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Screening for genes that underlie organs' 3D structure formation using fruit fly as a model organism

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Abstract:

Organ formation involves dynamic cell shape changes, adaptations and responses to signaling molecules. Despite numerous studies on the involvement of various cellular structures and signaling pathways in organogenesis, current knowledge regarding the genetic control over dynamic tissue morphogenesis remains limited. The pupal wing of *Drosophila* melanogaster has been shown to include cellular structures and pathways common to the organogenesis of other animals. This project utilized *Drosophila* wings to study specific candidate genes associated with organogenesis and their contributions to final wing development. The screening of genes of interest has been carried out by employing tissue- and stage-specific RNAi-mediated gene knockdown methods. The results show that seven candidate genes, which have been characterized as regulating cellular homeostasis and structures, play important roles in wing morphogenesis and vein formation. Furthermore, most RNAi-mediated wing phenotypes resemble the loss-of-function phenotypes of conserved signaling pathways such as Notch, Wg/Wnt and BMP. Therefore, these observations suggest that candidate genes may regulate conserved signaling pathway during *Drosophila* wing development.

Keywords:

Drosophila melanogaster, RNAi, organogenesis, pupal wing, wing imaginal disc

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

Organite 3D-struktuuri moodustamise aluseks olevate geenide sõeltest, kasutades mudelorganismina äädikakärbest.

Keerukas ja kompleksne organite kujunemine kätkeb dünaamilisi raku kuju muutusi, mis on reguleeritud erinevate signaaliradade poolt. Vaatamata üha täienevatele teadmistele molekulaarsete signaalimehhanismide ja rakuliste struktuuride osalemise kohta organogeneesis, on arusaam kuidas dünaamilised raku kuju muutused on geneetiliselt kontrollitud siiani ebaselged. Mudelorganismi, äädikakärbse (*Drosophila* melanogaster), nuku tiiva arengus osalevad rakulised struktuurid ja molekulaarsed signaalirajad toimivad sarnaselt teiste loomade organogeneesile. Seega oli antud magistritöö eesmärgiks välja selgitada spetsiifilised kandidaatgeenid, mis osalevad äädikakärbse tiiva arengu kujunemisel. Kasutades nii koe- kui arenguetapi spetsiifilist RNAi vahendatud geeni mahasurumise metoodikat, hinnati selle mõju tiibade arengule ja fenotüübiliste tunnuste kujunemisele. Tulemusena leiti seitse kandidaatgeeni, mis osalevad rakulise homöostaasi ja struktuuride regulatsioonis ja mängivad rolli tiiva morfogeneesis ja tiivasoone kujunemisel. Enamik RNAi-vahendatud tiiva fenotüüpe sarnanesid tiibadele, kus on häiritud Notch, Wg/Wnt ja BMP signaalmolekulide funktsioon. Kokkuvõtvalt näitavad tulemused, et analüüsitud kandidaatgeenid osalevad konserveerunud

raku signalisatsiooni masinavärgis, mis aitab reguleerida äädikakärbse tiiva ja tiivasoonte arengut.

Märksõnad:

Äädikakärbes, *Drosophila* melanogaster, RNAi, organogenees, nuku tiib, tiiva imaginaaldisk

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ABBREVIATIONS

BMP – Bone Morphogenic Protein

Dpp – Decapentaplegic

MTOC – Microtubule organising centers

PCM – Pericentriolar materials

PCV – Partial Crossveinless

RNAi – Ribonucleic acid interference

ROS – Reductive oxygen species

TGF-β – Transforming growth factor- β

TNT – Tunnelling Nanotubes

UAS – Upstream Activating Sequence

Introduction

Organ formation is a complex process that involves many gene expression, molecular mechanisms, and general cellular processes. Despite some research uncovering the existence

of signalling processes, the precise mechanisms and genetic controls aren't fully understood, specifically regarding how morphological changes affect organ growth and how it acquires its final 3D shape (Gui et al., 2019; Tran et al., 2024). To study this, *Drosophila melanogaster*, commonly known as the fruit fly, was used as a model organism. The choise of *Drosophila* is ideal due to its ethical acceptability, short life cycle, and the ease of genetic manipulation. Particularly, the wing serves as a significant model to study gene roles during embryogenesis and organogenesis. The wing of the fruit fly transitions from a single layer epithelial sheet to a two-layered 3D structure during the transition from the larval to the pupal/adult stage of development. Moreover, multiple signalling pathways regulate organ formation, and are involved in various processes essential for wing development, such as cell communication, proliferation, differentiation, and apoptosis within the 3D space. Communication through signal transduction is facilitated by specific proteins and cellular structures, including mitochondria and the microtubules. Consequently, the fruit fly, particularly its wing, is recognized as a powerful tool for genetic studies.

Therefore, the aim of the project was to determine the genetic control of mechanisms involved in wing development, with the goal of establishing the basis for understanding the functions of candidate genes and their contribution to tissue morphogenesis. Fruit flies in this work had certain candidate genes knockdown, and accordingly their phenotypes were analysed to understand the effects of gene knockdown on the morphological outputs.

1 Literature Review

1.1 *Drosophila melanogaster* as a model organism:

The fruit fly, *Drosophila melanogaster*, has long been recognized as one of the most valuable and widely used model organisms in scientific research. The initial discovery and molecular characterization of numerous gene products, known to play key roles in human physiology

and medicine, were first described in fruit flies (Beira & Paro, 2016), where genetic analyses of such organisms can give us the clear image of the genes relevant to the organs development and understanding the mechanisms that partake. Really, despite their small size and seemingly simple nature, fruit flies have provided tremendous insights into various biological processes and have contributed significantly to our understanding of disciplines ranging from fundamental genetics to the development of tissues and organs to reveal molecular functions of human disease-associated genes (Kuntz & Eisen, 2014) (Wangler et al., 2015)

The great interest in fruit flies which offers several significant advantages as a model organism across diverse areas of research is due to its ease of cultivation, well-defined genetics, and conservation of fundamental biological processes. *Drosophila* genome is 60% homologous to that of humans, less redundant, and about 75% of the genes responsible for human diseases have homologs in flies (Rubin et al., 2000; Ugur et al., 2016).

