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The functions of cytoskeleton, extracellular matrix, and signalling pathways in Drosophila melanogaster wing morphogenesis

Bachelor's Thesis (12 ECTS)

Curriculum Science and Technology

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Tartu 2022

The functions of cytoskeleton, extracellular matrix, and signalling pathways in Drosophila melanogaster wing morphogenesis

Abstract:

Components of the cytoskeleton, extracellular matrix, and signal transduction pathways are tightly interconnected and involved in the complex tissue morphogenesis process. It is important to create a whole picture of their functions not only on the cellular scale but in whole tissue. By utilizing GAL4/UAS/GAL80ts conditional gene knockdown and ectopic expression system in a fruit fly Drosophila melanogaster, it is possible to evaluate candidate gene functions specifically in epithelial wing tissue during earlier and later developmental stages. As the result of this work, the crucial genes participating in wing morphogenesis were found, and the mechanisms of their functions were proposed.

Keywords: *Drosophila melanogaster* wing, cytoskeleton, ECM, signal transduction pathway, gene screening, tissue morphogenesis

CERCS: B350 Development biology, growth (animal), ontogeny, embryology

Tsütoskeleti, rakuvälise maatriksi ja signaaliradade funktsioonid äädikakärbse Drosophila melanogaster tiiva morfogeneesis

Lühikokkuvõte:

Tsütoskeleti, rakuvälise maatriksi ja signaaliradade komponendid on omavahel tihedalt seotud ning osalevad keerukates koe morfogeneesi protsessides. Oluline on luua tervikpilt nende komponentide funktsioonidest mitte ainult raku, vaid kogu koe tasemel. Kasutades GAL4/UAS/GAL80ts süsteemi geeni konditsionaalseks alla surumiseks ja ektoopiliseks ekspressiooniks äädikakärbses (D. melanogaster), on võimalik hinnata kandidaatgeenide funktsiooni spetsiifiliselt tiiva epiteelikoes nii varasemates kui hilisemates arenguetappides. Selle bakalaureusetöö tulemusena leiti tiiva morfogeneesis osalevad olulised geenid, mille funktsioonide mehhanisme antud töös käsitleti.

Võtmesõnad:

Drosophila melanogaster tiib, tsütoskeleton, ECM, transduktsiooni signaalrada, geeni skriinimine, koe morfogenees

CERCS: B350 Arengubioloogia, loomade kasv, ontogenees, embrüoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

+TIP – Microtubule “plus,, end tracking protein

AF – Actin filament

Ap – *Apterous*. Gene driver that regulates expression in a dorsal layer of a wing

Arp – Actin-related protein

BM – Basement membrane

BMP - Bone Morphogenetic Protein

Bristles (Trichomes) – wing hairs

Brk – Brinker

Cnn – Centrosomin

Dn – Dominant-negative mutation that disrupts the functions of a wild-type gene

Dpp - Decapentaplegic

DsRNA – Double-stranded RNA

ECM – Extracellular matrix

Egfr - Epidermal growth factor receptor

Ena – Enabled

Fim – Fimbrin

hTau – Human Tau

Kat60 – Katanin 60 catalytic domain

LanB2 – Laminin B2 subunit

Miro – Mitochondrial Rho

Morphogen – Molecule regulating the patter of tissue development

MTOC – Microtubule-organizing center

ncMTOC – Non-centrosomal microtubule-organizing center

Nrt - Neurotactin

Nub – *Nubbin.* Gene driver that regulates expression in a whole wing

Pav – Pavarotti

Pbl – Pebble

PCM – Pericentriolar material

Pnut – Peanut

Rhea – Homolog of Talin in *Drosophila*

RNAi – RNA interference

Sas-4 - Spindle assembly abnormal 4

Sdb - SAXO downstream of blistered

Sep1 – Septin 1

Sfl - Sulfateless

Shot – Short stop

Sn – Singed

Sqh – Spaghetti squash

Zip – Zipper

-TIP – Microtubule “minus„ end tracking protein

Tsr – Homolog of Cofilin in *Drosophila*

Wg/*Wg* – Wingless protein/ Gene, coding for the protein

Wnt – Wingless signalling pathway

Yw – Wild-type fly genotype

αSpec – α- Spectrin

βSpec – β-Spectrin

INTRODUCTION

Cytoskeleton and extracellular matrix components have been long known to have crucial roles in various cellular processes, including cell division, adhesion, movement, shape regulation, and molecules trafficking, which are all necessary for the formation of complex organs and tissues in multicellular organisms (Fletcher & Mullins, 2010; Franz et al., 2013). Signal transduction pathways are also tightly connected with these components and allow rapid and efficient intercellular communication needed for coordinated tissue morphogenesis. While the function of many components is well studied on the scale of individual cells, their roles are much less understood on the whole-tissue scale. *Drosophila melanogaster* is a promising model to study the function of cytoskeletal, extracellular matrix, and signal transduction components in separate tissues for several reasons. First, it is one of the best-studied multicellular model organisms, in which many conserved signal transduction networks were discovered (Friedman & Perrimon, 2007). Second, flies have a fast life cycle and are very efficient in proliferation, which allows the rapid screening of many genes. Third, *Drosophila* has a larger genetic toolkit that allows efficient genetic screening. Some of these tools enable spatial and temporal regulation of target gene expression. In this thesis work, it was used to knockdown or overexpress the broad list of candidate genes in the *Drosophila* wing during earlier and later developmental stages. The genetic screening provides the results of interesting wing phenotypes that set the basis for evaluating candidate genes' function in tissue morphogenesis and studying various factors essential for epithelial tissue architecture.

1 LITERATURE REVIEW

1.1 *Drosophila melanogaster* as a model organism

1.1.1 The biological advantages of the *Drosophila* model

Drosophila melanogaster, also known as the fruit fly, is a frequently used model organism in various biological and biomedical studies. There are several advantages of the *D. Melanogaster* as a model. First, approximately 60 percent of the fruit fly's genome is homologous to human (based on review by Mirzoyan et al., 2019). Moreover, 75% of known disease-associated genes in humans have homologs in the fruit fly. Flies are easy to maintain and do not require advanced infrastructure or complex technical training. Fruit flies can be raised at room temperature; they have a fast generation time of roughly ten days and a short life cycle of 2 to 3 months, depending on the temperature and availability of the culture medium (Fernández-Moreno et al., 2007). The life cycle of a fly can be divided into 4 phases (Fernández-Moreno et al., 2007) (Figure 1a). It starts with an embryo, which transits into a worm-like larva stage after one day. The larval stage differentiates into first, second, and third instar larva. The first instar larvae feed on the surface of the growth medium. The second instar larvae go inside the medium, and the third instar larvae crawl out on the wall of a vial, where they finally start pupariation. During pupariation, body metamorphosis occurs for a larva to become an adult fly. Fruit flies reproduce fast, and an adult female can lay approximately 100 eggs per day. *Drosophila* flies are usually kept in plastic vials (Figure 1b), and their culture medium is relatively inexpensive and mainly consists of sugar and flour. At the same time, flies are complex multicellular organisms, in which the studies of tissue morphogenesis and development of intercellular communication can be done efficiently.

1.1.2 The use of *D.melanogaster* in biological and biomedical studies

D. melanogaster species has been thoroughly studied for more than 100 years. It started with crucial genetical research by Thomas Morgan (Morgan, 1910). He discovered the connection between the eye colour of a fly and its sex and formulated his theory of sex chromosome-linked traits. The study of Morgan set the basis for the chromosome theory of inheritance and initiated many other genetic studies in *Drosophila* (Miko, 2008). Besides genetics, research in *Drosophila* led to many important discoveries in developmental biology, neurobiology, and cancer biology. Thus, many evolutionary conserved signalling pathways were first identified in *Drosophila* (Bellen et al., 2010). The broadly studied signalling

transduction systems and the abundance of disease-associated genes that have human homologs allowed Drosophila to be an excellent model for researching various diseases, including cancer (based on a review by Mirzoyan et al., 2019). The presence of anatomically similar or close structures between the fruit fly and human made it possible to consider epithelial, brain, gut, lung, blood, and even prostate cancers within the *Drosophila* model. The fly model also has been used for chemical screening, leading to the efficient drug screening for Parkinson's disease, metabolic disorders and polycystic kidney disease (Gasque et al., 2013; Hofherr et al., 2016; Whitworth et al., 2006). Moreover, the analysis of the conjunctional application of anti-cancer drugs with radiation for cancer treatment can also be done in fruit flies (Edwards et al., 2011).

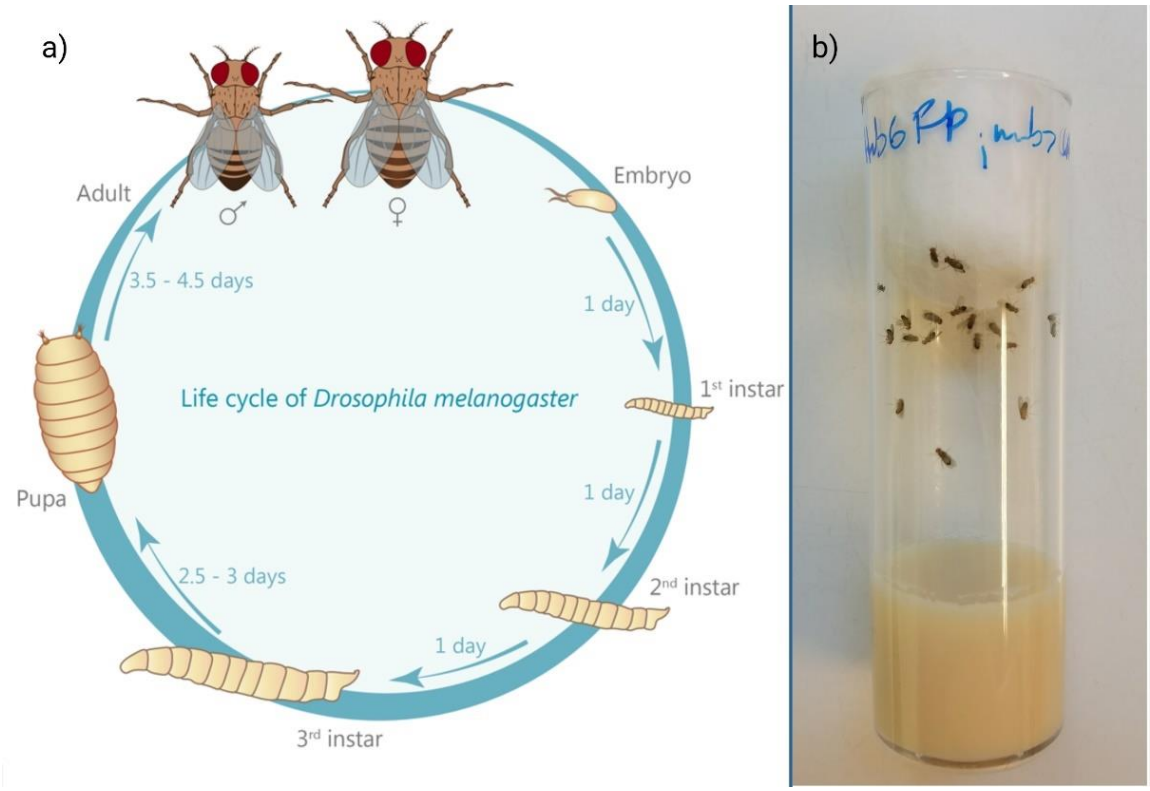

Figure 1. The life cycle of *Drosophila melanogaster.* a) The main stages of the life cycle of *D.melanogaster. Adapted from Methods — The Walter Lab, n.d.* b) The typical vial with culture medium (fly food) on the bottom where fruit flies are kept.

1.1.3 Signal transduction pathways discovered in *Drosophila*

Cell-cell communication through chemical signal transduction is crucial for the tissue development and homeostasis of multicellular organisms. Signalling networks first discovered and studied in Drosophila include Notch, Hippo, Wingless (Wnt), Hedgehog, and others (Bellen et al., 2010; a review by Misra & Irvine, 2018). The studies of signal

transduction systems in *Drosophila* lead to cancer research and neurobiology development. The Notch signalling is vital in neurogenesis and neural differentiation in vertebrates. Notch proteins in mammals can act as oncoproteins and are associated with epithelial tumours, breast cancers and T-cell leukaemia (Radtke & Raj, 2003). At the same time, in other cancers, including cervical cancer and small-cell lung cancers, Notch may act as a tumour suppressor. The Hippo network regulates cell proliferation and organ growth and involves tumour suppressor proteins (based on a review by Misra & Irvine, 2018). The component of the Hippo network, Yorkie protein in *Drosophila*, that has YAP homolog in mammals, maintains stem cells in various tissues, promotes cell growth and plays a key role in tissue regeneration. Defects in Hippo signalling can lead to tissue overgrowth and uplifted tumorigenesis. The Wnt signalling cascade has a significant role in neurogenesis and synaptic differentiation, as well as regulation of tissue morphogenesis and its repair (Inestrosa & Arenas, 2009; Sanz-Ezquerro et al., 2017). Malfunctioning in Wnt signalling is linked to several cancer types, including breast, cervical, colorectal cancer, and others. The Hedgehog signalling pathway is crucial in cell proliferation and differentiation and the process of ageing (Sasai et al., 2019). Abnormal levels of Hedgehog proteins can result in the enlargement of structures like the skeleton, limbs, and central nervous system or retardation of their growth. As all these signal transduction pathways are conserved between different species and play multiple significant roles from the beginning of the organism's development to the end, the study of the components of these systems in *Drosophila* can create complex connections between development, disease, and genetics, and elucidate the essential aspects of cell communication and its regulation in all multicellular organisms.

1.2 Genetic and molecular toolbox in *D. melanogaster*

1.2.1 Balancer chromosomes

It would be impossible to have *D. melanogaster* as such a powerful model without the versatile molecular and genetic tools that have been introduced to it since the discovery of this organism. First, gene mutations were introduced to *Drosophila* employing chemical and radiation treatment (Tolwinski, 2017). Mutagenic alkylating agents, specifically ethylmethane sulfonate (EMS), were the basis of the forward genetic screening and consequent gene mapping (Venken & Bellen, 2014). The study of X-rays and their mutagenic effect on *Drosophila* brought Hermann Muller the Nobel Prize in 1946 (H. J. Muller, 1928; Tolwinski, 2017). Hermann Muller also discovered the concept of the balancer chromosome (Hermann J Muller, 1918). In his experiments, Muller used heterozygous *D.*

melanogaster strain, in which female flies had one normal and one inverted X chromosome (Figure 2). The inverted X chromosome carried a recessive lethal mutation and was marked with Bar (B) dominant mutation, which produces a slit-like eye shape in flies (Venken & Bellen, 2014; Wolfner & Miller, 2016). The male progeny of the heterozygous females only had wild-type eyes. If the recombination occurred between the normal X chromosome and *Bar* mutation-carrying inverted chromosome, then some part of the male progeny would have slit-shaped eyes.

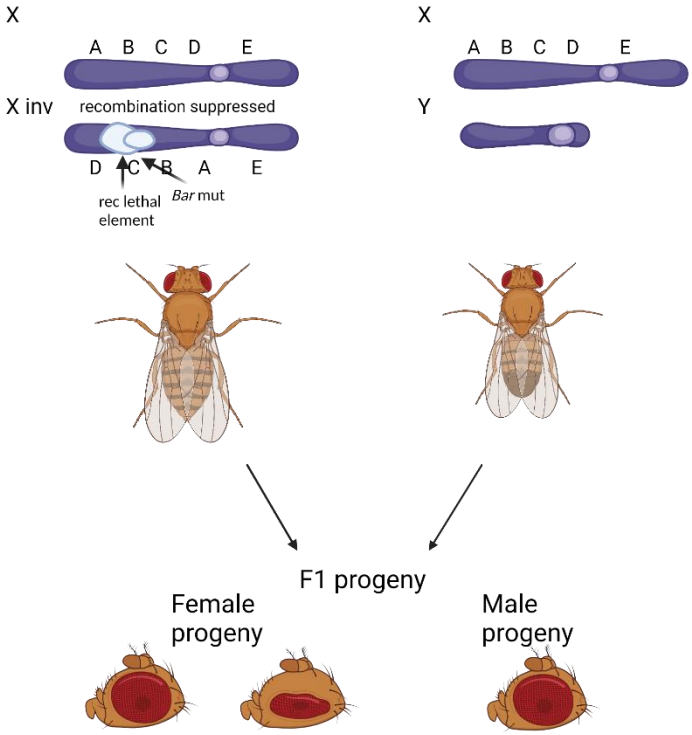

Figure 2. The plan of Morgan's experiment. Female flies are heterozygous and have one normal and one inverted X chromosome. Inverted X chromosome contains recessive lethal marker and *Bar* dominant mutation resulting in slit-shaped eyes. The resulting progeny have females with both normal and slit-like eyes and males with normal eyes only. The males that inherit inverted X are lethal. If recombination occurred between X chromosomes in females, the small percentage of males with slit-shaped eyes would survive. *The figure was created with BioRender.com.*

This and further studies confirmed that paracentric and pericentric inversions of chromosomes reduce the recombination between homologues (Hermann J Muller, 1918; Sturtevant & Beadle, 1936). It is important to note that recombination between chromosomes is not fully inhibited, but the progeny with recombined chromosomes will be lethal in the case of occurrence (Kaufman, 2017). In the case of a crossing over event, the DNA strands must make a loop to align properly (Figure 3). After the crossover, the resulting recombinant

chromatids will create inviable gametes with segmental aneuploidy. Previously, single inversions in balancer inverted chromosome fragments could be overcome by rare double crossover events. However, these chances were almost entirely eliminated by introducing overlapping inversions or several inversions within one fragment (Kaufman, 2017).

