentier, 2016). Accumulation of Tau proteins are considered to be linked with Alzheimer's disease (Bouge and Parmentier, 2016). Primary biochemical function of Tau is to maintain the stability of microtubules. It has been demonstrated that overexpression of hTau leads to impaired mitosis *in vivo* inducing a mitotic arrest, with the presence of monopolar spindles, and results in apoptotic cell death (Bouge and Parmentier, 2016).

In addition to γ-TuRC and CAMSAP/Patronin family, another proposed player in the organization and stabilization of MT minus-ends is Ninein (Bellett *et al.*, 2009). It is thought to act as an anchor by interacting with γ-TuRC, but a direct binding to the ncMT minus-ends has not yet been demonstrated (Akhmanova and Hoogenraad, 2015). Ninein was first identified to be an anchor for cMTOC minus-ends. During epithelial cell polarization, it can relocate from the centrosome to apical sites. This makes it plausible to speculate that Ninein may have a similar role at ncMTOC sites (Bellett *et al.*, 2009; Sanchez and Feldman, 2017). A reasons why the role of Ninein as a MT minus-end anchor at ncMTOC sites is under speculation is the fact that it is hypothesized that nucleators and stabilizers themselves may act as minus-end anchors (Akhmanova and Hoogenraad, 2015; Sanchez and Feldman, 2017).

MT severing proteins Katanin and Spastin destabilize MTs (Zhang *et al.*, 2007; Brodu *et al.*, 2010; Sharp and Ross, 2012). They regulate MT number and length through fragmenting pre-existing MTs along the length of the filament. Through the severing of existing MTs they initiate new MT growth thanks to the new ends lacking GTP-cap (Zhang *et al.*, 2007).

Site-specific adapters are necessary for MT minus-end proteins to anchor to specific subcellular sites. They differ depending on the cell type, but it has not been directly shown that adapter proteins and minus-end proteins are linked. So far transmembrane protein PioPio in *Drosophila melanogaster* tracheal cells has been shown to be necessary for γ-TuRC localization to the apical membrane (Nashchekin *et al.*, 2016).

It is more than likely that additional proteins regulating MT dynamics exist (Sanchez and Feldman, 2017). Identification of these proteins and mechanisms by which they interact with each other will give us much needed insight into MTOCs to better understand their function in different cellular aspects. Understanding how cells move from cMTOCs to ncMTOCs and back during morphogenesis according to the necessity (cell division for tissue growth and differentiation for correct cell function) will give us greater understanding of how morphogenesis takes place during development.

5. EXPERIMENTAL PART

AIMS OF THE THESIS

The overall goal of the project was to uncover cellular mechanisms underlying 3D structured morphogenesis. As is implied in the literature, membrane protrusions are involved in intercellular communication needed for proliferation, cell fate decision, and migration (Sherer and Mothes, 2008; Buszczak *et al.*, 2016). This led to the hypothesis, that cell protrusions may be involved in wing development. New kind of tubulin-based MT-protrusions, termed interplanar bridges, have been demonstrated to play a role during pupal wing development. The aims of the thesis are as follows:

1. To identify genes that regulate MT minus-end dynamics at ncMTOC sites.
2. To investigate dynamics of interplanar bridges underlying pupal wing morphogenesis.

To accomplish these goals, *in vivo* RNAi approach has been employed to knock down the genes during pupal stage and identify the candidates to govern MT minus-end dynamics. Additional time lapse imaging experiments were conducted to explore the molecular mechanism of interplanar bridges and their involvement in cell communication for mitosis and re-apposition.

MATERIALS AND METHODS

Fly genetics

The karyotype of *Drosophila melanogaster* is comprised by four chromosomes. They include the sex chromosomes X and Y, two large chromosomes 2 and 3, and a small chromosome 4. Females have two X chromosomes and males a single X and the Y chromosome (Celniker and Rubin, 2003; Hartmann and Sekelsky, 2017; Kaufman, 2017).

In addition to the small genome and having just four chromosomes, fruit flies possess balancer chromosomes, providing a valuable tool for genomic engineering (Morgan, 1910; Muller, 1918, 1927; Venken *et al.*, 2016). Balancers contain three crucial features. Firstly, they have inversions that eliminate the progression of meiotic recombination events. Secondly, they possess recessive lethal or sterile mutations that affect reproductive viability or fitness of homozygous flies. Thirdly, they carry an observable dominant marker that can be followed from one generation to another in heterozygotes (Morgan, 1910; Muller, 1918, 1927; Kornberg and Casso, 1999; Venken *et al.*, 2016). Balancers are available for all chromosomes except the small fourth and Y chromosome (Morgan, 1910; Roote and Prokop, 2013; Miller *et al.*, 2016, 2018). Information in more detail about *Drosophila melanogaster* genes, balancers, and more is available in the website http://flybase.org (Tweedie *et al.*, 2009).

Candidate genes were selected based on the previous knowledge about their role in regulating MT minus-end dynamics at ncMTOC sites (Bellett *et al.*, 2009; Tanaka *et al.*, 2012; Jiang *et al.*, 2014; Yau *et al.*, 2014; Nashchekin *et al.*, 2016). Fly stocks used in the thesis were obtained from two stock centers. *y;kat60* (B35634), *y;;kat60* (B28375), *y;;kat-60L1* (B32506), *y;kat-60L1* (B36866), *y;kat80* (B66000), *y;;klp10A* (B33963), *y;;γtub23C* (B42799), *y;;γtub23C* (B31204), *y;;spas* (B27570), *y;spas* (B53331), *y;;grip84* (B33548), *y;ninein/Cyo (y;bsg25D/Cyo)** (B62414), *y;;αSpec* (B42801), *y;αSpec* (B56932), *y;;αSpec* (B31209), *y;;par-1* (B32410), *y;;shot* (B28366), *y;shot* (B64041), and *y;;patronin* (B36659) were purchased from the Bloomington Drosophila Stock Center (BDSC). *y;;par-1* (V52553) was obtained from the Vienna Drosophila RNAi Center (VDRC). These RNAi stocks investigated in the experimental part express dsRNA for RNAi of a specific gene under the control of UAS (Perkins *et al.*, 2015). *yw* was used as a control. Fly stocks were maintained at 25°C unless otherwise mentioned. The information about the RNAi stocks used is summarized in Table 1.

Table 1. Fly stocks used for RNAi screening.

Name of the gene	Stock center	Stock number	Genotype	Inserted chromosome
katanin 60 (kat60)	BDSC	35634	*y;kat60*	2
katanin 60 (kat60)	BDSC	28375	*y;;kat60*	3
katanin p60-like 1 (kat60L1)	BDSC	32506	*y;;kat-60L1*	3
katanin p60-like 1 (kat60L1)	BDSC	36866	*y;kat-60L1*	2
katanin 80 (kat80)	BDSC	66000	*y;kat80*	2
kinesin-like protein at 10A (klp10A)	BDSC	33963	*y;;klp10A*	3
γTubulin at 23C (γTub23C)	BDSC	42799	*y;;γTub23C*	3
γTubulin at 23C (γTub23C)	BDSC	31204	*y;;γTub23C*	3
spastin (spas)	BDSC	27570	*y;;spas*	3
spastin (spas)	BDSC	53331	*y;spas*	2
gamma-tubulin ring protein 84 (grip84)	BDSC	33548	*y;;grip84*	3
*Ninein (nin)**	BDSC	62414	*y;ninein/Cyo (y;bsg25D/Cyo)*	2
αSpectrin (αSpec)	BDSC	42801	*y;;αSpec*	3
αSpectrin (αSpec)	BDSC	31209	*y;;αSpec*	3
αSpectrin (αSpec)	BDSC	56932	*y;αSpec*	2
par-1	BDSC	32410	*y;;par-1*	3
par-1	VDRC	52553	*y;;par-1*	3
short stop (shot)	BDSC	28336	*y;;shot*	3

short stop (shot)	BDSC	64041	*y;shot*	2
patronin	BDSC	36659	*y;;patronin*	3

12 genes of interest were investigated in the thesis. 20 stocks were obtained from two stock centers, BDSC and VDRC, with their respective stock numbers. The genotypes are written in a manner that homologous chromosomes are separated by slash (/) and non-homologous chromosomes are separated by semicolon (;). * *bsg* encodes the ortholog of *ninein*. Cyo is a second chromosome balancer.

RNAi screening

A widely used method to generate transgenic flies is the UAS/Gal4 system (Brand and Perrimon, 1993; Celniker and Rubin, 2003; Mondal *et al.*, 2007). Regulatory protein Gal4 is a transcription factor from budding yeast (*S. cerevisiae*) that activates genes downstream of upstream activating sequence (UAS) enhancer elements. By crossing Gal4-expressing flies to UAS construct lines, the genes downstream of UAS enhancers are activated (Fig. 6 A). Once crossed, the progeny will display expression of these UAS-coupled genes in the chosen Gal4 pattern (Brand and Perrimon, 1993; Rodríguez *et al.*, 2012). The spatial-temporal pattern of Gal4 activation can be further refined through the use of temperature-sensitive Gal80 ($Gal80^{ts}$) (Brand and Perrimon, 1993; Mondal *et al.*, 2007; Rodríguez *et al.*, 2012; Diaz-Garcia *et al.*, 2016). It acts as a Gal4 repressor by binding and masking the Gal4 transcription function depending on the temperature (Fig. 6 B). For example, at 18ºC $Gal80^{ts}$ is binds to Gal4 which leads to the inactivation of Gal4´s function and the gene of interest is not expressed. At 29ºC $Gal80^{ts}$ will disassociate from Gal4 and gene of interest will be expressed (Brand and Perrimon, 1993; Mondal *et al.*, 2007; Rodríguez *et al.*, 2012; Roote and Prokop, 2013). Thereby, using the UAS/Gal4/$Gal80^{ts}$ system provides a tool to express RNAi genes downstream of UAS in a spatial-temporal matter to study the functions of genes of interest in a specific tissue, in a precise time.

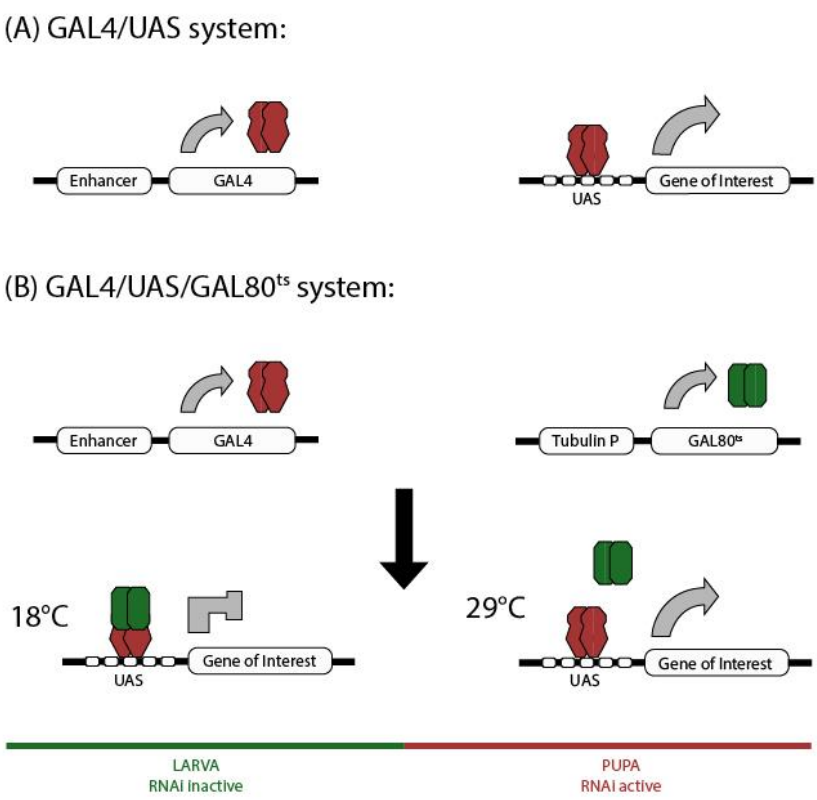

Figure 6. Gal4/UAS/Gal80^{ts} system. (A) Gal4/UAS system. (B) Gal4/UAS/Gal80^{ts} system. At 18ºC, Gal80^{ts} acts as an Gal4 repressor. This will lead to RNAi not being expressed. At 29ºC, Gal80^{ts} will dissociate from Gal4. It is then able to possess its active function and RNAi will be expressed. During the RNAi screening, vials were shifted to 29 ºC when third instar larva was apparent. Gal4 (red), Gal80^{ts} (green) (according to Classen *et al.*, 2008).

To identify genes important in the pupal wing development through their role in regulating MT minus-end dynamics, the RNAi-mediated gene knockdowns were implemented in a stage specific manner by using the UAS/Gal4/Gal80^{ts} system. Wing-specific driver *nubbin (nub)* was used to express transgenes in apical and basal tissue layers of the wing blade (Averof and Cohen, 1997).

w;nub>Gal4;Gal80^{ts} female flies were crossed with males containing gene of interest (Table 1). Wing phenotypes of F1 progeny were examined. Flies were crossed and grown at 25°C. Vials were shifted to 29°C approximately 3 days after egg-laying when third instar larval stage was apparent. Prepupae were collected after 16 hours and kept at 29°C till eclosing. Developmental timing was calculated based on previously published data (Buttitta *et al.*, 2007). Adult flies with proper genotype were put into 70% ethanol and kept in it for roughly 4 days. They were washed with PBS and kept in it for about 2 hours until dissection. Female and male flies were divided and one wing from each fly was dissected in PBS. 10 wings from both male

and female flies were placed onto the microscope slide. Wings were mounted using 70% glycerol, after which cover glass was placed onto the slide and fixed with transparent nail polish.