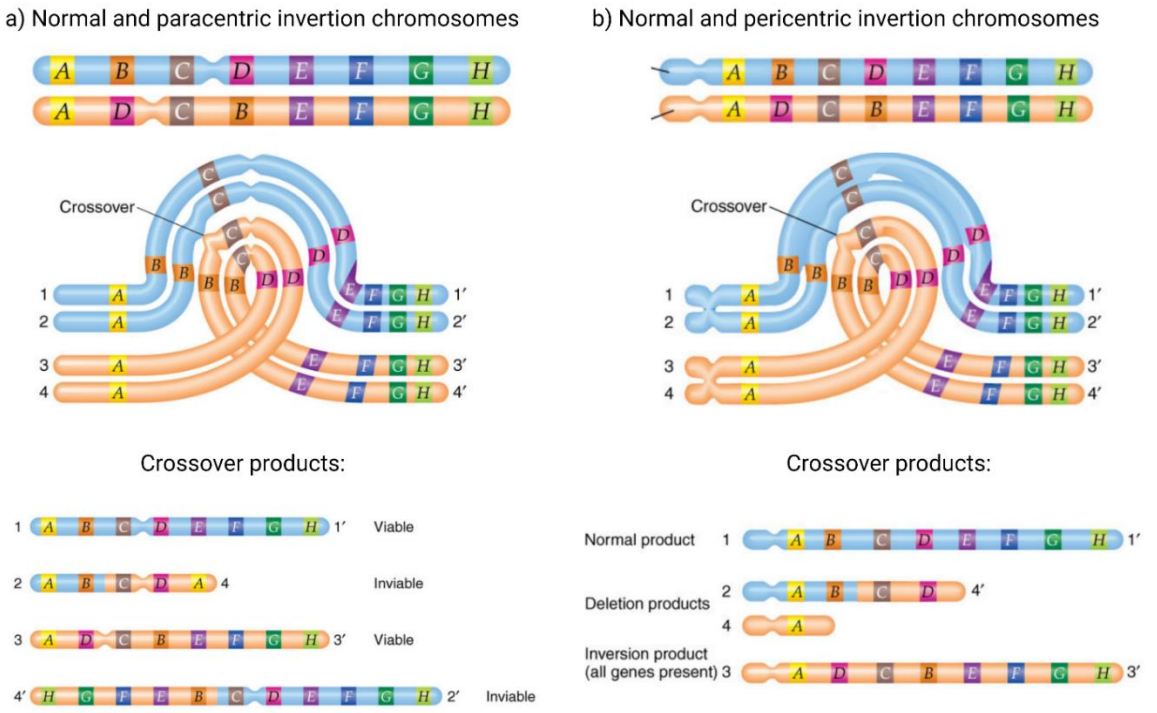

Figure 3. Recombination event between normal and inverted chromosomes. a) Recombination between normal chromosome and chromosome with paracentric inversion; b) normal chromosome and chromosome with pericentric inversion. The figure shows the loop forming during the recombination event and possible recombination products. *Adapted from Russell, 2009.*

The balancer chromosomes in *Drosophila* find two main applications. First, they allow maintaining recessive lethal mutations on the balancer chromosome without the elimination during crossing over in the same manner as in Muller's experiment. Second, balancers are crucial for creating stable inbred stocks, in which certain mutations and genetic elements are preserved by marking the balancer chromosome with dominant mutation having a recognizable phenotype and recessive lethal genetic element (Hales et al., 2015). In this case, all the inbred progeny will either be homozygous for a mutation of interest when inheriting two copies of the normal chromosome, heterozygous with visible dominant mutation when inheriting both normal and balancer chromosome, or lethal in the case of inheritance of two balancer chromosomes. The typical dominant balancer chromosomes include *CyO*, which carries *Curly (Cy)* dominant gene marker resulting in curly wings, *FM6* which has *Bar (B)* dominant mutation responsible for slit or bean-shaped eyes, and *TM6B* tagged with two

markers, *Tubby (Tb)* associated with the thicker and shorter body in flies, and *Humoral (Hu)* linked with the excessive humeral bristles (wing hairs, also known as trichomes). The choice of a balancer depends on the genomic location of the genetic elements that need to be preserved in the flies' population. For instance, *FM6* carries the inversion in the X chromosome, *CyO* is the second chromosome balancer, and *TM6B* is the third chromosome balancer.

1.2.2 GAL4/UAS/GAL80^{ts} system in *Drosophila*

Another important molecular tool for inducible tissue-specific foreign gene expression or native gene overexpression in *Drosophila* is GAL4/UAS/GAL80^{ts} system. GAL4 is a protein derived from the *Saccharomyces cerevisiae* yeast (Duffy, 2002). GAL4 acts as the transcription factor and regulates the expression of genes upon binding to specific 17 basepairs (bp) sites that are identified as the Upstream Activating Sequences (UAS) enhancer element. Using transposable *P*-element, scientists introduced *GAL4* and *UAS* elements into the *Drosophila* genome (Hales et al., 2015). *P*-element was used as a transformation vector, where *GAL4* or *UAS* sequences were cloned instead of the internal transposase gene. The transposase gene catalyzes the cutting and insertion of the *P*-element into the genome. If internal transposase is substituted with the sequence of interest and an independent source of transposase is used, they can be co-injected into embryos to facilitate the insertion of *GAL4* or *UAS* elements. *GAL4* and *UAS* are usually expressed in two different fly lines. Thus, in one line, *GAL4* expression can be spatially controlled by the associated tissue-specific promoters, also named drivers. In the other line, *UAS* is bound to the gene of interest, which stays in a transcriptionally silent state. The two lines are mated, and the progeny contains all elements needed for the regulated gene expression (Figure 4). Furthermore, GAL80 protein in this system allows conditional activation of gene expression under GAL4-UAS control. GAL80 acts as the inhibitor of GAL4 upon binding (*FlyBase Experimental Tool Report: GAL80ts*, n.d.). Temperature-sensitive GAL80^{ts} is active at 18-20°C but inactivated in temperatures of 29-30°C (Merkling et al., 2015). Therefore, flies kept at room temperature do not have the expression of GAL4-UAS controlled genes, and the expression is activated upon temperature shift.

Besides gene overexpression, GAL4/UAS/GAL80^{ts} system is used to track loss-of-function phenotypes in flies. The first method is the gene knockdown by RNA interference (RNAi) (Duffy, 2002). For RNAi-mediated gene knockdown, different techniques can be utilized. In the primary approach, the synthetic inverted repeat (IR) sequence or double-stranded

hairpin RNA (dsRNA) of the target gene is bound to *UAS* (Nishihara et al., 2004) (Figure 4). Upon expression, dsRNA is produced in both cases. Due to the later processing of dsRNAs by molecular complexes in a cell, they can mediate mRNA degradation and consequent target gene silencing. Another technique is the GAL4-UAS mediated overexpression of the dominant-negative mutated gene. The product of this gene disrupts the regular activity of wild-type protein or blocks its function (Duffy, 2002; Veitia, 2007). Both approaches are widely used for phenotype screening in mutant fly strains.

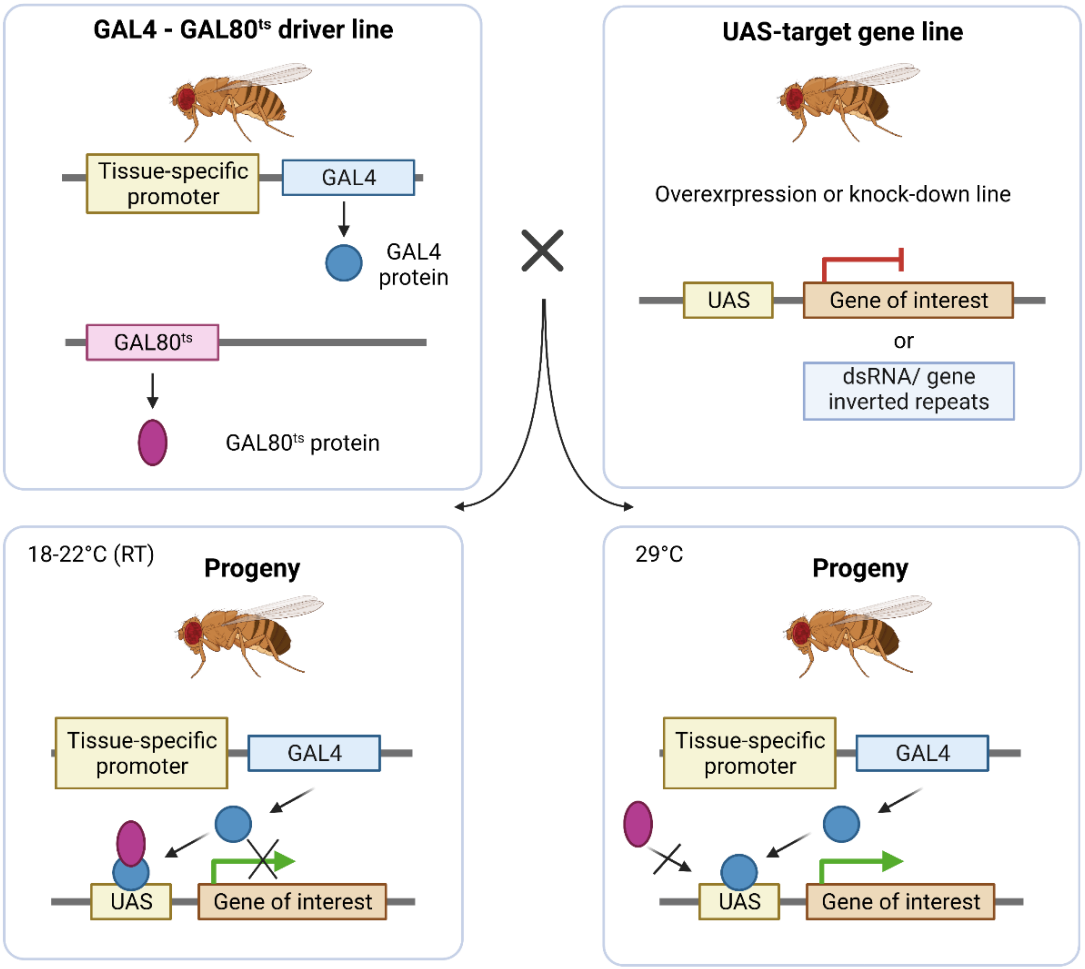

Figure 4. GAL4/UAS/GAL80^{ts} system in *Drosophila*. The fly line with GAL4-GAL80^{ts} elements is crossed with the UAS-target gene/gene dsRNA line. In progeny, all GAL4/UAS/GAL80ts system elements are present. When flies are kept at room temperature (RT), GAL80 protein binds GAL4 protein and inhibits gene/dsRNA expression. When the progeny is transferred to 29°C, GAL80 can not bind GAL4, and gene/dsRNA under UAS control is expressed. *The figure was created with BioRender.com.*

1.2.3 *D. melanogaster* genome

The modest chromosome content of the fruit fly also plays an essential role in the rapid genetic screening and preservation of mutations. The genome contains five chromosomes, specifically X and Y sex chromosomes, large polytene chromosomes 2 and 3, and the smallest dot chromosome 4 (Figure 5) (Kaufman, 2017). Both sexes have chromosomes from 2 to 4, and the determination of sex depends on the dosage of X chromosomes (Hales et al., 2015). Thus, the presence of two X chromosomes, like in XX and XXY flies, is the female determinant, while XY and X0 flies are males. The gene content of fruit flies reaches approximately 17800 genes, and among them, around 14000 are protein-coding (Kaufman, 2017). The initial annotated genome sequence of *D. melanogaster* was already available by the year 2000 and accounted for 180 Megabases (Mb) (Adams et al., 2000). The largest collection of genomic information about *Drosophila* species is available in the FlyBase project, which is regularly updated by a consortium of research facilities (*FlyBase Homepage*, n.d.).

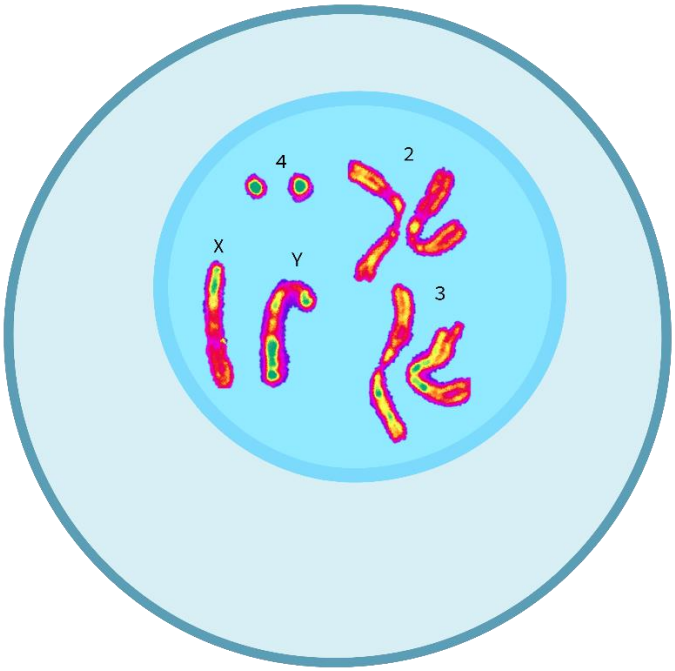

Figure 5. *D. melanogaster* chromosome content inside the graphic cell nucleus. *The figure was created with BioRender.com.*

Variable stocks of fruit flies with deleted and inserted genetic elements, different gene expression and silencing patterns, and balancer chromosomes are commonly found in several *Drosophila* stock centres, including Bloomington Drosophila Stock Center, NIG-FLY, Vienna Drosophila Resource Center and others (*Bloomington Drosophila Stock Center: Indiana University Bloomington*, n.d.; *NIG-Fly - Fly Stocks of National Institute of*

Genetics -, n.d.; *VDRC Stock Center: Main Page*, n.d.). The large availability of various fly lines allows accelerating gene screening in the flies and quick introduction of novel complex genetic systems.

1.3 *Drosophila* wing as a research tool

1.3.1 Wing development during the larval and pupal stage

The accessibility of spatial gene regulation in *Drosophila* makes it possible to perform studies on different tissues and organs. Thus, a fruit fly wing became a separate, broadly researched model. *Drosophila* wing starts its development from the structure known as an imaginal disc (Figure 6a). Imaginal discs are the precursor structures that give rise to the appendages in an adult fly, like wings, eyes, legs, halteres, and genitals (Beira & Paro, 2016). Imaginal disc forms from the cluster of epithelial cells in the embryo and morphologically transforms and grows during larval and pupal stages. Intensive growth and cell division in the wing imaginal disc during the larval stage are combined with the tissue patterning process with proper assignment of cell fates (Neto-Silva et al., 2009). The wing disc is compartmentalized and has planar polarity with anterior-posterior and dorsal-ventral organization patterns (Figure 6b). During the same period, the provein regions are formed, which later transform into an adult fly's veins. The morphogen molecules like Decapentaplegic (Dpp) and Wingless (Wg) provide signalling between cells in different compartments (Neto-Silva et al., 2009). Their concentration controls the patterning and boundaries of compartments and cell proliferation within them.

The morphology of a wing imaginal disc in the late larval stage includes locations from which notum, hinge, and wing parts emerge (Tripathi & Irvine, 2022) (Figure 6b,d). In the longitudinal section, distinct tissue folds can be observed. They separate wing (pouch), hinge, and notum regions (Figure 6c). The wing disc goes through substantial changes during the larval-pupal transition. First, larval disc everts, extends out of the body cavity so that the wing part elongates, and dorsal-ventral compartments form bilayer flattened wing structure (Tripathi & Irvine, 2022) (Figure 7a). In the first 5 hours after pupariation, neighbouring epithelial cells exchange places (cell intercalation) and change their shape from longer columnar to cuboidal. Wing enlargement is accompanied by tissue flattening and degradation of the extracellular matrix (basement membrane). During the first 10 hours after pupariation, dorsal and ventral layers of the wing stay adherent, but during the next 10 hours, the layers separate, forming an inflated structure. The hinge part contracts, and the wing

attaches to the secreted pupal cuticle. Wing continues elongation, and cells rearrange, proliferate, and change their shape to more isometric. By the 20th hour after pupariation, two layers appose again to form the final adult wing shape (Gui et al., 2019; Matamoro-Vidal et al., 2015; Tripathi & Irvine, 2022). The layers' adhesion, separation, and second opposition during the pupal stage are mediated by the formation and degradation of cell-cell protrusions or intercellular cables between cells (Sun et al., 2021). After the 35th hour, the wing epithelium gets covered in the adult cuticle. Cell division is arrested, and later wing area expansion happens due to the increase in cell size. After the hatching of an adult fly, epithelial cells go through epithelial-mesenchymal transition and apoptosis, and the adult wing is mostly made of the cuticle (Matamoro-Vidal et al., 2015; Tripathi & Irvine, 2022). The cuticle expands and flattens due to the pressure from the hemolymph in wing veins.