Adult wing images were taken with a Nikon ECLIPSE 90i microscope and processed by using CorelDRAW 2020 (22.1.0.517) software. Wings were measured with NIS-Elements 4.30 imaging software and statistical analysis was done using Prism 9.0.2.

Time lapse imaging

w,ubi>αTubulin:GFP;nub>Gal4,ubi>cnn:Cherry;Gal80^{ts} flies were used for further live imaging. GFP tagged α-tubulin was expressed under the *ubiquitin (ubi)* promotor to track the distribution of MTs (Hershko *et al.*, 1983; Desai and Mitchison, 1997). *centrosomin (cnn)*, a component of centrosome, was utilized to identify cells undergoing cell division (Heuer *et al.*, 1995).

Prepupae of indicated genotype were raised and collected at 25°C. Vials containing 3rd instar larvae were then transferred to 29°C incubators for 14 hours. Prepupae were collected, briefly washed with water, dried with a lint-free wipes (Kimwipe), and positioned on a double-sided tape, right wing facing up. Using a microknife (Fine Science Tools), orifice was dissected into the pupal case in the region of the wing (Classen *et al.*, 2008). A drop of halocarbon oil (Sigma Aldrich) was put onto the exposed pupal wing to prevent tissue from drying during imaging. The pupae on a double-sided tape were placed onto a 24 x 50 mm coverslip. Around 3-4 pupae were collected to the coverslip and kept in a Petri dish with moist Kimwipe until imaging. Time-lapse imaging was done using Leica SP8 STED confocal microscope. Optical antero-posterior cross sections of each wing were taken in 5-minute intervals using the xzyt-function. Time lapse images were taken from 14 – 18 hours AP.

Images were processed and analyzed using ImageJ 1.52p and Imaris v.9.1.2. software. In addition, ImageJ was used to process time lapse images into AVI-format videos.

RESULTS

Adult wing RNAi screening

Using the protocol for RNAi screening, genes shown to be involved with MT minus-ends dynamics were investigated to study their role in wing morphogenesis. F1 progeny female and male adult wings were analyzed separately. The anatomical and morphological features of mutant wings were examined in comparison to control wings. During RNAi screening, knockdowns of different genes resulted in a milder (Fig. 7, 9, 10) or more severe (Fig. 8-10) phenotypes. It is important to note, that different stocks containing the same gene RNAi had different phenotypes (Fig. 7 N-P; Fig. 8 E-F; Fig. 9, 10).

Knock-down of *katanin 60 (kat60), gamma-tubulin ring protein 84 (grip84),* and *ninein (nin)* did not exhibit noticeable wing phenotype compared to the control (Fig. 7 A-C, K-L; Fig. 9, 10).

katanin 80 (kat80), katanin p60-like 1 (kat60L1), spastin (spas), γTub23C (B42799, *kinesin-like protein at 10A (klp10A), αSpectrin (αSpec)* (B42801, B31209), and *par-1* (V52553) mutant flies had rather mild phenotype, which included additional detached vein formation alongside LV2 (Fig. 7 D-I, M-P; Fig. 9, 10). In addition, *klp10A* and *αSpec* mutants exhibited abnormal vein formation at the terminal tips at LV4 and LV5 compared to the control (Fig. 7 A, M-O). Knock-down of *γTubulin at 23C (γTub23C)* (B31204) resulted in a mild phenotype, where abnormal PCV formation was visible (Fig. 7 J; Fig. 9, 10).