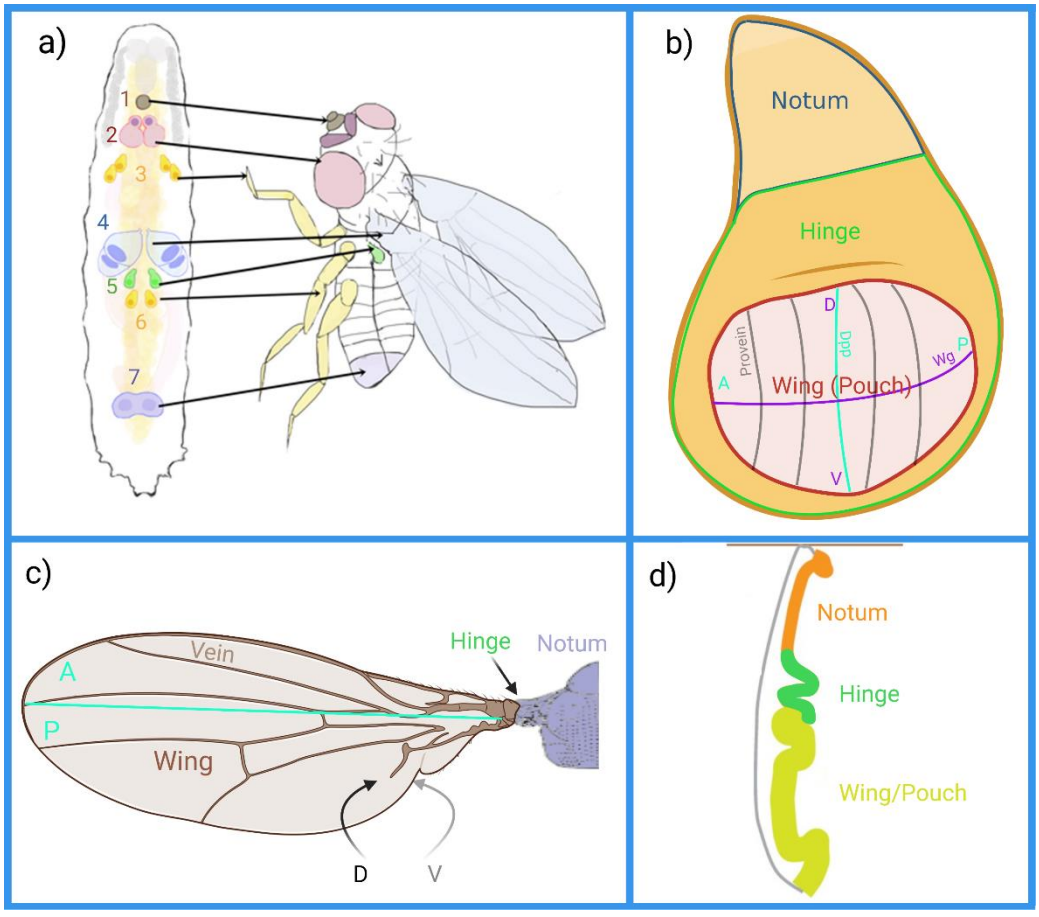

Figure 6. Morphology and location of wing disc and adult wing. a) Imaginal discs that give rise to the structures in an adult fly: 1 – Labial disc; 2 – Eye discs; 3 – Upper leg discs; 4 – Wing discs; 5 – Haltere discs; 6 – Lower leg discs; 7 – Genital disc. *Adapted from Jaszczak & Halme, 2016.* b) Wing imaginal disc during the late larval stage. It consists of notum, hinge and wing (pouch) regions. The wing region has a planar polarity: dorsal-ventral (D-V) compartments that are regulated by Wg morphogen diffusion, and anterior-posterior (A-P) compartments that are

regulated by Dpp morphogen diffusion. Provein regions are formed in the same development stage. c) Wing of an adult fly. It can be divided into the same regions as the wing imaginal disc. The D layer faces the viewer, and the V layer is behind the screen. The A-P sections are divided by a blue line. d) Longitudinal section of the wing disc. *Adapted from Tripathi & Irvine, 2022. The figure was created with BioRender.com.*

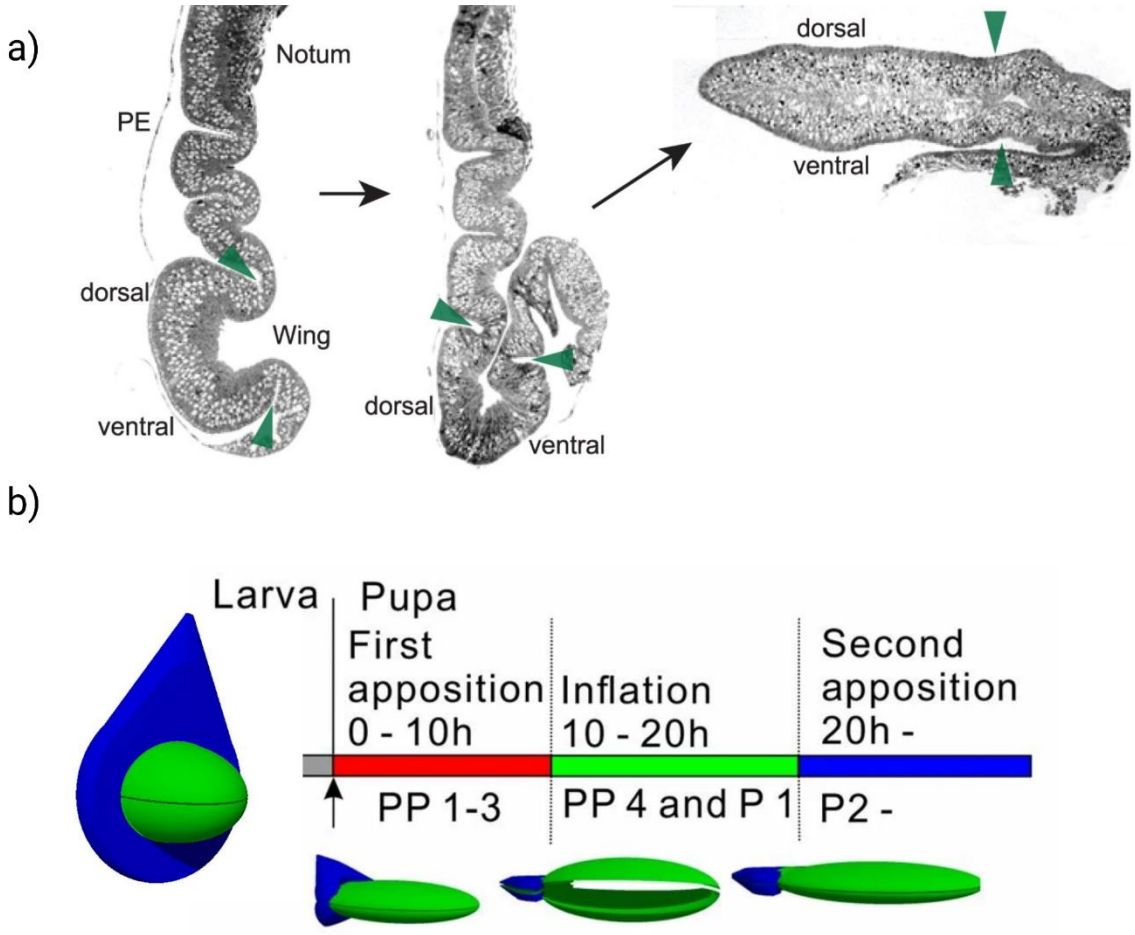

Figure 7. Metamorphosis during larval and pupal stages of development. a) Initiation of larval-pupal transition in the wing disc. The wing disc's dorsal and ventral compartments elongate and appose each other, forming a pupal wing. *Adapted from Tripathi & Irvine, 2022.* b) The eversion from imaginal disc and changes in the pupal wing: initial adhesion (5-10 hours after pupariation (APF)); separation and formation of cables between dorsal and ventral layer (10-20 hours APF); re-apposition and attachment of layers (after 20 hours APF). *Adapted from Gui et al., 2019.*

1.3.2 Adult wing morphology

The adult wing consists of the vein and intervein regions (Figure 8). The veins are ectodermal tubes that contain densely packed cells producing thicker and darker cuticle (Blair, 2007). Intervein cells are lost soon after flies hatch, and therefore vein region cells are the only living cells in the adult wing. The veins are responsible for wing rigidity and act as vessels

for hemolymph, nerves, trachea, and blood cells. The wing has five central longitudinal veins (L1-L5), two small longitudinal veins (L0 and L6), and three crossveins: anterior crossvein (ACV), posterior crossvein (PCV), and humeral crossvein (HCV). Additionally, closer to the hinge region, anal and radial veins are located, while proximal and distal marginal veins bound the anterior compartment of the adult wing. While longitudinal veins' patterns appear already in the larval stage, crossveins are specified in the later pupal stage (Tripathi & Irvine, 2022). The regulation of vein localization is dependent on many signalling pathways, including Hedgehog, bone morphogenetic protein (BMP), epidermal growth factor receptor (Egfr) signalling, Wnt, and Notch.

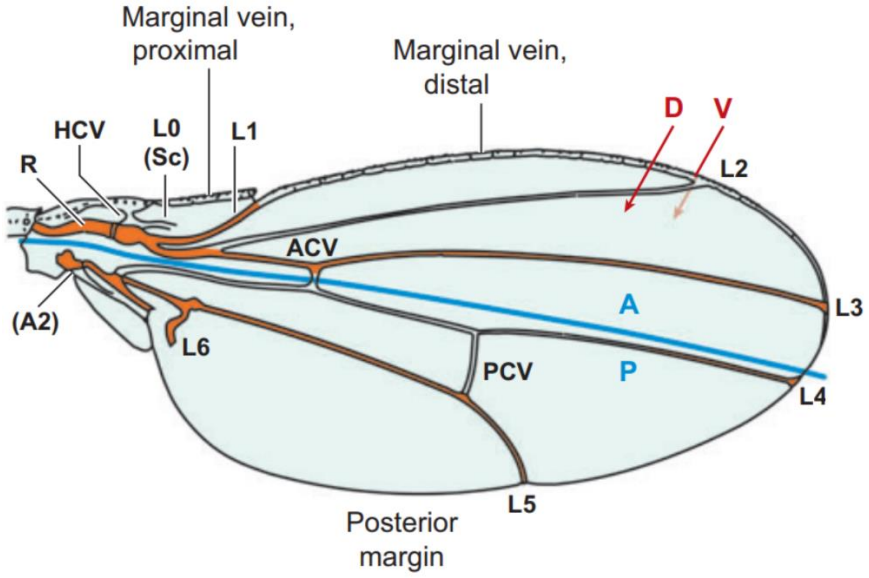

Figure 8. Drosophila adult wing. A-P blue stripe divides anterior and posterior planes. The adult wing has vein and intervein regions. The veins can be divided into longitudinal (L0-L6) veins, posterior, humeral, and anterior crossveins (PCV, HCV, and ACV), anal vein (A2), radial vein (R), and marginal veins. *Adapted from Blair, 2007.*

1.3.3 Usage of the wing in research

Both wing imaginal disc and adult wing are commonly used as a platform for developmental and morphological research. Imaginal disc and adult wing provide an understanding of tissue patterning, and wing discs are widely studied for understanding epithelial morphogenesis, their communication in tissue, and epithelial cancers (Tripathi & Irvine, 2022). The genome-wide studies of the relationships between genotype and phenotype are also commonly conducted in the *Drosophila* wing (Pitchers et al., 2019). Wing can be dissected in each stage of its development and separately analyzed by different microscopy methods. Wing imaginal discs are typically used for fluorescence microscopy, where separate proteins can be tracked

with antibody-based immunostaining or by attaching them to a reporter fluorescent protein, such as green fluorescent protein (GFP) (Maity et al., 2013; Snapp, 2005). Furthermore, the dynamics of wing disc development can be tracked with time-lapse live imaging under a confocal microscope (Aldaz et al., 2010). The adult wings can be studied with light microscopy, and morphological features can be examined with image processing software.

1.4 Cytoskeleton components in cellular processes and tissue morphogenesis

1.4.1 Functions and composition of the cytoskeleton

The cytoskeleton is a structure responsible for many functions in intracellular and intercellular processes. It regulates cell shape, localizes the organelles inside it, and connects the cell with its environment (Fletcher & Mullins, 2010). The cytoskeleton plays an important role in signal transduction, cell movement and adhesion, cell division, and tissue morphogenesis (J. Zhang et al., 2017). Therefore, studies of its components are necessary for understanding tissue architecture. The cytoskeleton consists of highly dynamic filamentous polymers and regulative proteins (Fletcher & Mullins, 2010). The main polymeric components of the cytoskeleton are actin filaments, tubulin-based microtubules, and intermediate filaments. Interestingly, intermediate filaments are not present in the cells of *Drosophila*, while microtubules and actin filaments have very conserved structures and associated proteins across species (Cho et al., 2016). Components of the cytoskeleton are organized, mobilized, assembled, and disassembled in response to external factors and the function of regulatory proteins. The regulative proteins can be divided into nucleating-promoting factors that create filaments; capping proteins, stopping the assembling; polymerases, responsible for faster growth; depolymerization factors, serving disassembling functions; and crosslinker or stabilizing proteins, which provide the structural support for the cytoskeletal network. The filamentous cytoskeleton networks are differentiated based on mechanical rigidity, polarity, dynamics of assembling, and association with proteins driving intracellular trafficking, known as molecular motors (Ayloo & Holzbaur, 2015; Fletcher & Mullins, 2010).

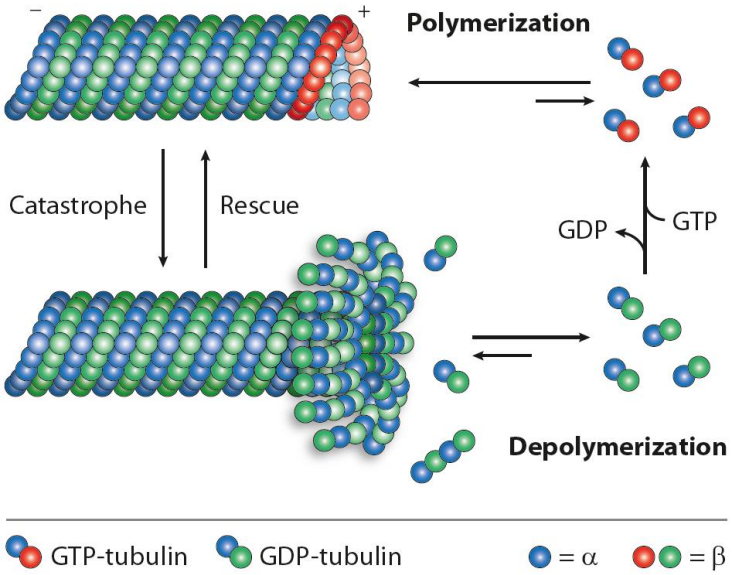

Figure 9. Structure and assembly of microtubules. Microtubules consist of αβ-tubulin dimers. They can go through rapid cycles of polymerization (also known as a rescue) and depolymerization (also known as a catastrophe). These processes are mediated by GTP hydrolysis. *From Microtubules, Post-Translational Modifications of Tubulins and Neurodegeneration, n.d.*

1.4.2 Structure, role and assembly of microtubules and centrosomes

Microtubules are the most rigid out of three types of cytoskeletal polymers. These are hollow rods with a diameter close to 25 nanometers (nm) (Figure 9) (*Microtubules - The Cell - NCBI Bookshelf*, n.d.). Microtubules undergo continuous assembly and degradation, while their persistence length can reach 5 millimetres (mm). They consist of tubulin dimers, α-tubulin and β-tubulin. Microtubules play critical roles in the separation of chromosomes during mitosis. They are also essential for cell movement, intracellular organelles transport and determination of cell shape. Most microtubules in a cell extend and initiate from microtubule-organizing centres (MTOCs). In most proliferating or migrating cells, the centrosome acts as the main MTOC (Muroyama & Lechler, 2017). However, many non-centrsosomal MTOCs (ncMTOCs) can be found in differentiated cells. To fastly reorganize themselves from one MTOC to another, microtubules can go through rapid cycles of polymerization and depolymerization (*Microtubules - The Cell - NCBI Bookshelf*, n.d.). They exist in a condition known as "dynamic instability" and always have a subset of them growing and another portion shrinking in the cell. Microtubules also have polarity within the cell and grow by the attachment of tubulin to their "plus" end, while the "minus" end is usually attached to the centrosome or other MTOC. The dynamic behaviour of microtubules is controlled by α- and β-tubulin-bound guanosine triphosphate (GTP) hydrolysis (Figure 9).