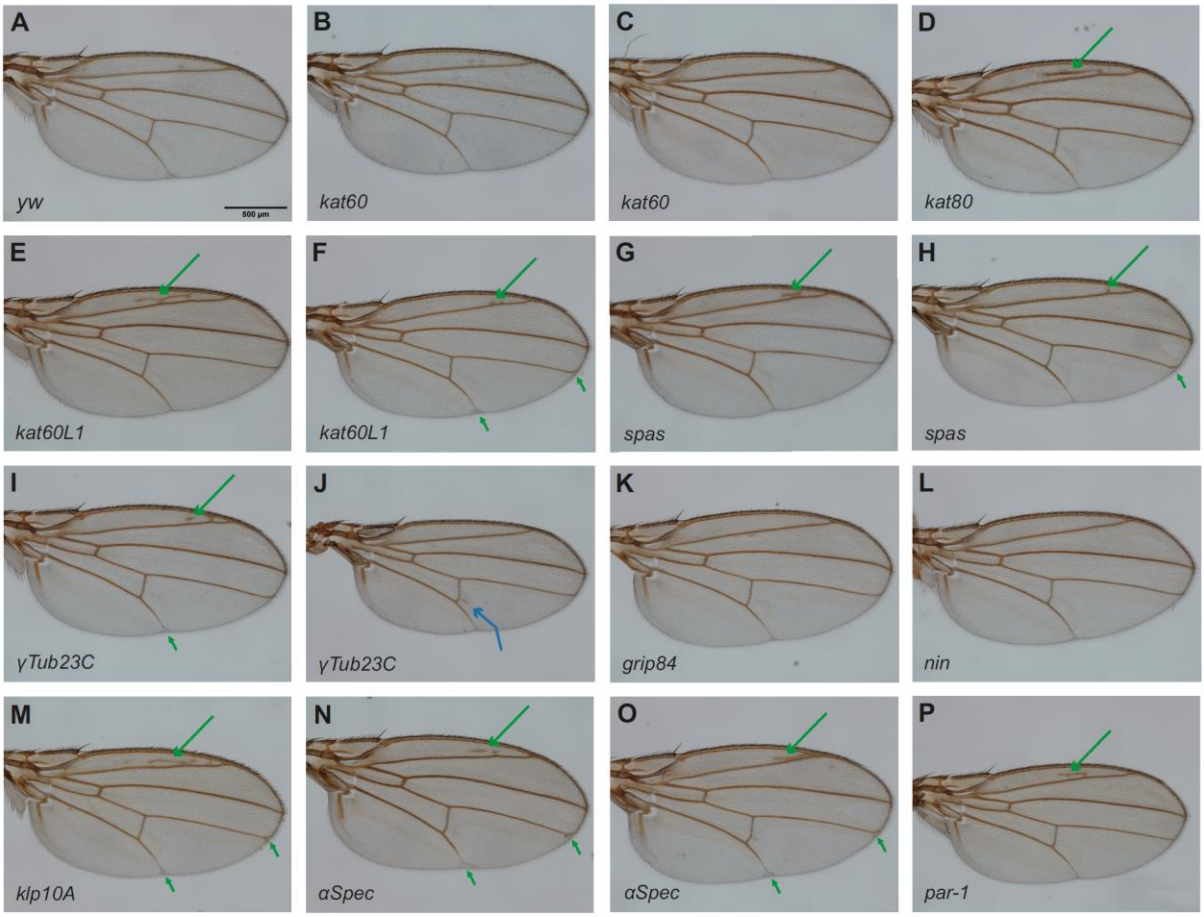

Figure 7. Adult wings with milder phenotypes. (A) *yw* was used as control. (B-P) RNAi mutants wings of *kat 60* (B28375, B35634), *kat 80* (B66000), *kat60L1* (B36866, B32506), *spas* (B53331, B27570), *γTub23C* (B42799, B31204), *grip84* (B33548), *nin* (B62414), *klp10A* (B33963), *αSpec* (B31209, B42801), and *par-1* (V52553). Green arrow shows additional detached vein formation alongside LV2. Blue arrow implies to PCV formation abnormality. (A-P) Images of female wings (male and female wing phenotypes were similar). Axes are as follows: proximal left, distal right, anterior up, posterior down. N=10

shot, *patronin*, *par-1* (B32410), and *αSpec* (B56932) mutants showed a severe adult wing phenotype (Fig. 8-10). Smaller wing sizes and slight to severe wing shape changes were apparent in all three gene knockdowns (Fig. 8 B-F, Fig. 9-10). *shot, patronin* and *αSpec* mutants showed noticeable blistering (Fig. 8 B-D, F). Inaccurate *par-1* expression led to evident wing shape change and vein patterning abnormalities, which included additional crossvein formation between L2/L3 and LV4/LV5 (Fig. 8 E). hTau is MT-binding protein. The knock-down of *hTau* leads to adult wing phenotype, which is characterized by smaller wing size, wing shape change, and patterning abnormality alongside LV2 (unpublished data) (Fig. 8 G).

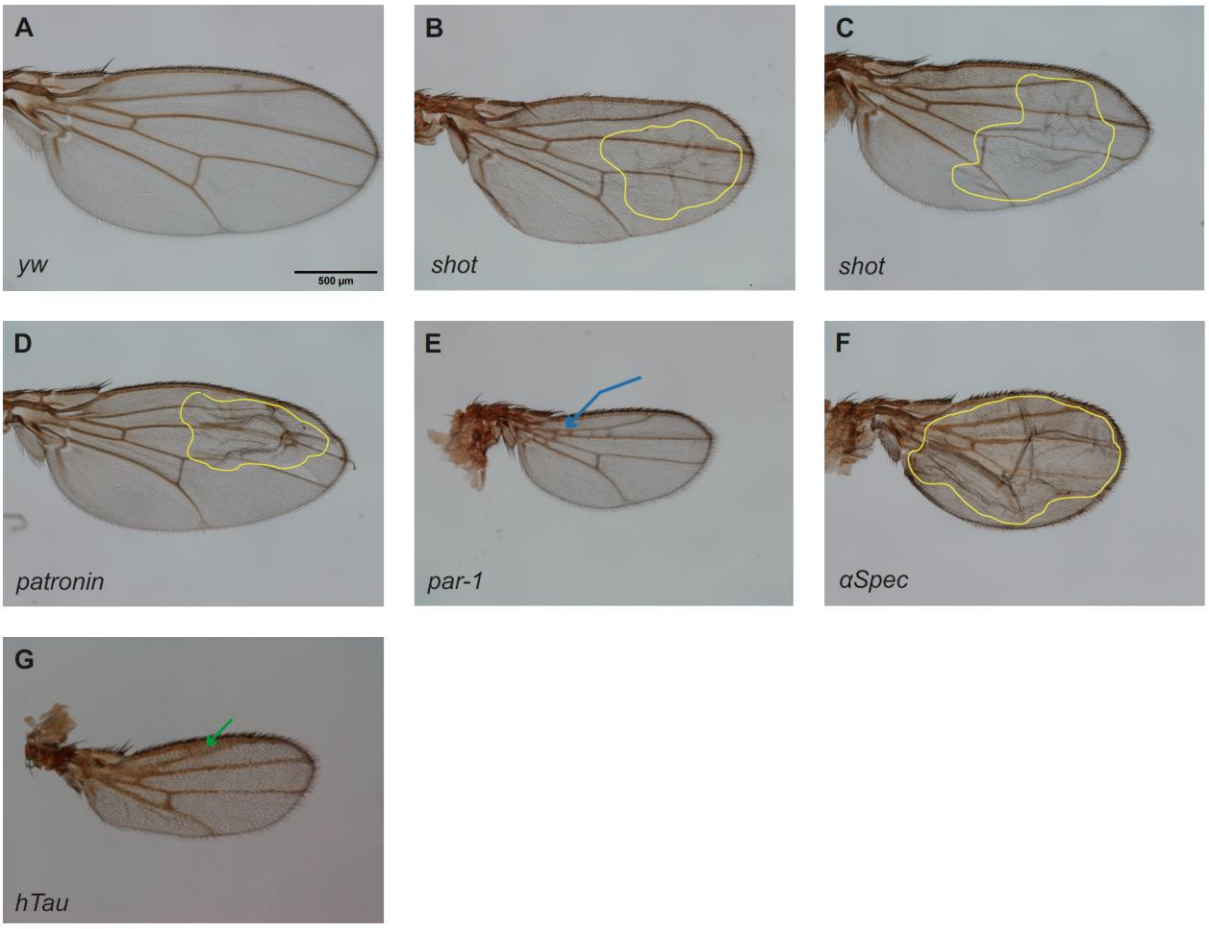

Figure 8. Adult wings with evident phenotypes. (A) *yw* was used as a wild type. (B-F) RNAi mutants wings of *shot* (B64041, B28336), *patronin* (B36659), *par-1* (B32410), *αSpec* (B56932). (G) Overexpression mutant wing of *hTau* (unpublished data). Yellow dotted line shows region of blistering. Blue arrow indicates additional crossvein formation. Green arrow signifies vein patterning anomaly alongside LV2. (A-F) Images show female wings (male wings correlated to female wing phenotypes). Axes are as follows: proximal left, distal right, anterior up, posterior down. Scalebar 500µm. N=10

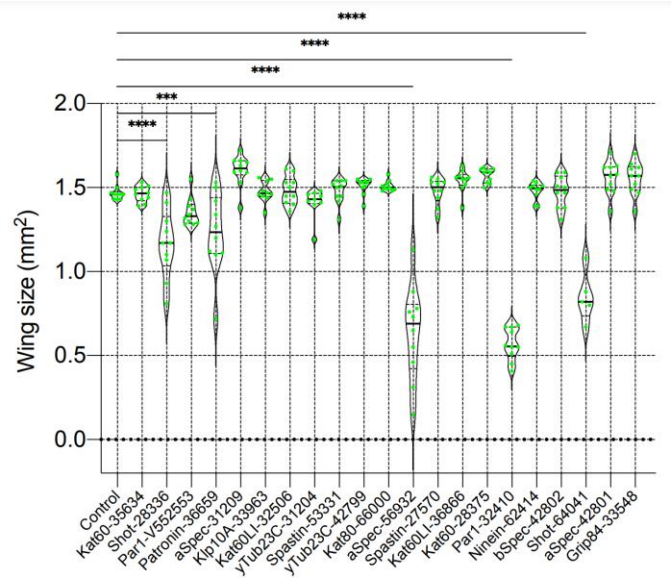

Figure 9. The female adult wing sizes for RNAi screened genes compared to the control (*yw*). *** p<.0001, **** p<.00001. N=10

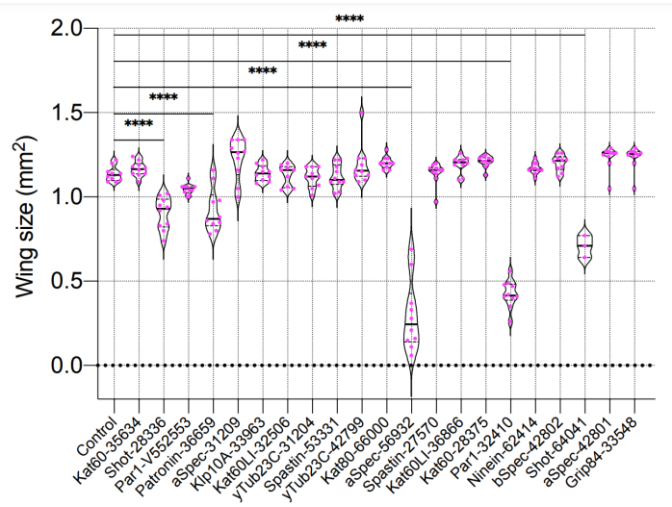

Figure 10. The male adult wing sizes for RNAi screened genes compared to the control (*yw*). **** p<.00001. N=10.

From this data it was concluded that Shot, Patronini, Par-1, and αSpectrin are important in pupal wing morphogenesis in the context of having a role in MT minus-end dynamics at ncMTOC sites. To understand the role of MT minus-end dynamics in communication and coordinated mitosis at tissue level, *w,ubi>αTubulin:GFP;nub>Gal4,ubi>cnn:Cherry;Gal80^{ts}* pupal wings were examined.

Time lapse imaging

MT-based protrusions can be marked with GFP-α-tubulin and membrane-bound-GFP (Buszczak *et al.*, 2016). In addition, *cnn* is used to visualize cells undergoing mitosis (Heuer *et*

al., 1995). Exposed part of the pupal wing was investigated in real time under the confocal microscope (Fig. 11 A). At 14 hours AP, individual cells have long MT-based protrusions, hereby termed interplanar bridges (IpBs). They originate from the apical side of both dorsal and ventral epithelial layer and are extended to the basal domain (Fig. 11 B, E, H). At 16 hours AP, IpBs start to assemble (Fig. 11 C, F, I). From then on, gradual decrease in the number of protrusions is apparent. At 18 hours AP, many of the cells have lost their IpBs (Fig. 11 D, G, J).

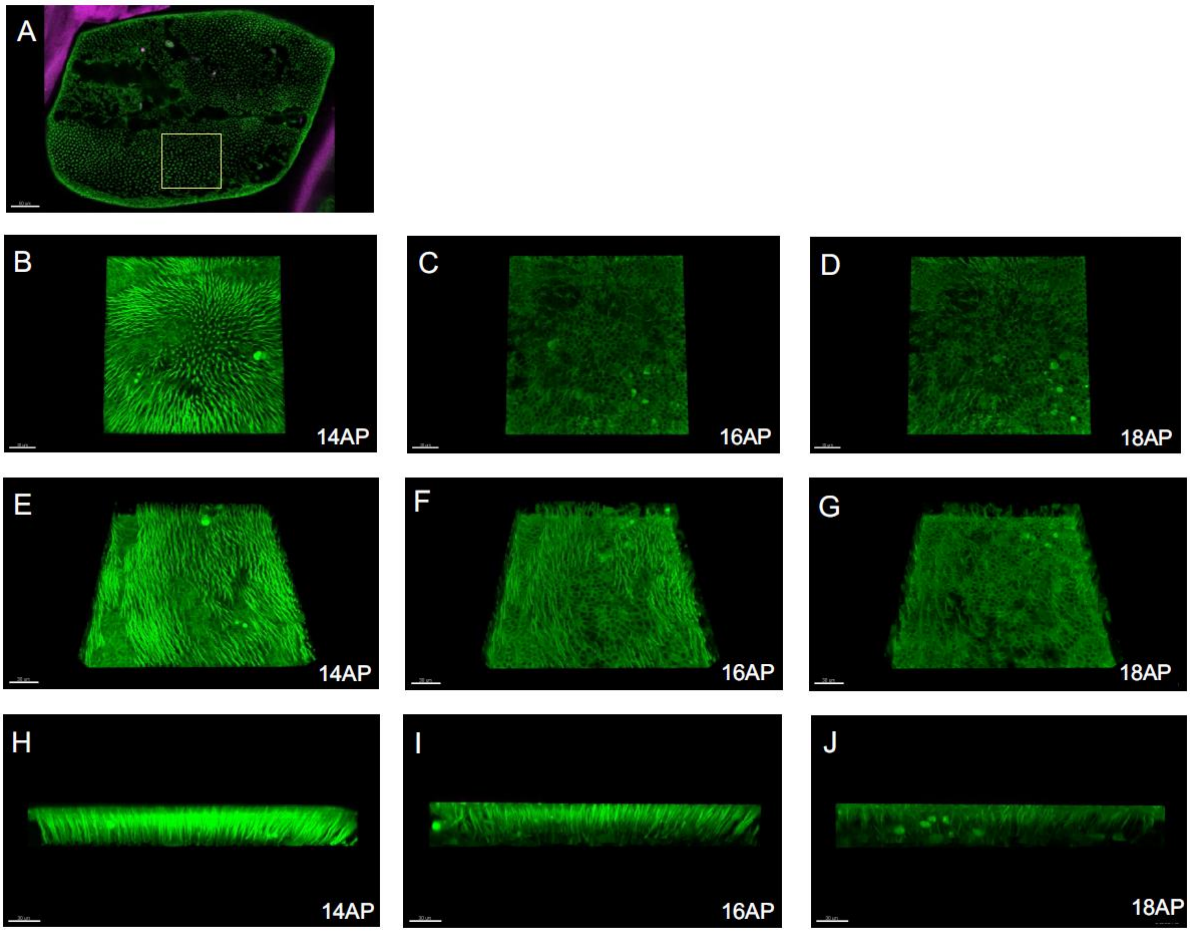

Figure 11. Dynamics of MT-based interplanar bridges. (A) Exposed pupal wing. (B-D) Dorsal view of the dorsal epithelia. (E-G) Ventral view of the dorsal epithelia. (H-J) Lateral view of the dorsal and ventral epithelia. (B, E, H) 14 hours AP. (C, F, I) 16 hours AP. (D, G, J) 18 hours AP. Yellow box indicates the zoomed in region shown in B-J. Scalebar 30µm. N=5.