If GTP-tubulin complex concentration is high, polymerization is favoured. Once GTP-bound tubulins are associated with microtubules, GTP is hydrolyzed to guanosine diphosphate (GDP), and the released energy favours the deformation of tubulin subunits (Burbank & Mitchison, 2006).

1.4.3 Centrosomal MTOC and its components

The centrosome is a MTOC located adjacent to the cell nucleus (*Microtubules - The Cell - NCBI Bookshelf*, n.d.). It consists of two cylindrical centrioles surrounded by pericentriolar material (PCM) that initiates microtubules assembly. γ-tubulin is the main protein in centrosome composition, which builds into the γ-tubulin ring complex. This complex contains γ-tubulin and γ-tubulin ring proteins known in *Drosophila* as Grips proteins (Tillery et al., 2018). Centriole and, hence, centrosome assembly in *Drosophila* is mediated by Sak kinase (Saurya et al., 2016). The centriole assembly proteins are conserved across species but known under different names. For example, Sak is an ortholog of Polo-like kinase 4 (Plk4) in mammals. Sak engages Spindle assembly abnormal 6 (Sas-6) and Anastral spindle 2 (Ana-2) to the mother centriole to form a cartwheel structure that initiates daughter centriole building. Sas-6 and Ana-2 then recruit Spindle assembly abnormal 4 (Sas-4) that helps to mobilize centriolar microtubules. Together with Sas-4, Centrosomal protein 135kDa (Cep135) also contributes to centriolar microtubules assembly. Centriolar coiled-coil protein 110kDa (Cp110) regulates the length and duplication of centrioles (Franz et al., 2013). Stabilization of PCM and centrosomal loading of Sak are maintained by Asterless (Asl), while centriole-to-centrosome conversion is mediated by Anastral spindle 1 (Ana-1) (Dzhindzhev et al., 2010; Saurya et al., 2016).

Asl and Sak also play essential roles during mitosis. During cell division, centrosome duplicates, and the microtubule cytoskeleton transforms into a chromosome-segregating structure called the mitotic spindle (Figure 10) (Fletcher & Mullins, 2010; *Microtubules - The Cell - NCBI Bookshelf*, n.d.). Several proteins, including Microtubule-associated protein 60 (Map60), Centrosomin (Cnn), and kinesin-6 motor Pavarotti (Pav), are important for correct spindle aggregation (Edzuka & Goshima, 2019; Tillery et al., 2018). The formation of the mitotic spindle and the ability to find chromosomes also depend on the assembly dynamics of microtubules.

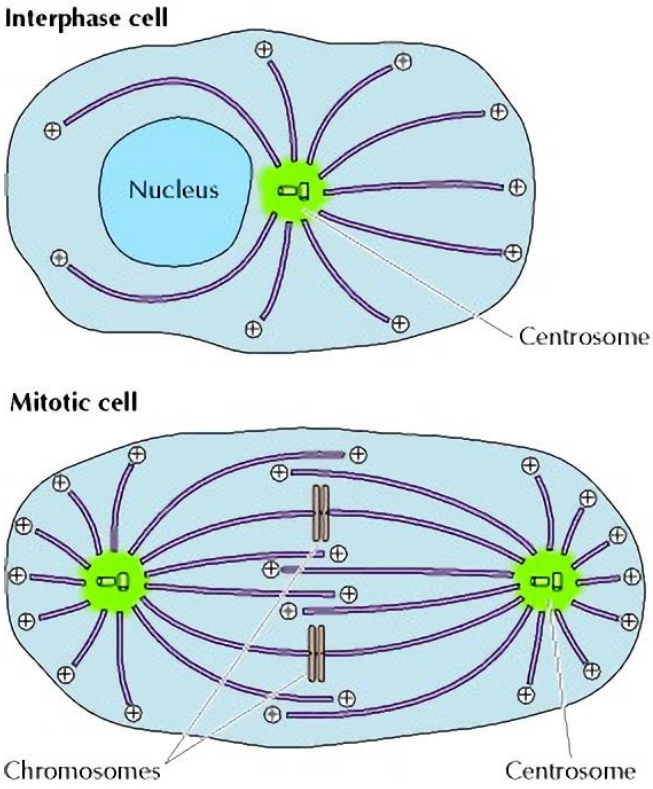

Figure 10. Centrosomal MTOCs location in a non-dividing and dividing cell. a) Centrosome location in interphase before division. b) Centrosome duplication and location in mitosis. Centrosomes consist of two centrioles surrounded by PCM. Microtubules extend from the centrosome with their "minus" end attached and "plus" end growing. During mitosis, centrosome duplicates and two centrosomes move to the cell poles. Microtubules form mitotic spindle and stick to chromosomes to allow their segregation. *From Microtubules - The Cell - NCBI Bookshelf, n.d.*

Together with centrioles congregation and centrosomes reorganization during mitosis, PCM organization is also a necessary process (Tillery et al., 2018). Cnn plays a crucial role in organizing PCM scaffold and recruiting γ-tubulin ring complex to the centrosome, while Spindle defective 2 (Spd-2) can promote Cnn functions. Asl is needed to recruit Cnn and Spd-2 to centrioles. Maturation of centrosomes and Cnn regulation are performed by Polo kinase. Aurora-A kinase (AurA) shares its functions with Polo and location in an inner region of PCM (Richens et al., 2015). PCM assembly during interphase is also regulated by Pericentrin-like protein (Plp). Plp is concentrated at centrioles and the outer region of PCM, where it co-localizes with Cnn.

1.4.4 Non-centrosomal MTOCs

In most differentiated cells, including epithelial, neuronal, and muscle cells, microtubules have ncMTOCs (Figure 11) (Bartolini & Gundersen, 2006; Sanchez & Feldman, 2017). In epithelial cells, noncentrosomal microtubules are specifically crucial for establishing apical-

basal polarity (Buckley & St Johnston, 2022). NcMTOCs, or noncentrosomal arrays, can be generated by three main processes: discharge from centrosomes, nucleation at noncentrosomal locations, and breakage of pre-existing microtubules. The generation of nc-microtubules by the release from centrosomes is found explicitly for epithelial and neuronal cells. Katanin (Kat) and Spastin (Spas) are involved in the process of centrosomal release in neuronal cells and, presumably, epithelial cells (Bartolini & Gundersen, 2006). Kat60 catalytic subunit was also shown to be involved in microtubule depolymerization (Díaz-Valencia et al., 2011). After release from the centrosome, microtubules are moved to the assembly sites with the help of motor proteins, where they are built into higher-order structures. At the sites of assembly, ncMTOCs have proteins that interact with microtubule "minus" ends and adapter proteins that create the connection with the site (Sanchez & Feldman, 2017). γ-tubulin ring complex also helps to enhance microtubule assembly at ncMTOCs and acts as the nucleator. The stabilization of microtubule "minus" end and protection from degradation after nucleation are associated with the CAMSAP/Patronin family, and anchoring at the MTOC is mediated by Blastoderm-specific gene 25D (Bsg25D), also known as Ninein in mammals.

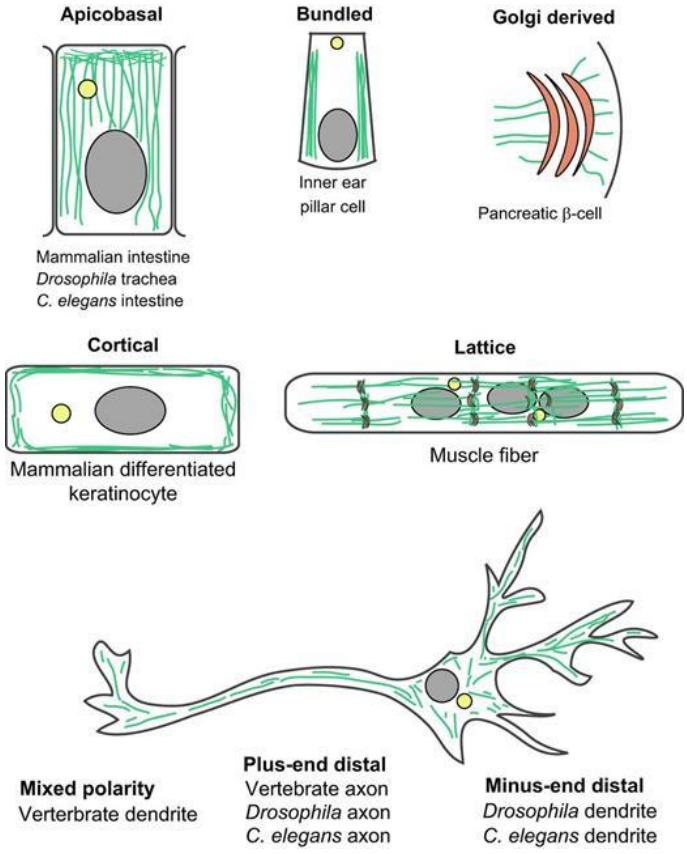

Figure 11. Noncentrosomal microtubule arrays in the different cell types. Microtubule arrays are marked with green, centrosomes are yellow. *From Muroyama & Lechler, 2017.*

1.4.5 MAPs and microtubule motor proteins

The organization of microtubules inside the cell is also regulated by many other microtubule-associated proteins (MAPs) (De Forges et al., 2012). Besides GTP, MAPs also can bind microtubule "plus" end (+TIPs) or "minus" end (-TIPs). They act to stabilize, generate or depolymerize microtubules. The most studied stabilizers in mammals are Tau, MAP2 and MAP4, while in *D. melanogaster,* they include Tau and Futsch (Bolkan & Kretzschmar, 2014; De Forges et al., 2012). Tau also has essential roles in the nervous system in both humans and *Drosophila.* The loss of Tau in *Drosophila* leads to neurodegenerative processes and aberrations in photoreceptor development (Bolkan & Kretzschmar, 2014). One of the recently discovered microtubule stabilizers in *Drosophila*, SAXO downstream of blistered (Sdb), was studied in a wing (Sun et al., 2021). The well-known +TIPs that track "plus"-end are End-binding proteins (EBs) and Short spindle 2 (Ssp2), or Sentin. Previously mentioned CAMSAP/Patronin protein family include the major "minus"-end stabilizers. Another set of proteins regulates the disassembly of microtubules. Kat60 is one of these. Kinesin-like protein at 10A (Klp10A), the member of the Kinesin motor proteins family, also promotes disassembly (Tillery et al., 2018). Motor proteins associated with microtubules like Kinesin and Dynein play an important role in the intracellular transport of cells with polarity and establishment of this polarity, like in epithelial cells (Januschke et al., 2002). Dynein is the "minus"-end-directed protein, while Kinesin is the "plus"-end-directed motor. These proteins participate in the transport of proteins, mRNAs, and organelles. They also have helper proteins; for instance, CG14763 in *Drosophila* is responsible for dynein binding activity, while Mitochondrial Rho GTPase (Miro) forms Miro/Milton/Kinesin transport complex that regulates neuronal mitochondrial transport (*FlyBase Gene Report: Dmel\CG14763*, n.d.; Lee & Lu, 2014). Interestingly, the novel studies suggest that Miro plays a role in microtubule stabilization and may interact with proteins that directly control microtubule dynamics, for example, Tau and Par-1 kinase (Iijima-Ando et al., 2012; Lee & Lu, 2014). Aside from motor proteins, polarized organization and dynamics of microtubules are regulated by several components like Fat (Ft), Dachsous (Ds) and Par-1 (Tillery et al., 2018). These proteins are also needed for planar cell polarity regulation.

1.4.6 Structure, assembly and functions of actin filaments

Actin filaments (AFs) are the second type of cytoskeletal structure. They are much less stiff than microtubules and reach approximately 7 nm in diameter and several micrometres in length (Fletcher & Mullins, 2010; *Structure and Organization of Actin Filaments - The Cell*

- *NCBI Bookshelf*, n.d.). AFs play a significant part in cell shape determination, cell migration, mechanical support, cell adhesion and division. AFs are thin and flexible, but the abundant presence of crosslinkers helps assemble them into higher-order structures. The AFs were first isolated from the muscle cells, where they slide along myosin filaments to make the cells and, consequently, muscles contract. The monomer of AFs, globular (G) actin, has binding sites in the "head" and "tail" parts and forms filamentous (F) actin upon binding to other monomers (*Structure and Organization of Actin Filaments - The Cell - NCBI Bookshelf*, n.d.). Due to the rotation of G-actin monomers inside the filaments, they form a double-strand helix structure. All actin monomers become oriented in one direction and make actin helix polar. As well as microtubules, AFs have "plus" and "minus" ends (*Structure and Organization of Actin Filaments - The Cell - NCBI Bookshelf*, n.d.). However, unlike microtubules, AFs do not go through polymerization-depolymerization cycles. They steadily elongate with regulation by adenosine triphosphate (ATP) hydrolysis and assemble or disassemble in response to local signals (Figure 12). AFs aggregation or disassembly, as well as crosslinking into networks, are controlled by actin-binding proteins. Cofilin, also known as Tsr in Drosophila, is the critical protein in the disassembly process (*Structure and Organization of Actin Filaments - The Cell - NCBI Bookshelf*, n.d.). It binds to AFs and increases the rate of dissociation of G-actin. Profilin reverses this effect and stimulates the incorporation of monomers. Enabled (Ena or VASP) protein in *Drosophila* also acts as actin polymerase, stimulating actin addition at the "plus" end (Chen et al., 2014). The growth of AFs is also under the control of Capping proteins (Cpa and Cpb) that inhibit the addition or loss of actin monomers from the "plus" end (Fernández et al., 2011).

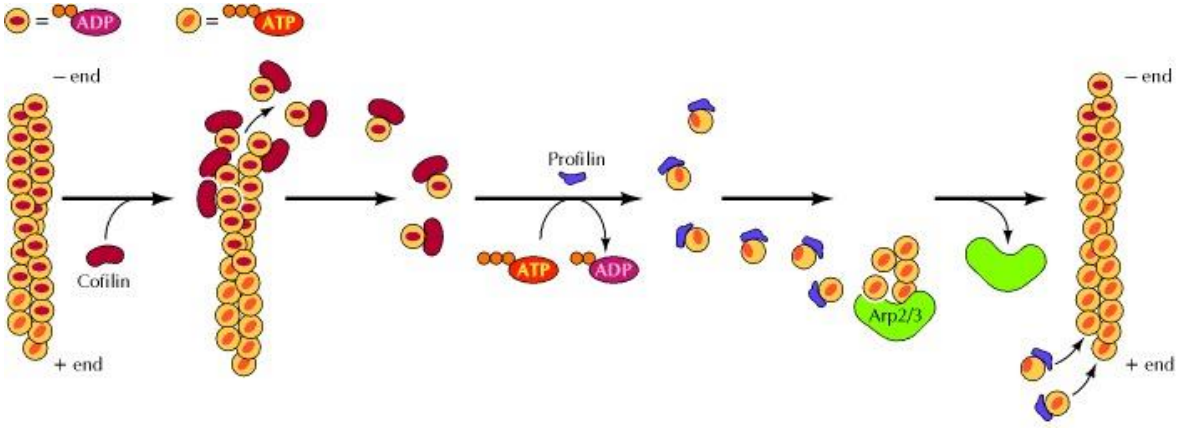

Figure 12. Assembly and turnover of AFs by the set of involved proteins. *From Structure and Organization of Actin Filaments - The Cell - NCBI Bookshelf, n.d.*

1.4.7 Actin-related proteins

Actin-related proteins (Arps) form a big, conserved protein family specialized early in eukaryotic evolution (Schroeder et al., 2020). They perform a broad range of functions. Thus, the Arp2/3 complex can initiate the formation of actin filaments, and together with Cofilin and Profilin, it remodels the actin cytoskeleton (*Structure and Organization of Actin Filaments - The Cell - NCBI Bookshelf*, n.d.). Arp2/3 complex in *Drosophila* includes seven components: Arp2, Arp3 and Arpc1 to Arpc5. Other Arps are involved in microtubule-based transport, including Arp1 and Arp10, which promote the activation of the Dynein microtubule motor protein (Schroeder et al., 2020). Several Arps, Arp4 to Arp8, are involved in chromatin remodelling (Schroeder et al., 2021). Aside from conserved Arps, many eukaryotic genomes also have rapidly evolving non-canonical Arps. The first non-canonical Arp discovered in *D. melanogaster* was Arp53D. The whole range of its actions is unknown, but it is highly expressed in the actin structures of *Drosophila* flies' testes.