Additionally, it became apparent, that IpBs and mitosis are coupled (Fig. 12). At 14 hours AP, individual cells have long and well-defined IpBs (Fig. 12 A, D). During wing development, these protrusions start to bundle and disassemble (Fig. 12 E-F). This leads to the eventual loss of the IpBs (Fig. 12 G). This process is coupled with mitosis, as the loss of cell protrusion leads

cells to go into cell division (Fig. 12 G-M). It is important to note, that some cells that do not lose their IpBs do not go into mitosis (Fig. 12 D-M).

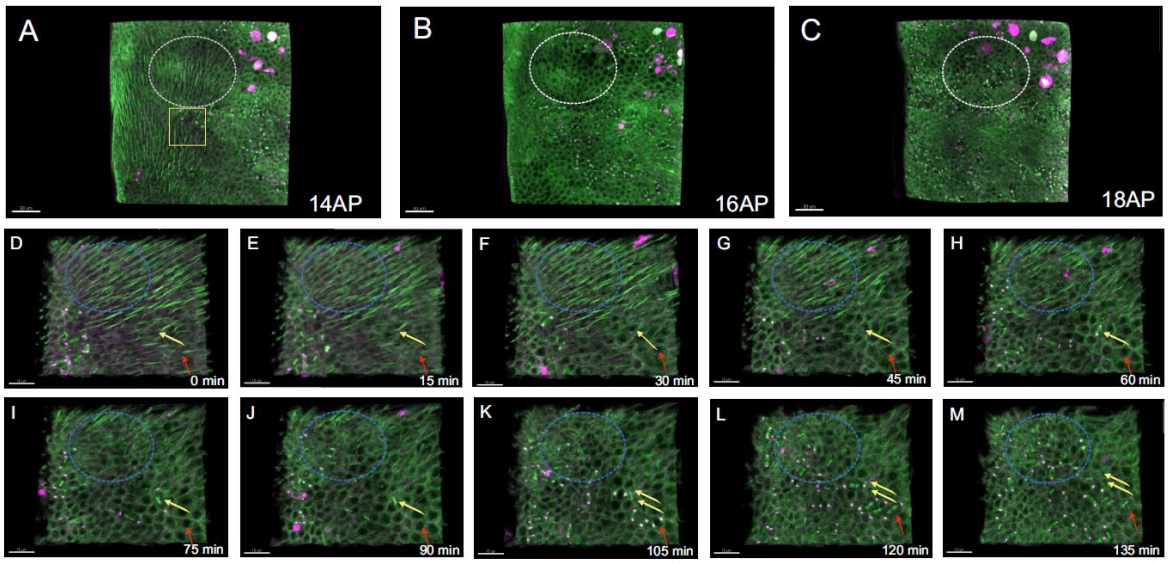

Figure 12. Coordination between interplanar bridges and mitosis. (A-C) Apical view of epithelia from 14 to 18 hours AP, respectively. (D-M) Basal view of epithelia from 0 – 135 minutes. 0 minutes implies 14 hours AP. Grey dotted circle indicates the loss of IpBs and increase of mitotic cells at tissue level. Yellow box signifies the region showed in D-M. Blue dotted circle shows the progression of loss of IpBs and increase of cell division in a specific region at cellular level. Yellow arrows indicate the coordination between IpBs and cell division as the loss of protrusion leads to mitosis. Red arrows show another neighboring cell going through the process in a delayed manner. (A-C) Scalebar 30µm. (D-M) Scalebar 15µm. N=5.

DISCUSSION

During pupal wing development, simple epithelia converts into two-layered 3D wing containing two epithelial layers (Waddington, 1940; Garcia-Bellido and Merriam, 1971). During these processes, dorsal and ventral epithelia appear to undergo coordinated growth (Waddington, 1940; Etournay *et al.*, 2016; Gui *et al.*, 2019). But what kind of mechanisms might there be to explain these processes?

It has been shown that *Drosophila melanogaster* BMP-type ligand Dpp coordinate 3D tissue architecture formation by regulating tissue growth and pattern formation/differentiation (Gui *et al*., 2019). Although this study nicely explains how pattern formation (wing vein or intervein) is coordinated between the two layered epithelia, it was still puzzling how coordinated mitoses take place when two layered epithelial sheets are separated during inflation stage. To shine a light to these questions, pupal wing was used as a model.

Considering that development requires fine coordination, it was hypothesized that different kinds of protrusions might play a role during wing development. Indeed, tubulin-based MT structures, proposed to be termed interplanar bridges (IpBs), were found during pupal wing development (unpublished data).

In the thesis, time lapse imaging was used to examine the pupal wing of *Drosophila melanogaster* from 14 to 18 hours AP during inflation stage. Around 14 hours AP, individual cells have long and clearly distinguishable IpBs where MT minus-ends are anchored to the apical side, while plus-ends grow basally (Fig. 11 B, E, H; Fig. 12 A, D). During a next few hours majority of IpBs disassemble and eventually many cells lose their protrusions (Fig. 12 D-F). Remaining IpBs start bundle and appear to form the extended bundled structure prior to apposition. Interestingly, as was seen from the live imaging, after loss of IpBs, centrosomal cnn:Cherry was apparent (Fig. 12 F-M). Thereby it was concluded that loss of MT-based IpBs leads to mitosis (Fig. 12). We hypothesize that dynamics of IpBs coordinate tissue growth between the two-layered epithelia.

Recent observations suggest that MTs are regulated by two distinct MTOCs (Lopez-Fanarraga *et al.*, 2001; Brodu *et al.*, 2010; Sanchez and Feldman, 2017). Our data reveal that ncMTOCs govern the MT dynamics of IpBs. Therefore, the thesis work set up a screening of co-factors of ncMTOC by using UAS/Gal4/Gal80 system to identify candidate genes involved in the regulation of MT minus-ends (Brand and Perrimon, 1993; Rodríguez *et al.*, 2012).