1.4.8 Actin bundles and networks

Arp2/3 complex and Formin/Frl participate in actin polymerization and assembly of branched or sparse AFs. They can be modelled into actin bundles and actin networks (Dehapiot et al., 2020; *Structure and Organization of Actin Filaments - The Cell - NCBI Bookshelf*, n.d.). In bundles, filaments are packed closely into parallel arrays, while in networks, they are loosely crosslinked in orthogonal arrays. Actin-bundling proteins are mostly small rigid proteins that constrain filaments into close alignment, while proteins organizing AFs into networks are usually large and flexible. The important examples of actin-bundling proteins that regulate dense bundles are Fimbrin (Fim) and Fascin/Singed (Sn). A(alpha)-actinin cross-linker is responsible for the looser structure of the bundle. Actin networks are organized by Filamin cross-linker.

1.4.9 Actin interaction with the cell membrane

The association of AFs with the plasma membrane is crucial to cell structure (*Structure and Organization of Actin Filaments - The Cell - NCBI Bookshelf*, n.d.). The complex of AFs with actin-binding proteins forms a structure beneath the plasma membrane, known as the cell cortex. Actin-binding protein Spectrin, related to Filamin, is the major protein in this complex. Spectrin was first investigated as a critical component of the membrane skeleton in erythrocytes that protects cells from mechanical stress. Spectrin consists of α and β chains. Aβ-Spectrins form tetramers that bind to AFs and establish the spectrin-actin network.

Ankyrin creates the link between the spectrin-actin network and the plasma membrane. Several other proteins serve functions analogous to Spectrin, such as Ezrin, Radixin, Moesin (ERM) protein family, and Dystrophin, which links AFs in the cell cortex. In humans, Spectrin mostly performs its functions in erythrocytes, while Spectrin-related proteins are present in other cell types. However, Spectrin distribution is quite complex and is not restricted by one tissue type (Pesacreta et al., 1989).

The plasma membrane of many cells has specific sites of contact with adjacent cells, tissue, or substrate (*Structure and Organization of Actin Filaments - The Cell - NCBI Bookshelf*, n.d.). AFs support these contacts and are anchored to these areas of the plasma membrane, which are called focal adhesions. The connections between AFs and focal adhesions are mediated by A-actinin, Talin (Rhea in *D. melanogaster*) and Vinculin. AFs in the cell cortex and focal adhesion sites are organized into contractile actin-myosin (actomyosin) networks, also known as stress fibers (Lehtimäki et al., 2021). Besides adhesion and cell form shaping, they can also participate in cell morphogenesis and migration. Stress fibers can be divided into dorsal, ventral stress fibers, and transverse arcs (Figure 13a). Ventral stress fibers are dense actomyosin networks located at focal adhesions at the cell bottom. In contrast, dorsal stress fibers are non-contractile AF bundles located at focal adhesions at the front of a cell. Transverse arcs are thin contractile actomyosin bundles that associate with dorsal stress fibers. Actomyosin bundles consist of actin and Non-muscle myosin II (NMII) filaments. In *Drosophila,* NMII ortholog is *zipper (zip*), and *spaghetti squash (sqh)* encodes the light chain of non-muscular myosin (L. Zhang & Ward IV, 2011). The contractility of actomyosin is generated through NMII filaments sliding along actin. The amount, turnover and contractile forces of actomyosin are regulated by the Rhomboid (Rho) signalling pathway (Rho-family GTPases), kinase-phosphatase pathways, and Ca^{2+} influxes (Lehtimäki et al., 2021; Schmidt et al., 2021). The crucial molecules in the Rho pathway include Rho, Rho guanine nucleotide exchange factor proteins (Pebble (Pbl), RhoGEF2, RhoGEF3), Rac, Cdc42 GTPases, Protein kinase N (Pkn), Diaphanous (Dia) and Rho kinase (Rok) (Barmchi et al., 2005; Lu & Settleman, 1999; Schmidt et al., 2021). Actomyosin and associated proteins are also crucial for generating an actomyosin-based contractile ring that separates two cells after division (Miller, 2011). Besides, essential roles in a contractile ring formation and cytokinesis are played by Anillin (Scraps/Scra) and proteins from Septin complex - Peanut (Pnut), Septin 1 (Sep1) and Septin 5 (Sep5) (Field et al., 1996; Goldbach et al., 2010). Septins are sometimes recognized as a separate component of the cytoskeleton and are highly conserved across eukaryotes (Mostowy & Cossart, 2012). They remind cytoskeletal components because of

their filamentous structure and association with microtubules, AFs, and plasma membranes. However, their functions and mechanisms of assembly are less understood.

1.5 Cytoskeleton, extracellular matrix, and cell interactions in cellular processes

Focal adhesions form a contact point between the cytoskeleton and extracellular matrix (ECM). ECM is the non-cellular structure present within all tissues (Frantz et al., 2010). It is crucial for physical scaffolding and biochemical and biomechanical signalling. This signalling is necessary for cell differentiation and tissue morphogenesis (Frantz et al., 2010). The biochemical cues can reach cells through interaction at focal adhesions mediated by transmembrane receptor Integrin that binds Talin and A-actinin associated with AFs (stress fibers) (Figure 13b) (Frantz et al., 2010; *Structure and Organization of Actin Filaments - The Cell - NCBI Bookshelf*, n.d.). ECM is generally formed from water, proteins, and polysaccharides, but each tissue has an individual composition of ECM. This structure is very dynamic and can be remodelled enzymatically and non-enzymatically. Epithelial tissue, like the *Drosophila* wing, is in direct interaction with one type of ECM, known as basement membrane (BM). BM is a 60 to 120 nm matrix network that consists of Laminins (LanA, LanB1, LanB2) and Collagen type IV (Cg25C) interlinked by Nidogen (Ndg) and the heparan sulfate proteoglycan (HSPG) Perlecan (Trol) (Isabella & Horne-Badovinac, 2015; Kozyrina et al., 2020). BMs maintain tissue integrity and shape and can be involved in cell-cell communication, cell migration and contraction. Laminins are the key regulators of BMs assembly and are required for cellular layers adhesion and coordinated cell movement. Aside from cell-ECM and cytoskeleton-ECM contacts, cell-cell adhesion also plays a vital role in tissue morphogenesis. The main molecules in cell-cell adhesion are Cadherins (Bulgakova et al., 2012). They also participate in cell proliferation and apoptosis. Aside from Cadherins, several other molecules participate in cell-cell adhesion. They include Neurotactin (Nrt), Neuroglian (Nrg), Fasciclin II (FasII), Klingon, and), and Fasciclin III (FasIII) (Speicher et al., 1998).

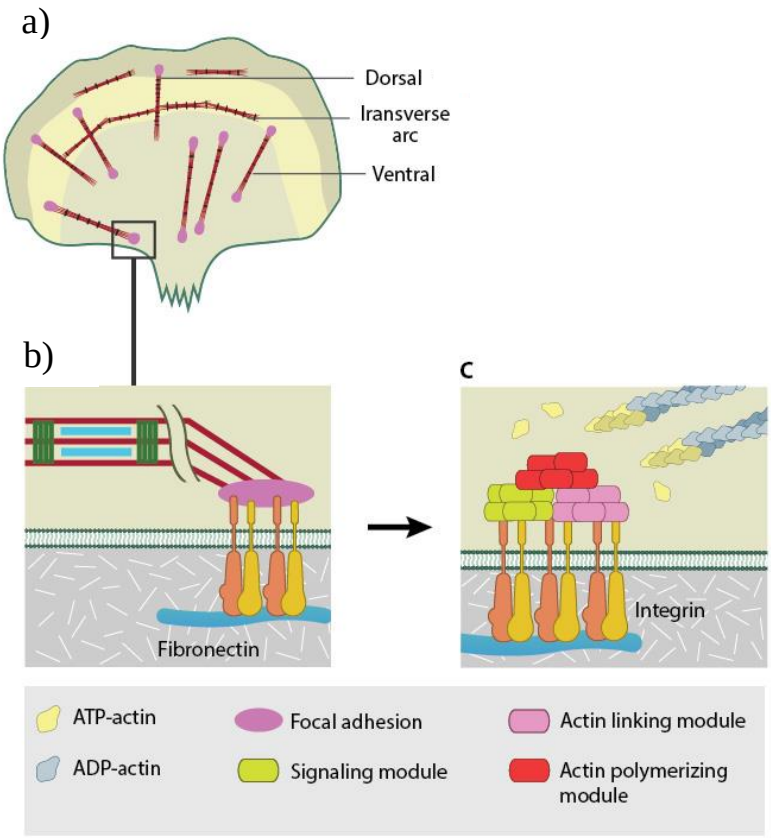

Figure 13. Stress fibers types and their interaction with ECM. a) Types of stress fibers inside a cell: dorsal, ventral, and transverse arc. b) Interaction of AF and its modules with ECM component Fibronection through Integrin receptor. *Adapted from What Is the Function of Stress Fibers? | MBInfo, n.d.*

Cytoskeleton components interact not only with ECM and cell membrane but also with each other. Many cytoskeletal integrators are involved in these processes. Spectraplakins are the central family of cytoskeletal crosslinkers (J. Zhang et al., 2017). Spectraplakin genes produce various isoforms that associate with all cytoskeletal filaments. In *Drosophila*, Short stop (Shot) belongs to Spectraplakins. However, not only Spectrplakins can interact with different cytoskeletal components. For example, Spectrin also interacts with microtubules and AFs (Pesacreta et al., 1989). ERM family protein Moe can also interact with both components (Solinet et al., 2013). The multifunctionality of many cytoskeletal proteins suggests their complex roles in cellular processes and organogenesis.

1.6 Other components regulating tissue morphogenesis

Several molecules considered in my work do not belong to previously mentioned structures. Among them are proteins involved in signalling pathways, like the Notch receptor and its

ligand Delta that participate in cell differentiation and proliferation, and transcription factor Brinker (Brk) that repress targets of Dpp (part of BMP) signalling (Campbell & Tomlinson, 1999; Go et al., 1998). Epsin (Iqf) contributes to endocytic network stability and Notch signalling regulation (Langridge & Struhl, 2017). Another set of proteins screened in this thesis is involved in heparan sulfate proteoglycans (glypicans) synthesis. They include Dally, Dally-like protein (Dlp), and Sulfateless (Sfl). Glypicans play an important role in the morphogen diffusion of signalling pathways like Wnt, Hedgehog, and Dpp (Fujise et al., 2003; Han et al., 2005; Toyoda et al., 2000). One more protein screened in this work is the Deubiquitinating apoptotic inhibitor (Dubai) that antagonizes cell death signals (Yang et al., 2013).

2 THE AIMS OF THE THESIS

Cytoskeleton and ECM proteins are essential for processes that happen on a scale of a single cell or a whole tissue. Understanding their roles is crucial for the recognition of molecular mechanisms that mediate intercellular communication and tissue morphogenesis. *D. melanogaster* wing represents an epithelial tissue model in which these mechanisms can be studied. The molecular toolbox of *Drosophila* allows for performing overexpression or RNAi mediated knockdown of target genes with both spatial and temporal control. Thus, it becomes possible to regulate gene expression specifically in the *Drosophila* wing during developmental periods of interest, for example, during the pupal stage when dynamic morphological changes in wing 3D structure happen. The aims of this thesis are therefore following:

- Utilize GAL4/UAS/GAL80^{ts} system to carry out time and tissue-specific screening of candidate genes
- Analyze phenotypes of the mutant flies using light microscopy and statistical analysis
- Discuss the possible mechanisms and the role of genes in tissue morphogenesis based on the results

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Drivers

Tissue-specific promoters, known as drivers, spatially control the expression of the genes in GAL4/UAS/GAL80ts system. One of the used drivers is transcription factor *nubbin* (*nub-Gal4/Gal80^{ts}*), which promotes the expression in the whole wing (Figure 14a) (Ng et al., 1995). Another driver, *apterous (ap-Gal4/Gal80^{ts})*, drives gene expression in the dorsal region of the wing disc, which then specifies the dorsal layer of the adult wing (Figure 14b) (Bejarano et al., 2008; Milán & Cohen, 2003).

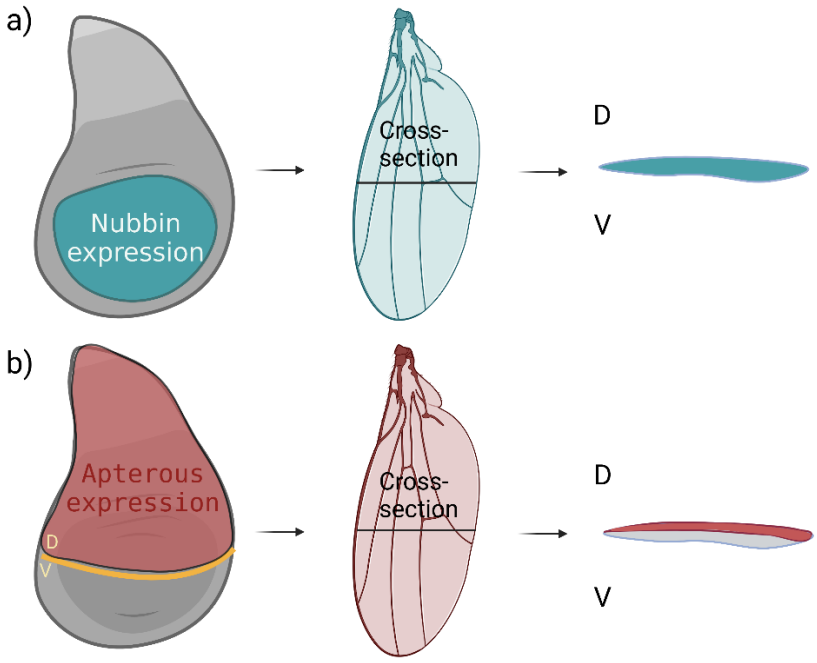

Figure 14. *Nub* and *ap* drivers controlled expression in a wing. a) *Nub* driver leads to the expression in the whole wing (pouch) part of the wing disc and pupal wing, which then affects dorsal and ventral (D and V) layers of the adult wing. b) *Ap* driver leads to the expression in the dorsal part of the wing disc and pupal wing, which then affects only the dorsal (D) layer of the adult wing. *Based on Ruiz-Losada et al., 2018. The figure was created with BioRender.com.*

3.1.2 Fly stocks

Fly lines containing candidate gene, dominant-negative (dn) mutated gene (disrupts the activity of the wild-type gene) or dsRNA (for RNAi-mediated knockdown) under the *UAS* enhancer element were obtained from Stock Centres, including Bloomington Drosophila Stock Center and Vienna Drosophila Resource Center, or previously provided to Prof.

Shimmi's research group. The full list can be found in Table 1. The primary function of each gene for which RNAi or overexpression stock was obtained, is discussed in the literature review.

Table 1. The names of genes that were overexpressed or silenced under UAS control.

Gene name	Knockdown (KD) or Overexpression (OE)	Stock number in the Stock Centre
Ninein	KD	B62414
Brk	OE	-
Kat60	OE	B64117
hTau	OE	B64389
LanA	KD	B28071
Rhea	KD	B39648
Rhea(2)	KD	B32999
αSpec	KD	B42801
βSpec	KD	B30533
Dubai	KD	V28960
Dubai (2)	KD	V330402
Miro	KD	V106683
Shot	KD	B28336
Ndg	KD	B62902
Nrt	KD	B28742
Nrt (2)	OE	B29879

Polo	KD	B33042
Klp10A	KD	B33963
Asl	KD	B35039
AurA	KD	B35763
Cnn	KD	B57149
Sak	KD	B57221
Plp	KD	B65231
Grip84	KD	B33548
Pav	KD	B42573
Scra	KD	B53358
Scra (2)	OE	B51348
Arp10	KD	B64570
Arp3	KD	B53972
Arp1	KD	B67932
Arpc5	KD	B63621
Arpc1	KD	B31246
Arpc1 (2)	OE	B26692
Arp5	KD	B33009
Arp6	KD	B65155
Arp53D	KD	B44580
Arp8	KD	B31202

Arp2	KD	B27705
CG14763	KD	B64649
Moe	KD	B33936
Moe (2)	OE	B31776
Dia	OE	-
Dia (2)	KD	B33424
Dia (3)	KD	B80437
Zip	OE (dn)	-
Zip (2)	KD	B38259
Zip (3)	KD	V7819
Pbl	KD	B28343
RhoGEF2	KD	B34643
RhoGEF2 (2)	OE	B9386
Sqh	KD	V109495
Sqh (2)	KD	V7916
Sqh EE (3)	OE	B64411
Rok	KD	-
Rok (2)	OE	B6671
Tsr	KD	B65055
Tsr (2)	KD	B38226
Ena	KD	B39034

Ena (2)	OE	B58731
Frl	KD	B32447
Sn	KD	B42615
Epsin	KD	B58130
Fim	KD	B33977
Cpa	KD	B41685
Cpb	KD	B41952
LanB1	KD	B42616
LanB2	KD	B55388
LanB2 (2)	KD	B62002
Sdb	KD	B57820
Sdb (2)	KD	B61925
FasIII	KD	B77396
Delta	KD	V109491KK
Notch	KD	V100002KK
Sep1	KD	B27709
Pnut	KD	B65157
Par-1	KD	B32410
Sas-4	KD	B35049
Cep135	KD	B65357
Ana1	KD	B61867

Dlp	KD	B34089
Dally	KD	B33952
Sfl	KD	B34601

Note. The stock numbers that start with «B» were obtained from Bloomington Drosophila Stock Center, and the stock numbers that begin with «V» were obtained from Vienna Drosophila Resource Center. The stocks with no number were obtained from independent sources.