Results showed that while some gene knockdowns showed mild phenotypes (Fig. 7, 9, 10), others had more severe anatomical and morphological changes compared to the control (Fig. 8, 9, 10). In specific, knock-down of *patronin*, *short stop*, and *α-spectrin* resulted in smaller wing size, wing shape change, and blistering (Fig. 8 B-D, F; Fig. 9, 10). Moreover, *par-1* mutants exhibited in addition to the wing size and shape change extra crossvein formation between LV2/LV3 and LV4/LV5 (Fig. 8 E; Fig. 9, 1).

The results from time lapse imaging indicated that at 14 hours AP, MT minus-ends are anchored to the apical side with MT plus-ends growing toward basal domain (Fig. 11 B, E, H; Fig. 12 A, D). This further confirmed that MTs are governed by ncMTOCs at that stage. After loss of IpBs, cnn:Cherry, which is a marker for detecting centrosomes indicating cell division, was detectable, accordingly majority of GFP tagged Tubulin was observed in the spindle formation under the control of centrosome (Fig. 12 D-M). We also tested our hypothesis that degeneration of MTs are required for coordinated mitoses by ectopic expression of MT-binding protein Tau (Gustke *et al.* 1994; Bouge and Parmentier, 2016). Our data showed that disassembly of MTs are significantly delayed by Tau protein expression, resulting in decreasing numbers of mitotic cells (unpublished data). Furthermore, these observations suggest that after the loss of IpBs, MTs are regulated by different organizing centers from ncMTOCs to cMTOCs. Previous studies indicate that MTs under the ncMTOCs are only observed in differentiated cells, e.g. neurons (Sanchez and Feldman, 2017). Our data clearly suggest that MTs regulation between ncMTOCs and cMTOCs are reversible and provide flexible modules in context dependent manner.

Additionally, it became apparent, that while some cells lost their IpBs and underwent mitosis, other cells retained their membrane protrusions and did not go into cell division (Fig. 12). This indicates that there is a cell face decision making step during wing development. It is more than needed to understand, which kind of signaling pathways may be involved in this process. Different pathways have been characterized to be involved in intracellular communication. For example, cytonemes have been shown to sustain long-range BMP signal in wing imaginal disc and MT-nanotubes have implied to transduce short-range BMP signal in male GSCs (Haglund *et al.*, 2011). Understanding the role of different signaling pathways in the context of MT-based IpBs will give much needed insight into the mechanisms now shown to have a role in mitosis.

As was seen from the live imaging, IpBs seem to be means for cell communication for coordinated mitosis on both epithelia. The correlation between IpBss and mitotic cells has been quantified (unpublished data). It shows, that during 14 – 18h AP, the number of IpBs decreases

while the number of mitotic cells increases in both epithelia. This implies that IpBs and mitosis appear to be coordinated in spatial-temporal manner.

In addition to their role in mitosis, it is possible, that IpBs are responsible for the re-apposition of D-V cell layers during inflation stage which results in the apposition of two layers, except from the vein regions (Waddington, 1940; Gui *et al.*, 2019).

Results of RNAi screening led to two conclusions. Firstly, that Patronin, Shot, Par-1, and α-Spectrin have a crucial role in wing development (Fig. 8-10). s*hot*, *patronin*, *par-1*, and *αSpec* knock-down adults exhibited smaller wings compared to the control, which makes it plausible to hypothesize that MT minus-end dynamics may be linked to proliferation (Fig. 9-10). Furthermore, three of the gene knockdowns exhibited wing blistering (Fig. 8 B-D, F). This makes it possible to consider that the dynamics of MT minus-ends at ncMTOC sites impact the functioning of IpBs. This makes it possible to assume that IpBs have a role in the communication between two epithelia to start their coordinated re-apposition. Secondly, other genes known to be involved in the governance of the MT minus-end dynamics at ncMTOC sites showed milder phenotypes (Fig. 7, 9-10). It is possible, that these components may have a less crucial role in the regulation of MT minus-ends, or they may be molecularly compensated by other MT minus-end proteins. It might be likely, that these genes which did not show noticeable wing size difference after the knock-down may still have a role in MT minus-end governance (Fig. 9, 10). This may come from the fact that even though the number of cells may be fewer, because of the lesser rate of mitosis, the sizes of individual cells may be bigger. That is the reason why in the future it is important to count adult wing cells through counting the bristles of the wing, as it is known from the literature, that one cell contains one bristle (Etournay *et al.*, 2016).

It is important to note, that different stocks containing the same gene RNAi had different phenotypes (Fig. 7 N-P, Fig. 8 E-F, Fig. 9, 10). The reason might come from the fact, that the penetrance of RNAi differs between the different fly stocks.

The identification of the CAMSAP/Patronin protein family was an important milestone to better understand the mechanisms that regulate MT minus-end dynamics (Jiang *et al.*, 2014; Yau *et al.*, 2014; Feng *et al.*, 2019). These proteins have been shown to interact with MT minus-end directly and have a role in stabilizing the minus-ends (Goodwin and Vale, 2010; Akhmanova and Hoogenraad, 2015). These results coincide with the previous findings that Patronin is an important MT minus-end stabilizer at ncMTOC sites (Goodwin and Vale, 2010; Jiang *et al.*, 2014; Yau *et al.*, 2014). Thereby it is possible to hypothesize that as a ncMTOC associated

protein governing the MT minus-end dynamics, it is necessary for the correct formation and functioning of the IpBs. Disruption of Patronin might lead to the instability of MTs and inadequate formation of protrusions leading to reduced mitosis and loss of correct apposition of D-V layers resulting in wing size reduction and blistering.

Shot is shown to be involved with MT minus-ends. In specific, in *Drosophila melanogaster*, Shot has been demonstrated to recruit Patronin to the apical domain to form ncMTOCs (Nashchekin *et al.*, 2016; Khanal *et al.*, 2017). RNAi screening results imply that as Patronin is not recruited to the ncMTOC by Shot, the possibility of reduction of mitosis and instability of IpBs will lead to the wing size reduction and disrupted apposition because of the inadequate intercellular communication between dorsal and ventral epithelia.

Another players, for example the Par-complex proteins and Spectrins, are shown to be needed for Shot/Patronin proper localization (Suzuki and Ohno, 2006; Khanal *et al.*, 2017). They act as apico-basal polarity determinants to control the location of Shot and thereby Patronin (Fletcher *et al.*, 2015; Khanal *et al.*, 2017). The results from adult wind screening imply that both are necessary for correct MT dynamics through their apico-basal polarity determination ability.