3.1.3 RNAi and UAS overexpression screening

The fly lines containing gene construct under UAS control were crossed with driver lines containing *nub-Gal4/Gal80^{ts}* or *ap-Gal4/Gal80^{ts}*. The crosses of driver lines with *yw* (wild-type) flies were used as the control. The controls were processed in the same conditions as knockdown or overexpression crosses. Most of the crosses were made with *nub-Gal4/Gal80^{ts}*, which regulate the expression in the whole wing. Virgin female flies were collected from driver lines and crossed with male flies from UAS-RNAi or UAS lines. 2-3 replicas were made for each cross with around 20 female and 10 male flies. The crosses were kept at room temperature (RT) (20-22°C) and transferred to the new vial with fly food (culture medium) every 3-4 days. In each vial, flies left a large number of embryos, and vials were kept in RT till the appearance of third-instar larvae on the walls of a vial (Figure 15b). To induce GAL4-UAS activation and GAL80^{ts} inhibition in F1 progeny of the flies, vials were transferred to a 29°C incubator. For RNAi screening, vials were transferred to 29°C for 16 hours. After 16 hours, they were taken to RT, and white pupae (pupae that only started larval-pupal transition) were collected to the new vials, which then again were transferred to a 29°C incubator till the hatching of adult flies. As sufficient knockdown of genes takes a longer time than overexpression, the protocol for overexpression screening was slightly different: vials were kept in RT till the appearance of white pupae, which were then collected to the new vials and put at 29°C until the adult flies emerge. Adult flies were collected to the Eppendorf tubes with 96% ethanol for tissue fixation. When enough flies for statistical analysis were collected (10+ females and 10+ males), they were washed with Phosphate-buffered saline (PBS) 3 times to eliminate residues. The wings were dissected with forceps and mounted in 75% glycerol on glass slides (Figure 15a).

The wings were analyzed under the Olympus light microscope with a 4x objective. The area of the wings was measured with ImageJ software.

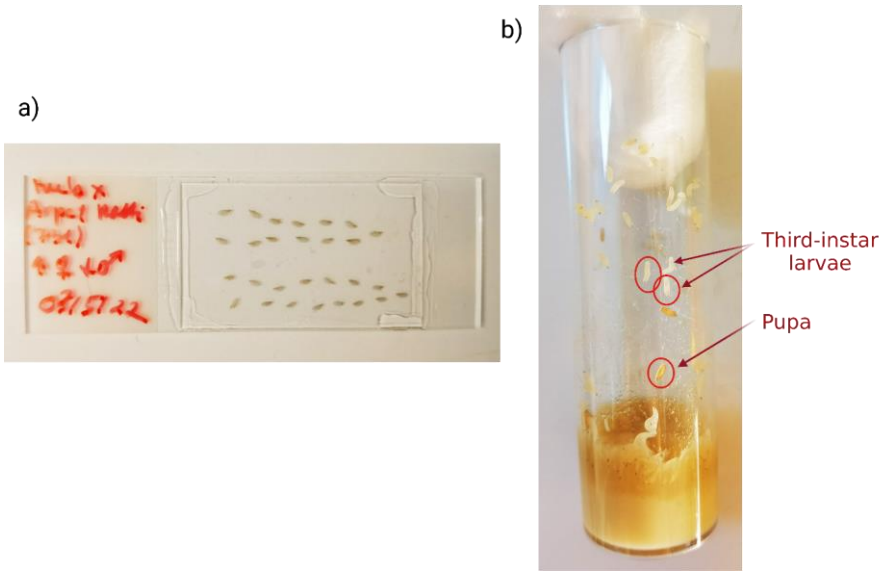

Figure 15. a) Example of a final slide with mounted wings. b) Vial with many third-instar larvae and few pupae.

3.2 RESULTS AND DISCUSSION

The phenotypes of adult wings were analyzed, and the resulting changes were observed in the following categories: abnormal wing size; abnormal vein patterning and size; formation of blisters – space between the dorsal and ventral layers of the wing and improper adhesion; formation of excessive bristles (trichomes wing hairs); incomplete spreading out of wings or failure to spread out; failure to hatch from the pupae; the appearance of melanotic tumours. Many of the screened genotypes did not have any noticeable phenotypic changes, several had moderate mutations, and several had drastic phenotypes.

3.2.1 Variations in wing size

The notable variations were observed and statistically analyzed for wing size (Figures 16 to 21). Wing areas were grouped separately based on the sex, used driver, and knockdown or overexpression screening. The crosses that resulted in significant wing area change and/or morphological deviations were highlighted with stars in Figures 16 to 21. All the controls were wild-type lines (*yw*) crossed with driver lines and stored under the same conditions as the screened crosses. Most crosses were performed with the whole-wing expression driver *nub* because it allows an overall assessment of gene function in the wing. A few crosses were done with *ap* driver to evaluate if expression in one layer may affect the phenotype: rescue it or, contrary, lead to the appearance of more drastic aberrations. The choice of candidates that were crossed with the *ap* driver was based on the observation of severe phenotype in the

same candidate crossed with the *nub* driver, or based on the vital function of a gene in tissue morphogenesis.

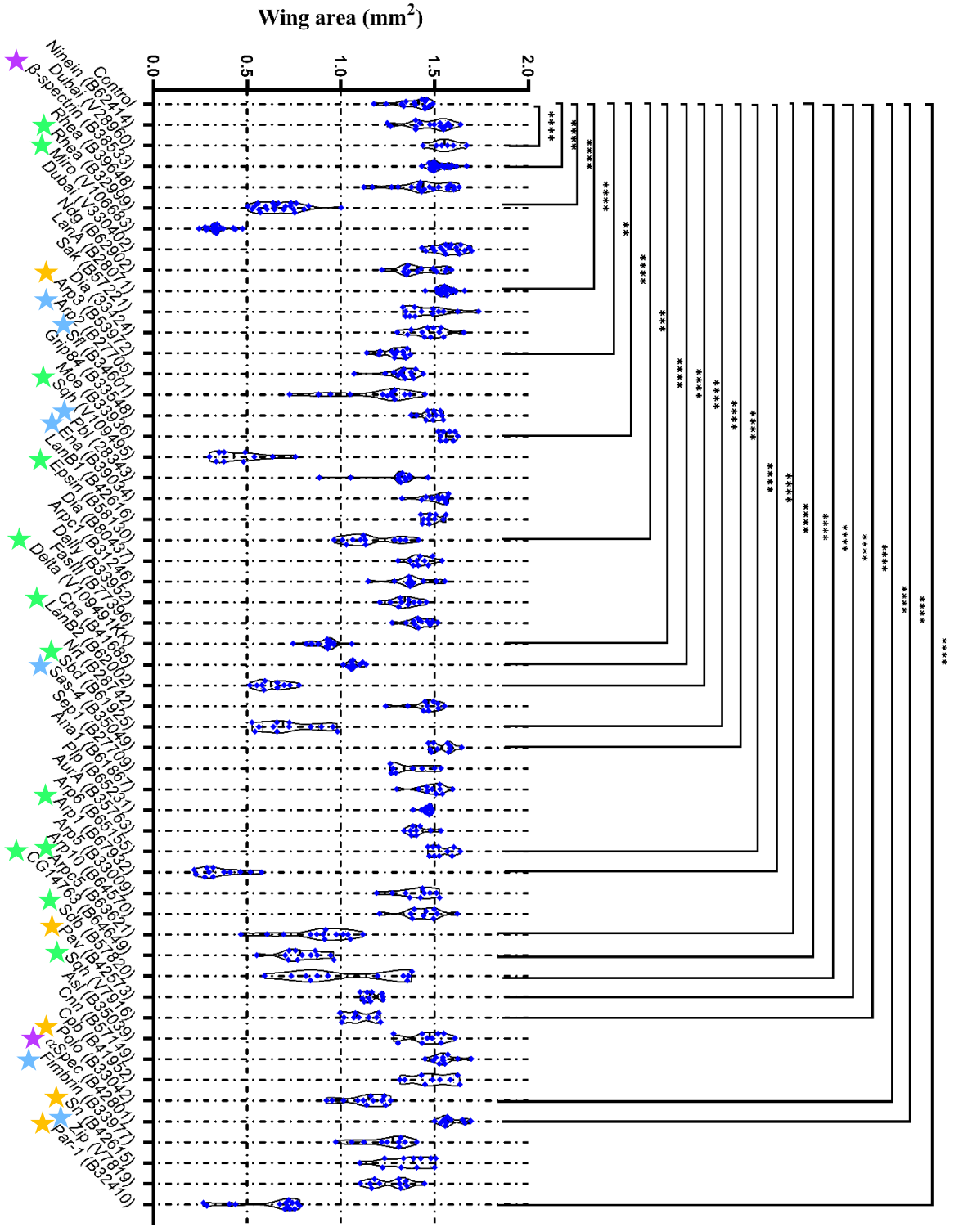

Figure 16. Wing areas (Y-axis) of adult female flies with RNAi (knockdown) of the candidate gene (X-axis) compared to a control. *Nubbin* driver. ** P<0.01,*** P<0.001, **** P<0.0001. N ≥ 10. Purple stars indicate crosses in which both sexes had a statistically significant change in area

with no changes in morphology. Green stars indicate crosses in which both sexes had a statistically significant change in the area and severe changes in morphology. Yellow stars indicate crosses in which both sexes had a statistically significant change in the area and moderate changes in morphology. Blue stars indicate crosses in which both sexes had no significant change in the area and moderate changes in morphology.

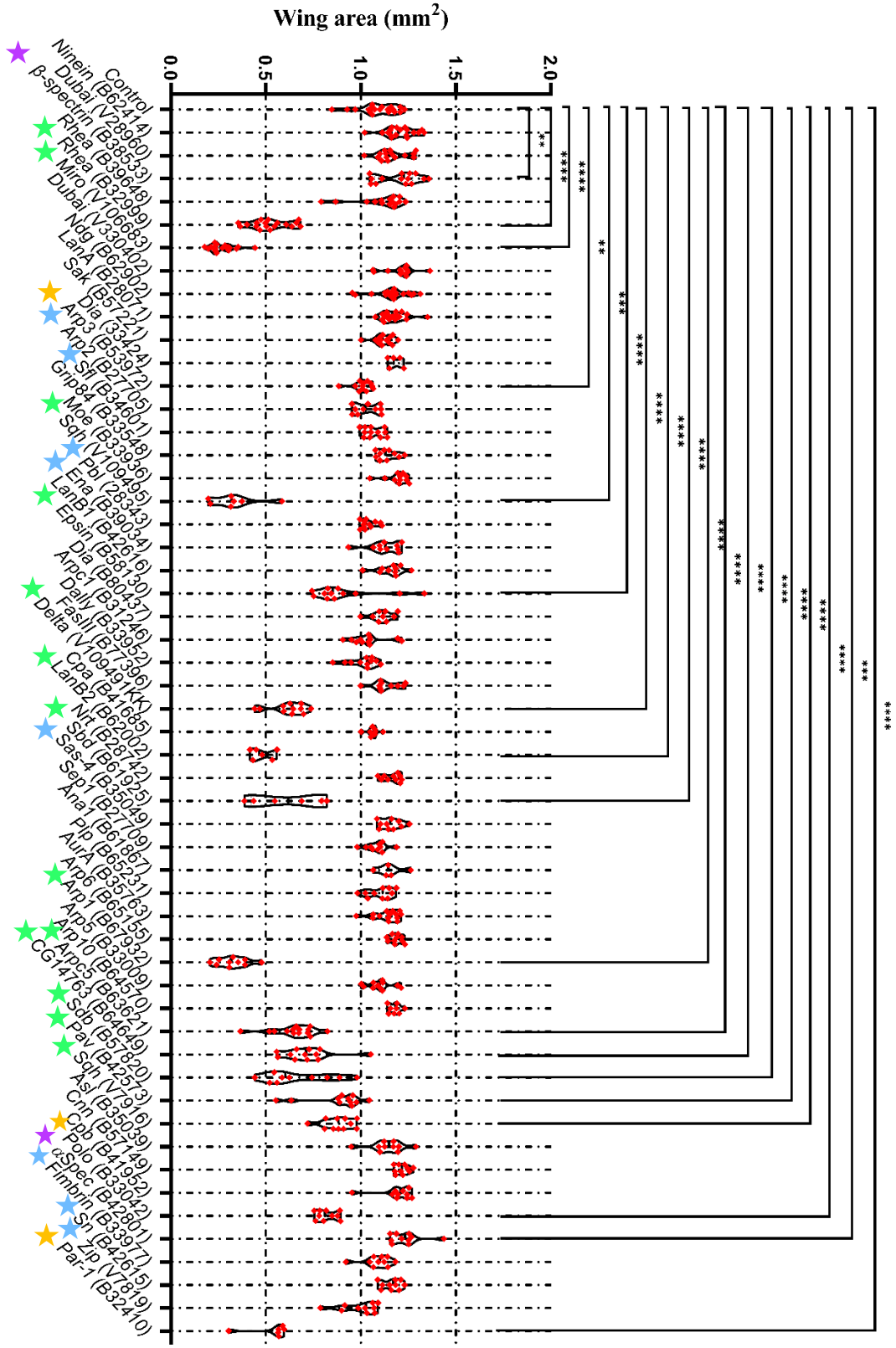

Figure 17. Wing areas (Y-axis) of adult male flies with RNAi (knockdown) of the candidate gene (X-axis) compared to a control. *Nubbin* driver. ** P<0.01,*** P<0.001, **** P<0.0001. N ≥ 10. Purple stars indicate crosses in which both sexes had a statistically significant change in area with no changes in morphology. Green stars indicate crosses in which both sexes had a statistically significant change in the area and severe changes in morphology. Yellow stars indicate crosses in which both sexes had a statistically significant change in the area and moderate changes in morphology. Blue stars indicate crosses in which both sexes had no significant change in the area and moderate changes in morphology.

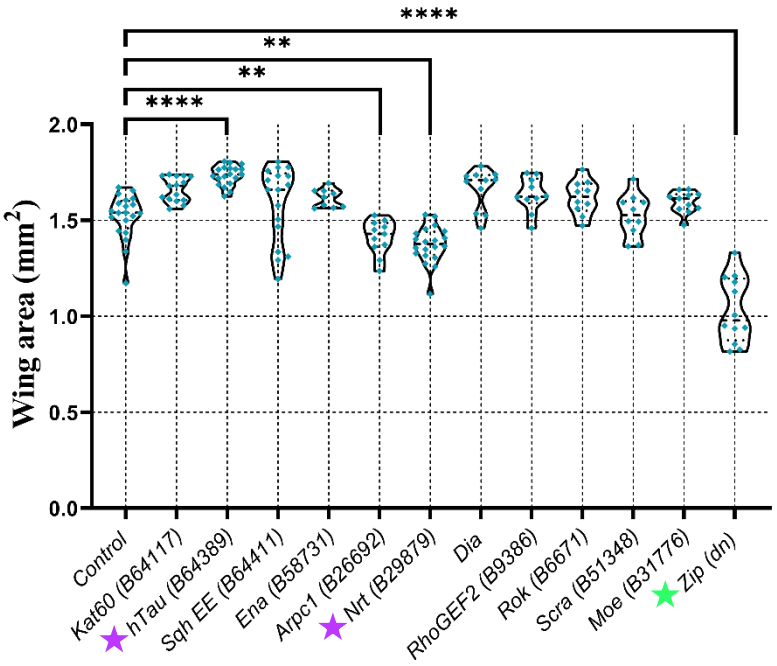

Figure 18. Wing areas (Y-axis) of adult female flies with overexpression of the candidate gene (X-axis) compared to a control. *Nubbin* driver. ** P<0.01, **** P<0.0001. N ≥ 10. Purple stars indicate crosses in which both sexes had a statistically significant change in area with no changes in morphology. Green stars indicate crosses in which both sexes had a statistically significant change in the area and severe changes in morphology.

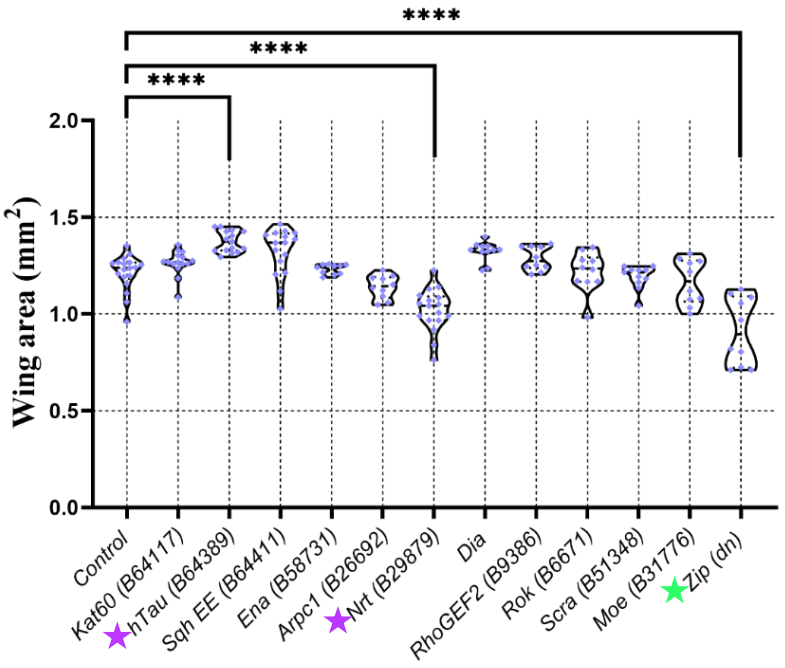

Figure 19. Wing areas (Y-axis) of adult male flies with overexpression of the candidate gene (X-axis) compared to a control. *Nubbin* driver. **** P<0.0001. N ≥ 10. Purple stars indicate crosses in which both sexes had a statistically significant change in area with no changes in morphology. Green stars indicate crosses in which both sexes had a statistically significant change in the area and severe changes in morphology.

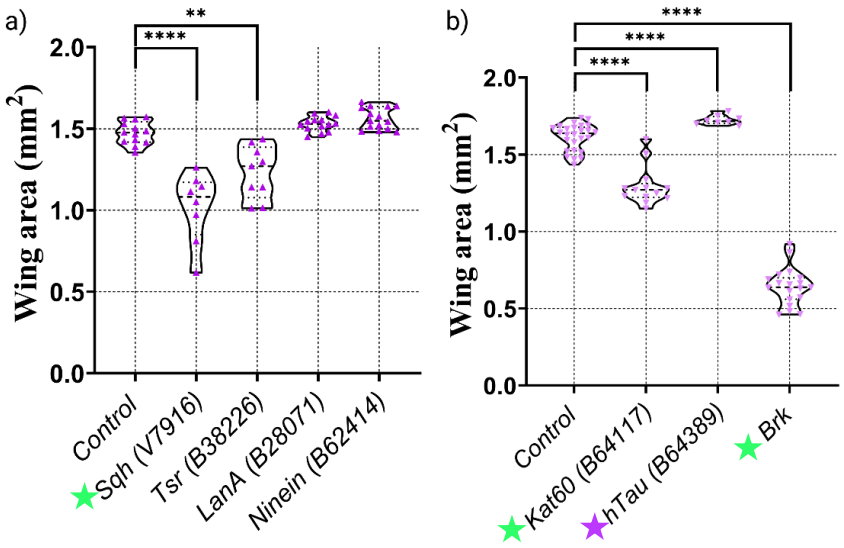

Figure 20. Wing areas (Y-axis) of adult female flies with a) RNAi (knockdown) and b) overexpression of the candidate gene (X-axis) compared to a control. *Apterous* driver. ** P<0.01, **** P<0.0001. N ≥ 10. Purple stars indicate crosses in which both sexes had a statistically significant change in area with no changes in morphology. Green stars indicate crosses in which both sexes had a statistically significant change in the area and severe changes in morphology.

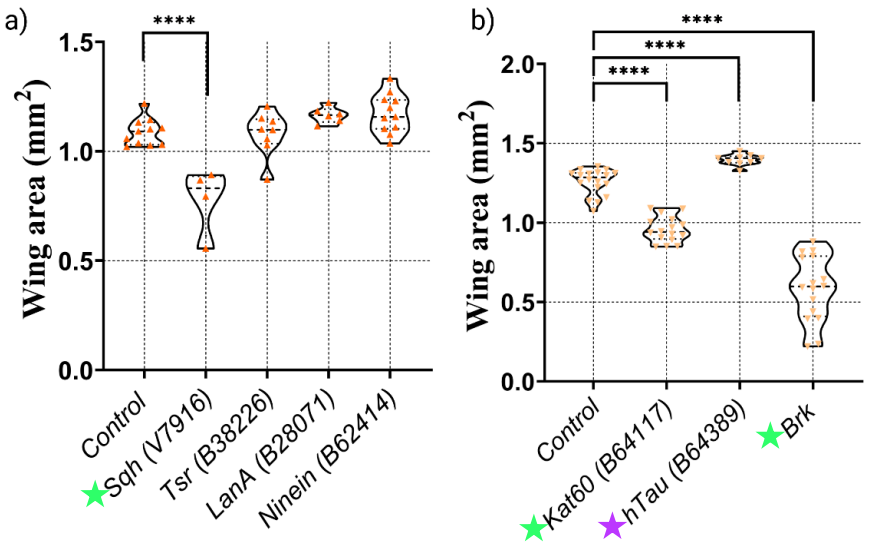

Figure 21. Wing areas (Y-axis) of adult male flies with a) RNAi (knockdown) and b) overexpression of the candidate gene (X-axis) compared to a control. *Apterous* driver. ** P<0.01, **** P<0.0001. N ≥ 10. Purple stars indicate crosses in which both sexes had a statistically significant change in area with no changes in morphology. Green stars indicate crosses in which both sexes had a statistically significant change in the area and severe changes in morphology.

As shown in Figures 16-21, the most drastic changes in the wing area were coupled with the severe changes in wing morphology. While in some cases, moderate defects in wing appearance were correlated with the change in wing size, in most instances, they did not result in a change in the size. In most examples, a considerable difference in wing area was due to the reduction in wing size. The opposite event, an increase in wing area, was observed only in a few cases, as will be discussed later.

The substantial variations in wing size may have several explanations. Some part of this variation is due to the difference in body size and epistatic effects (Gilchrist & Partridge, 2001). A more considerable variation of wing sizes was observed in female flies. In some crosses, a statistically significant change in the wing size was observed in females but not males.

The difference in wing areas can also be linked to the function of a candidate gene in a cell and tissue. Many screened genes are directly connected with cell division event, and therefore their deletion can affect the efficiency of this process. Components of actin filaments are also involved in cell shape maintenance. (*Structure and Organization of Actin Filaments - The Cell - NCBI Bookshelf*, n.d.). When complex changes in cell shape occur during later stages of wing development, these genes can play an important role in forming proper tissue shape.

In a few cases, the change in size was the only alteration in a wing phenotype. However, in most screenings, it was coupled with other external deviations. This observation can suggest the multifunctionality of many screened components, the interplay between the candidate gene and signalling transduction pathway, for instance, Hippo, which plays a significant role in cell proliferation and tissue size (Mo et al., 2014), and tight connections between cell division event and other processes in tissue morphogenesis.

An example of a protein involved in both microtubule skeleton organization and Hippo pathway regulation is Par-1 (Doerflinger et al., 2003; Huang et al., 2013). Consistent with a previous study by Huang et al. (2013), the knockdown of Par-1 results in downregulation of the Hippo pathway and drastic contraction of wing tissue (based on the data in Figures 16-17, Figure 22L). Interestingly, during this screening, ectopic crossvein formation was also observed in *par-1* mutants. It may suggest the link between Par-1 and BMP signalling involved in crossveins formation (Blair, 2007) or the connection between antagonized genes in the Hippo pathway and BMP signalling. The overexpression of the cell-cell adhesion component *Nrt* also decreases tissue area (based on the data in Figures 18, 19) (Speicher et al., 1998). It is known that cell adhesion molecules can regulate cell proliferation and apoptosis. Perhaps, *Nrt* overexpression may disrupt these processes.

While the reduction of wing size was quite drastic with some candidates, only a mild increase in tissue size was observed in several crosses. A more considerable variation was again observed in female flies. However, two knockdown genotypes with *nub* driver, *α* and *β spectrins*, resulted in increased wing area in both males and females (based on the data in Figures 16, 17). The αβ-spectrin complex is primarily known to be involved in cell cortex construction (Pesacreta et al., 1989). As the cytoskeleton component, it should act to limit and regulate cell size in a tissue. Therefore, the elevated tissue growth resulting from knockdown of its part indicates the importance of Spectrin in cell size limiting. Spectrin was also recently found as the upstream regulator of the Hippo pathway (Deng et al., 2015).

The enlarged wing tissue was also found in hTau overexpression with both *nub* and *ap* drivers (based on the date in Figures 18-19 (*nub)* and 20-21 (*ap*)). While the loss of Tau results in the decrease of microtubule number (Bolkan & Kretzschmar, 2014), its overexpression may promote microtubule stabilization and MTOC formation to, possibly, speed up the cell division process.

3.2.2 Moderate and severe aberrations in wing phenotypes

Developed phenotypes can be divided into moderate (Figure 22) and severe (Figure 23). Phenotypic abnormalities were observed in crosses with candidates with different functions. These candidates can be combined into several groups that are differentiated in Figures 22 and 23 and include: regulators of actin filaments growth; actomyosin complex regulators; actin-bundling proteins; microtubule-associated motor proteins and their regulators; microtubule assembly regulators and stabilizers; signalling pathways regulators; ECM components and their linker proteins; cytoskeleton crosslinkers.

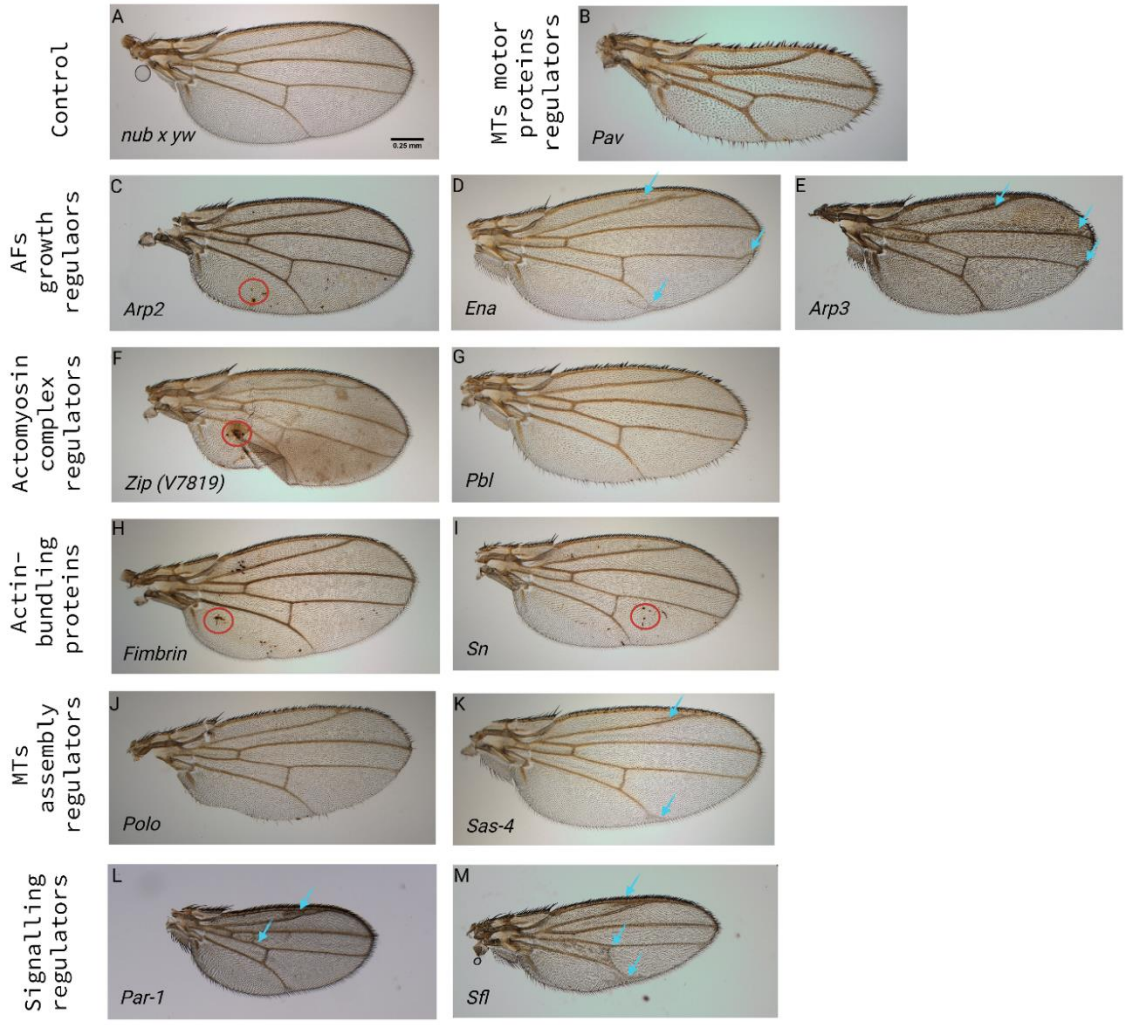

Figure 22. Moderate phenotypes of adult female wings in RNAi screening with *nub* driver. Genes are grouped based on their actions. (A) *Nub* x *yw* was used as a control. All pictures have the same dimensions. Examples of morphological deviations include ectopic veins expression marked with a blue arrow (D, E, K, L, M); hemocytes-based melanotic tumours are marked with red circles (C, F, H, I); over-growth of wing bristles (B, G); aberrations in shape and size (B, F, J, L, M). Numbers are indicated for gene candidates with several stocks. MT – microtubule. Scale bar: 0.25 mm.

While in the case of moderate phenotypic anomalies, flies usually do not have trouble with flying function, severe phenotypes result in partial or complete inability to fly.

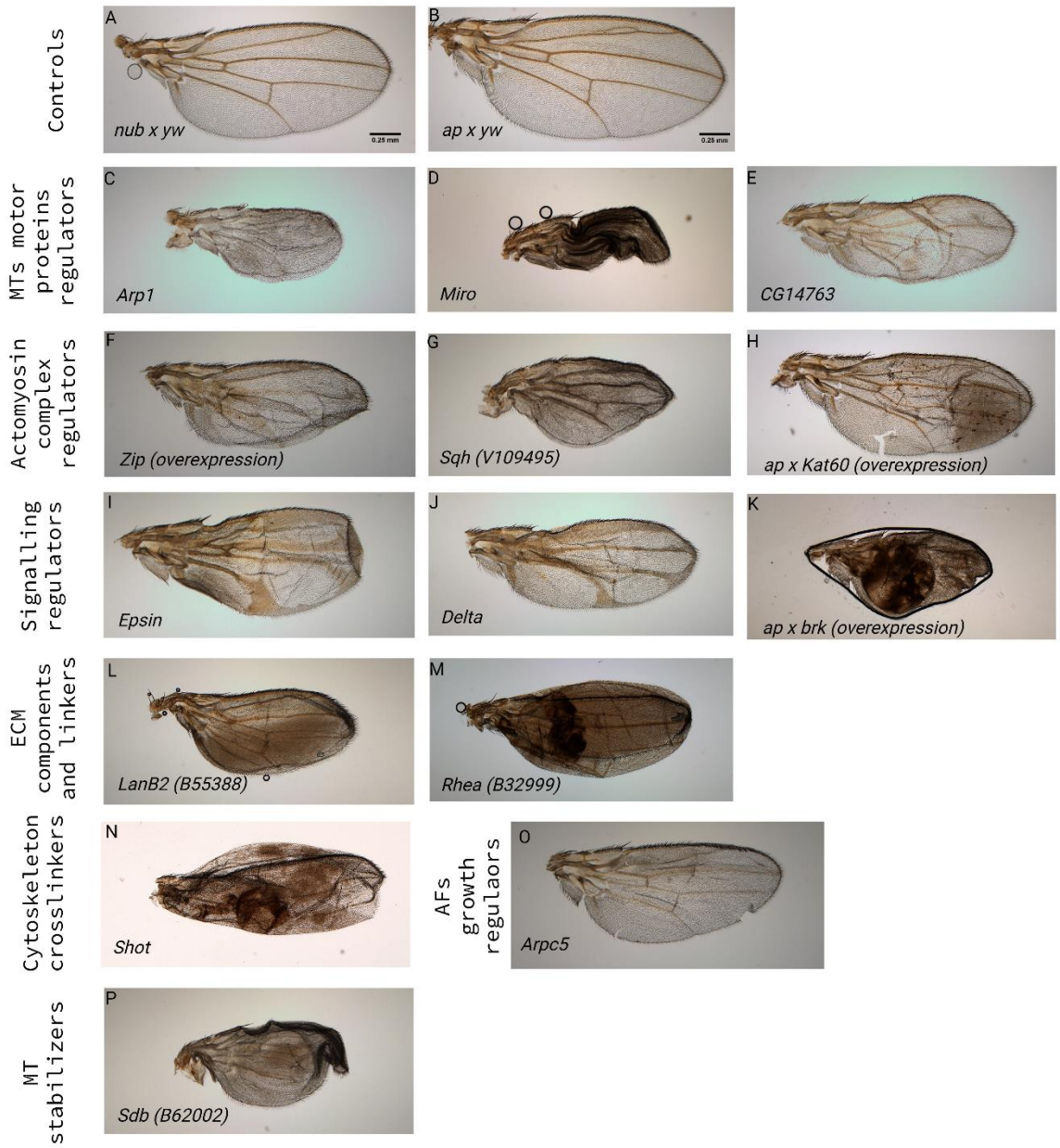

Figure 23. Severe phenotypes of adult female wings in RNAi and overexpression screening with *nub* and *ap* driver. Genes are grouped based on their actions. (A) *Nub* x *yw* and *ap x yw* were used as a control. All pictures have the same dimensions. Flies with *ap* driver and overexpression genotype are marked; all others have *nub* driver and RNAi. Examples of morphological deviations include significant size reduction (C-N, P); formation of blisters (E-F, H-J, O, P); construction of bubble-like wing - no adhesion between layers (C, K, G, L-N); formation of melanotic tumours (H, K, M, N); over-proliferation of vein regions and blurring of borders (I, J); incomplete spreading out or failure to spread out (D, P). Numbers are indicated for candidates with several stocks. MT – microtubule. Scale bar: 0.25 mm.