Lastly, it is more than likely that additional proteins regulating MT minus-end dynamics exist (Sanchez and Feldman, 2017). Identification of these proteins and mechanisms by which they interact with each other will give us much needed insight into ncMTOCs to better understand their function in different cellular aspects. During morphogenesis, both proliferation and differentiation of cells are important for organ development. These processes must occur according to the necessity, where cell division is needed for tissue growth and differentiation for correct cell functioning. It would be interesting to know what mechanisms and molecular players are involved in the switch from cMTOC to ncMTOC and back for the applicable wing development. This information will give us greater understanding of how morphogenesis takes place during organ development.

Through the results gathered from the thesis, it is proposed that there is a new mechanism that regulates MT dynamics coupled with spatial-temporally coordinated mitosis on both dorsal and ventral epithelia. As the results coincide with previously published data about membrane protrusions playing a vital role in cell proliferation, cell fate decision, and migration, makes it clear that interplanar bridges are another form of membrane protrusions responsible for intercellular communication (Rustom et al., 2004; Önfelt et al., 2006; Sartori-Rupp et al., 2019). From the data of time lapse imaging, we now have an idea of how MT dynamics is regulated

during 14-18 AP. Nonetheless, additional functions of interplanar bridges are still unclear. As is shown in other membrane protrusions, the role of MT-based interplanar bridges in vesicle transport, bundling mechanisms for coordinated cell migration and more is yet to be revealed. In the future, live imaging with gene manipulation of candidate genes (*shot*, *patronin*, *par-1*, and *α-spectrin*) is yet to be conducted. This experimental approach will allow us to understand how dynamics of interplanar bridges contribute to tissue morphogenesis. Finally, I presume that our findings are not specific to *Drosophila melanogaster* wing but rather general mechanisms underlying tissue morphogenesis.

6. SUMMARY

To better our understanding about cellular mechanisms underlying 3D structured morphogenesis, *Drosophila melanogaster* pupal wing was used as a model. The thesis focused on identifying genes known to regulate MT minus-end dynamics at ncMTOC sites. To achieve this objective, RNAi knock-down flies were generated and examined. From the results it was found that several genes including *patronin, short stop, α-spectrin,* and *par-1* play a role in fruit fly pupal wing development. These gene knockdowns exhibited adult fly phenotypes including reduced wing size, blistering, and additional crossvein formation indicating their role in proper functioning and dynamics of MTs. This led to the hypothesis, that MT-based protrusions might have a role in tissue growth through regulating mitosis and re-apposition.

MT-based protrusions, termed interplanar bridges, were shown to play a role in cell division. In specific, the results implied that these tubulin-based structures govern the coordinated mitosis necessary for tissue growth. During pupal wing development, cells possess protrusions on both dorsal and ventral epithelia. Their minus-ends are anchored to the apical domain, while plus ends grow into basal side. These protrusions from both epithelia seem to connect with each other. During wing development, IpBs assemble into bundles, after which some cells will lose their MT-based protrusions and will lead them to going into cell division. Other cells are able to retain their IpBs and will not go into mitosis.

Thereby it is proposed that MT-based IpBs are coupled with mitosis in spatial-temporal manner during *Drosophila melanogaster* pupal wing development. Taking into account the adult wing RNAi screening results it is suggested that several MT minus-end proteins shown to regulate MTs at ncMTOCs in epithelial cells, have a role in functioning of IpBs and thereby during pupal wing development.

7. RESÜMEE

Interplanaarsete sildade dünaamika ning koe morfogenees *Drosophila melanogaster* nuku tiivas

Hanna Antson Resümee

Käesoleva magistritöö eesmärgiks oli välja selgitada mehhanism, mis on aluseks 3D organi arenemisel. Selleks kasutati laialt kasutusel olevat arengumudelit, äädikakärbse *Drosophila melanogaster* nuku staadiumi tiiba.

Kuigi erinevaid intertsellulaarse kommunikatsiooniga seotud väljakasve on iseloomustatud mitmete mudelorganismide kudedes, on nende roll organismi arengus suuresti teadmata. *Drosophila melanogaster* on heaks mudeliks selgitamaks, milline on nende raku väljakasvete bioloogiline funktsioon ja dünaamika organismi arengus. Antud töös käsitleti teadaolevalt uudseid mikrotorukesi sisaldavaid raku väljakasve – interplanaarseid sildasid, äädikakärbse arenevas tiivas. Kuna varasemalt on teada, et mikrotorukeste miinus otsaga interakteeruvad valgud on olulised mikrotorukeste organisatsiooni keskuste regulatsioonil, siis püstitati hüpotees, et need valgud mängivad rolli ka interplanaarsete sildade kujunemisel ja dünaamikal. Sellest tulenevalt seati töö eesmärgiks välja selgitada millised mikrotorukeste miinusotsaga seotud valgud on olulised äädikakärbse areneva tiiva morfogeneesis. Kasutades eluskoe 5D mikroskoopiat ja biokuvamist seati teiseks suuremaks eesmärgiks iseloomustada interplanaarsete sildade morfoloogiat ja dünaamikat nuku tiiva arengus 14-18h pärast nuku moodustumist.

Eksperimentide tulemusena selgus, et mitmed mikrotuubulite miinus otsaga interakteeruvad valgud nagu Patronin, Short stop, α-Spectrin ja Par-1 on olulised äädikakärbse tiiva morfogeneesis. Häired nende faktorite töös põhjustavad tiibade märkimisväärselt väiksemat kasvu, tiivasoonte defekte ning villilaadsete struktuuride teket tiiva epiteelide vahel. Väiksem tiivasuurus võrrelduna kontrolliga viitas häiretele rakkude jagunemises ja võimalikule interplanaarsete sildade seosele mitoosiga. Selle tõestamiseks markeeriti mikrotorukesed (Tubulin:GFP) ja tsentrosoomid (cnn:Cherry) ning vaadeldi interplanaarsete sildade ja mitootiliste rakkude dünaamikat. Katsete tulemusena leiti, et interplanaarsete sildade dünaamika on oluline lüliti reguleerimaks koordineeritud mitoosi dorsaalse ja ventraalse tiiva epiteelirakkudes.

Lisaks iseloomustati antud töös detailsemalt interplanaarsete sildade dünaamikat 14-18h peale nuku moodustumist. Leiti, et osad epiteelirakud kaotavad arengu jooksul koordineeritult

väljakasvud ning lülituvad mitootilisse rakujagunemisesse. Teistel rakkudel interplanaarsed sillad säilivad ja koonduvad naaberrakkude väljakasvudega moodustades kimbulaadseid struktuure.

Kokkuvõtvalt magistritöö tulemustest lähtuvalt jõuti järeldustele, et mikrotorukeste miinus otstega seotud valgud on olulised interplanaarsete sildade mikrotorukeste korrektses dünaamikas ja funktsioneerimises ning seeläbi tiiva morfogeneesil. Lisaks järeldati, et interplanaarsed sillad mängivad rolli koordineeritud mitoosi regulatsioonis äädikakärbse nuku tiiva arengus.

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