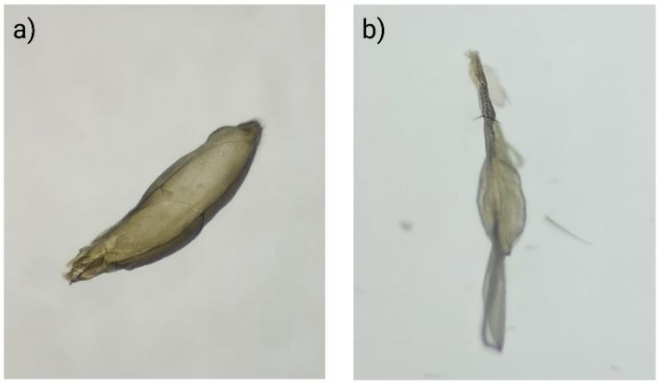

Figure 24. The side profile of a wing with a) bubble-like structure and b) blister.

Most of the phenotypes in Figures 22-23 have a combination of several defects. The pictures show the intermediate phenotype of wings in the crosses. However, some variation of phenotypes with more or less pronounced effects of RNAi or overexpression was always observed. This variation might result from many internal and external factors. While the approximate time points for screening protocol are the same, it is very complicated to predict precisely how long the white pupa stage lasts, account for the transferring time of white pupae to the new vials, and slight temperature differences inside an incubator, which may affect GAL4/UAS/GAL80^{ts} system. Moreover, even with homozygous stocks, epigenetic variations may influence the experiment and level of the *UAS* activation. This is one of the limitations of the GAL4/UAS/GAL80^{ts} system for a screening procedure because even with the consistent results, variation among them may be quite significant.

For a number of candidate genes, several stocks were used. They were ordered from different stock centres or had the *UAS-gene* construct on different chromosomes. Interestingly, quite drastic differences might be recognized in crosses with varying stocks of one gene. For instance, the cross of *nub* with *rhea* (B39648) (See Appendix) did not have any significant changes in phenotype, while *nub* x *rhea* (B32999) (Figure 23M) had bubble-like wings and melanotic tumours. The *nub* x *sqh* (V7916) (See Appendix) cross had an incomplete attachment of dorsal and ventral layers of a wing, while *nub* x *sqh* (V109495) (Figure 23G) resulted in the failure of a wing to spread out or bubble-like wing structure. A similar case was observed for *nub* x *zip* crosses: while *nub* x *zip* (B38259) (See Appendix) did not have any deviations, *nub* x *zip* (V7819) (Figure 22F) had melanotic tumours and blisters. The overexpression of the dominant-negative *zip* mutant resulted in the most substantial phenotype with longer blisters, melanotic tumours and ectopic veins emergence (Figure 23F). This is also a limitation of this screening because, possibly, the ordered stocks had more or less successful integration of the RNAi construct into the genome.

Some of the screened gene mutations resulted in the inability of the flies to emerge from the puparium. Thus, *nub* x *Notch*, *nub* x *tsr (Cofilin)* and *ap* x *tsr (Cofilin)* (B65055), *ap* x *rhea* and *ap* x *shot* all had puparium from which adult flies couldn't emerge. This observation suggests that gene knockdown, even on the scale of a wing, may affect the viability of a fly.

The crosses with both nub and ap drivers gave identical phenotypes in most cases. However, in the case of *Kat60* overexpression, the cross with *nub* driver did not have any phenotype aberrations (See Appendix), while the cross with *ap* driver wings had blisters and melanotic tumours (Figure 23H). Therefore, the connection of different drivers with separate layers or regions of a wing tissue allows for determining the gene function and regulation more precisely.

3.2.3 Wing veins in tissue morphogenesis

Knockdown or overexpression of many studied genes resulted in the ectopic expression of veins, the lack of veins or overexpression, and the indefinite veins' boundaries. Ectopic vein formation can have several underlying hypotheses. As vein cells are the only living cells in the adult wing, their development can be modulated by components specifically involved in cell migration or proliferation. For instance, ectopic vein genesis was observed in AFs growth regulator mutants *ena* and *Arp3* (Figure 22 D, E). Ena protein is involved in cell migration and actin polymerization, while Arp3 is mainly involved in actin polymerization (*Structure and Organization of Actin Filaments - The Cell - NCBI Bookshelf*, n.d.; Tucker et al., 2011).

Vein formation is regulated by several signalling pathways, as was discussed in the literature review (for the vein names and locations, please look at Figure 8, page 18). Early proveins formation is regulated by Egfr and BMP networks (Blair, 2007). Egfr and BMP (Dpp) signalling regulate longitudinal veins maintenance in pupal stages, while crossveins development is controlled by BMP and Wnt pathways. Wnt ligand Wg is also expressed in the distal marginal vein (Blair, 2007). If candidate genes are engaged in regulating the signalling pathways, their mutants may disrupt vein patterning. For instance, Sfl controls Wg diffusion (Lin & Perrimon, 1999). The *sfl* mutant has ectopic crossveins and a wider distal marginal vein (Figure 22M). Some *sfl* mutant wings also have small bristles and abnormal shapes. It may be due to the significant multifunctionality of Wg in tissue morphogenesis.

Notch signalling plays a vital role in refining the boundaries between the vein and intervein regions of a wing (Blair, 2007). The resulting phenotype of the *Delta* (Notch ligand) mutant

that has extensive veins with indefinite borders is consistent with this function (Figure 23J). *Epsin* mutant has similar vein patterning (Figure 23I). Epsin is the positive regulator of Notch and BMP signalling pathways (Langridge & Struhl, 2017; Vanlandingham et al., 2013). *Epsin* and *Delta* mutants with abnormal vein patterning also have reduced tissue size and blisters – space between dorsal and ventral layers.

Expansion and flattening of tissue in fly pupal and adult stages are partly mediated by vein cells and hemolymph (blood analogue in insects) pressure inside vein regions (Matamoro-Vidal et al., 2015). Moreover, during the pupal stage, the veins act as signalling centres. Dpp, a major regulator of wing growth, is produced by the vein cells in pupae. Improper vein formation may affect these processes, while aberrations in Dpp signalling may have a drastic outcome. Thus, overexpression mutant of Dpp regulator *brk* had no vein formation, bubble-like wing and melanotic tumour (Figure 23K) (Campbell & Tomlinson, 1999).

3.2.4 Motor proteins and cellular transport in tissue morphogenesis

Interestingly, the knockdown of candidate genes involved in motor proteins' regulation or components of motor proteins resulted in harsh effects. It is specifically true for microtubule-associated motor proteins Dynein and Kinesin. Several mutants include *Arp1,* which activates Dynein, *CG14763,* which enables dynein binding, *Miro,* which mediates both dynein and kinesin binding, and *pav,* which belongs to the Kinesin family. They all had pronounced phenotypical changes, mainly the formation of blisters (Figure 23C-E) (*FlyBase Homepage*, n.d.). *Miro* mutant wing failed to spread out (Figure 23D). Pav is also involved in regulating Wnt signalling, and its mutant did not have blisters formation. However, *pav* mutant had longer bristles, abnormal shape and vein overexpression (Figure 22B). The appearance of blisters in several mutants may be linked to the role of motor proteins in cell polarity (Januschke et al., 2002). Polarity is specifically crucial for the adhesion of epithelial cells, and its disruption may influence the sticking of wing cells or aberrations in development.

The ability of cells to exchange their neighbours (cell intercalation) is the key feature of epithelial tissue undergoing morphogenesis (Tetley & Mao, 2018). It performs an essential function in the pupal stage (Matamoro-Vidal et al., 2015). Actomyosin complex is vital for cell intercalation. Myosin motor protein binding and activity are regulated by Sqh, Zip and Kat60 proteins (*FlyBase Homepage*, n.d.). Their mutants have improper layer adhesion (Figure 22F, Figure 23F-H). This outcome could be due to the improper alignment and connection of the neighbouring cells in a tissue.

3.2.5 Hemocytes and melanotic tumours

As can be observed in figures 22 to 23, many mutants had brown-red dots and circular structures either on the surface of a wing (Figure 22C, F, H) or between the layers (Figure 23H, K, M, N). It was recently suggested that such patterns are melanotic tumours, which appear due to the incorrect functioning or leakage of hemocytes (hemolymph cells) from veins (Kiger et al., 2001). Imprecise vein diffusion and formation may result in vein leakage and hemocyte propagation. At the same time, hemocytes persist between wing layers after fly hatches. They phagocytose apoptotic epithelial cells and produce ECM components needed to bind dorsal and ventral sheets. The RNAi interference of several candidate genes involved in AFs growth regulation and bundling, including Arp2, Arpc5, sn and Fim, possibly leads to the improper migration or proliferation of hemocytes and the appearance of melanotic tumours (Figure 22C, H, I; Figure 23O). However, the exact mechanisms by which they could control hemocyte spreading and proliferation are unknown.

3.2.6 ECM and intercellular cables in wing adhesion

Reorganization of cellular shape during pupariation is associated with cytoskeleton remodelling and adhesive properties of the ECM structure – basement membrane (Kozyrina et al., 2020). The BM components also mediate proper folding of the wing epithelium and establish contact between wing surfaces. Integrin mediates the communication of ECM and cells. ECM component LanB2 and integrin-binding rhea (Talin) knockdowns significantly reduce tissue size and bubble-like wing structure (Figure 23 L, M).

During the pupal stage, the adhesion of wing layers is directed by intercellular cables of epithelial cells (Sun et al., 2021). These cables include ECM components, cytoskeleton components (microtubules and AFs), Septins, Spectraplakin Shot, Patronin, and Sdb. Contrary to the study by Sun et al. (2021), the knockdown of *Sep1,* and *pnut* genes coding for Septins did not result in phenotypic changes (See Appendix). *Shot* (Figure 22), *Sdb* (Figure 22N, P), and *Patronin* (not shown in this study) mutants all lacked wing layers connection.

3.2.7 The complex basis of wing morphogenesis

The results of the screening procedure suggest the complex roles of many cytoskeletal and ECM components, as well as signal transduction regulators in wing morphogenesis. Many of them have multiple roles in processes of vein regions formation, wing layers adhesion, hemocyte regulation, cell shape, communication, and proliferation control, tissue polarity organization, and many other functions. While having many limitations, for instance, the

difference in expression, substantial phenotype variability, imprecise conditions control and possible influence on the viability of flies, spatially and temporally controlled GAL4/UAS/GAL80^{ts} systems allows to elucidate the important processes in epithelial tissue morphogenesis during specific developmental stages, analyze the candidate genes function in these processes, and enlighten novel hypothesis about functions of these genes.

While the precise mechanisms of gene action can not be grasped from the analysis of phenotype aberrations, it is still possible to estimate gene functions and elucidate novel hypothetical gene actions on the scale of epithelial tissue. The preliminary determination of essential candidates based on phenotype screening can later eliminate the laborious processing of non-essential components. The best candidates can be subsequently studied with live imaging, where the precise cellular mechanisms and interactions of a candidate can be followed in time with immunostaining in certain developmental stages, like larval and pupal. Different time protocols can be additionally used to set the shorter or longer time frames for gene silencing or overexpression and assess the effects or determine specific time boundaries during which gene expression is important. The morphological changes in adult wing can also be analyzed more precisely with other tools that allow estimation of relative sizes of different wing compartments, vein locations and lengths, amount of bristles. Moreover, spatial regulation allows studying candidates in other tissues to compare the resulting effects and combine functions. Thus, it opens many possibilities for discussion and analysis of various genetic components in developmental processes and tissue architecture.

SUMMARY

By utilizing a spatially and temporally regulated gene screening method with GAL4/UAS/GAL80^{ts} system, it becomes possible to determine important factors in tissue architecture in a stage-specific manner. In this work, this method was specifically used to determine the functions of cytoskeletal, ECM components, and signalling pathway regulators in epithelial tissue morphogenesis in the example of the *Drosophila* wing. While a lot of components did not have any effect on adult wing phenotype, many candidates were determined to be involved in essential cellular processes, including proliferation, migration, intercalation, shape formation, and adhesion, tissue polarity and integrity formation. All these processes are necessary for the building of complex organ structures.

As the result of phenotypic screening and statistical analysis, several important findings were made.

- Wnt signalling regulator Sfl, BMP and Notch signalling regulators Epsin, Delta, and Brk were found to be crucial in the correct wing vein formation and regulation.
- In turn, the veins were found to be essential for the proper alignment of wing layers and adhesion, and mutants with disrupted vein patterns also had aberrations in wing size and adhesion.
- The mutants of microtubule-associated motor proteins and their regulators Arp1, CG14763, Miro, and Pav had significant deviations in wing shape and adhesion, which may be due to the aberrations in cellular polarity that is regulated by motor proteins.
- Myosin motor regulators Sqh, Kat60 and Zip are essential for cell intercalation processes in tissue morphogenesis, and their mutants have abnormal adhesion and size aberrations in wing.
- The relatively novel and not yet broadly discussed appearance of hemocyte-based melanotic tumours was found during the screening in AFs growth and bundling mutants Arp2, Arpc5, Fim and Sn. This may be due to the improper migration or proliferation of hemocytes, or vein leakage in these mutants.
- Basement membrane components and linkers Rhea and LanB2 were found to be critical in epithelial layers adhesion in pupal, and, lately, adult wing, and their mutants had bubble-like wings with no layer adhesion.

APPENDIX. The additional adult wing photos.

Many screened mutants did not have any wing abnormalities and were not considered in the figures, or only one stock out of several was shown. In this appendix, the pictures of the mutants that were discussed in this work, but not shown in the figures, are provided.

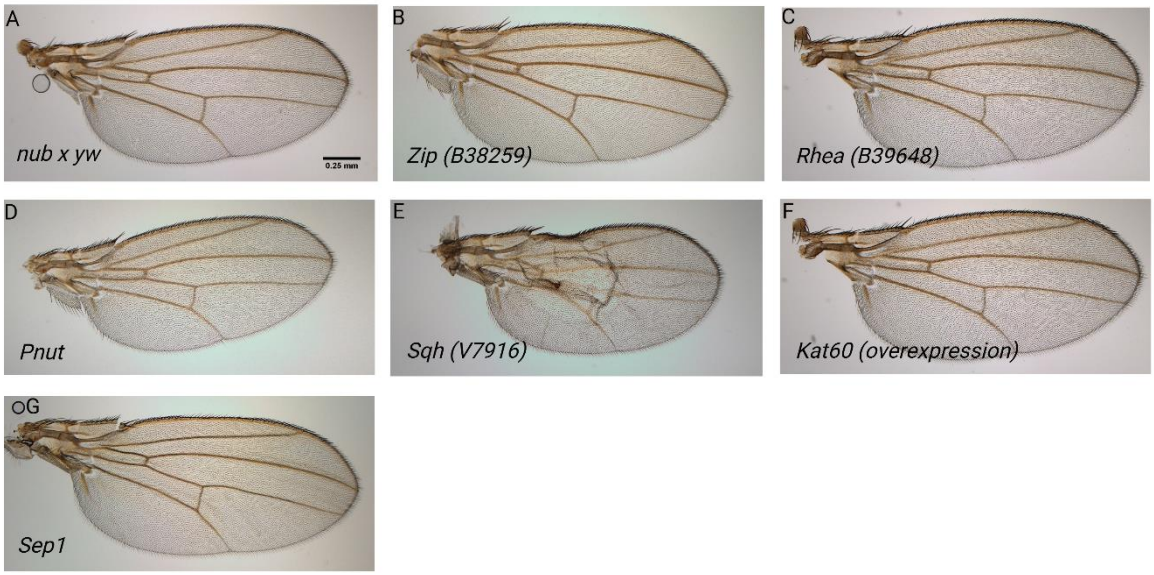

Figure 25. The additional adult wings. As the control, *nub* x *yw* was used. All mutants were done with *nub* driver. Except the mutant that is marked with as "overexpression", the mutants have RNAi of a gene. All pictures have the same dimensions.

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