Moreover, fruit flies have a relatively short lifespan ranging from 2 to 3 months, allowing researchers to observe multiple generations in a short period. They also reproduce quickly, where (at 25 degree Celsius) it takes 10 days for an embryo to become adult, with each female capable of laying hundreds of eggs in her lifetime (Bier, 2005; Schäfer & Jäckle, 2000).

Additionally, the fruit fly's genetic tools are highly developed, allowing for precise manipulation of gene expression and the ability to introduce specific genetic changes into the fly's genome. The fruit fly's genetic similarity to humans is another significant factor in its use as a research tool. Many genes involved in human diseases have homologs in *Drosophila*, making it possible to study these diseases in a more controlled and cost-effective manner (Mirzoyan et al., 2019; Schäfer & Jäckle, 2000). About 75% of human disease-causing genes have a functional equivalent in the fruit fly genome, and with this similarity it makes fruit flies an excellent model for studying human genetic disorders, such as neurodegenerative diseases, cancer, and cardiovascular disorders to better understand human diseases and develop treatments.

Furthermore, the fruit fly's ease of maintenance and breeding makes it an attractive choice for researchers. Flies are inexpensive to maintain and can be easily kept in controlled environments, reducing the costs associated with large-scale animal studies. They can be housed in small vials or bottles and fed with a simple diet consisting of yeast, sugar, and agar.

This low-cost and straightforward maintenance make fruit flies highly accessible for scientific research, even in smaller laboratories as well as to quickly generate and test large numbers of genetic mutations, accelerating the discovery process.

1.2 Life cycle of the *Drosophila melanogaster*:

As mentioned earlier, one of the special characteristics of these small organisms is their short life cycle, a feature that allows researchers to rapidly generate progenies for the intended purposes. Not only one can have progenies in a short amount of time, but also have a large number of intended progenies (Ashburner 1989). The duration of the development of the *D. melanogaster* from a fertilized egg to adult takes only 10 days on average, provided the environmental degree is 25 degree Celsius.

Provided that the temperature is 25 Celsius, here are the four developmental stages:

1.2.1 Embryogenesis:

The process of oocyte fertilization initiates embryogenesis. Female flies use specialized organs called seminal receptacles and spermathecae to store their sperm for up to two weeks after mating (Lefevre and Jonsson 1962). Sperm storage is supposed to lower ecological costs related to multiple matings and enable the synchronization of ovulation with sperm release (Wolfner 2003). Females typically wait a few days to mate again after mating, but multiple males' sperm can be stored at one time. Hence, sperm preference and competition have been noted based on the genetics of both the male and female. The process of fertilization doesn't start until the egg is prepared for laying. When mature eggs exit the ovaries, they pass through the oviduct, which a portion of the sperm storage is. One or more sperm enter the egg as it travels through the oviduct through the micropyle, a tiny, anterior aperture in the chorion. It's interesting to note that during fertilization, sperm fully penetrates the egg in flies instead of undergoing membrane fusion, and the sperm plasma membrane then breaks down in the egg's cytosol. Furthermore, fertilization is limited to the area that will develop into the developing embryo's anterior pole. The embryogenesis period lasts approximately 24 hours (Allocca et al., 2018). In this short time the embryo undergoes a series of nuclear divisions without cell division, creating a syncytium, after which the embryo undergoes gastrulation, a process in which the embryo is subdivided into segments along its anteroposterior axis. The embryo hatches out of the eggshell to become a larva (Alberts et al., 2002).

1.2.2 Larval stage:

The larvae progress through three stages. The first instar for 24 hours, the second instar for another 24 hours, and the third instar for 48 hours. During this time, the larvae eat and grow, and small groups of cells remain undifferentiated, forming structures called imaginal discs. These groups of cells will give rise to most of the structures of the adult body (Allocca et al., 2018). The larvae also undergo a process called molting, in which they shed their exoskeleton and grow a new one. The larvae grow rapidly during this stage, and their size and shape can vary depending on the availability of food and other environmental factors.

The first instar larva is the stage between hatching and the first molt, lasting one day. It is characterized by limited growth, feeding behavior, and the presence of jaw hooks for burrowing and feeding. The larva has a pair of spiracles for tracheal breathing and is actively feeding to support growth and development (H. -M. Li et al., 2008).

The second instar larva is the stage between the first and second molts, lasting another day. It exhibits increased size compared to the first instar and continues to feed and grow. The larva prepares for further growth and eventual pupation during this stage.

1.2.3 Pupal stage:

After the third larval instar, the larvae find a dry place to pupate. During the pupal stage, the larval body shortens and hardens, and the imaginal discs develop into adult structures. The pupal stage lasts approximately 4 days. The pupae are immobile and do not eat or grow during this stage. The pupal stage is a critical period in the development of *Drosophila melanogaster*, as the imaginal discs undergo a complex process of morphogenesis and differentiation to form the adult structures.

1.2.4 Adult stage:

After the pupal stage, the adult fly emerges. The adult fly has a typical insect anatomy, including compound eyes, three-part bodies (head, thorax, and abdomen), wings, and six jointed legs. The adult fly is capable of flight and can live for several weeks, depending on environmental conditions. The adult fly is also capable of reproduction, with females laying eggs that hatch into larvae and begin the developmental process again.

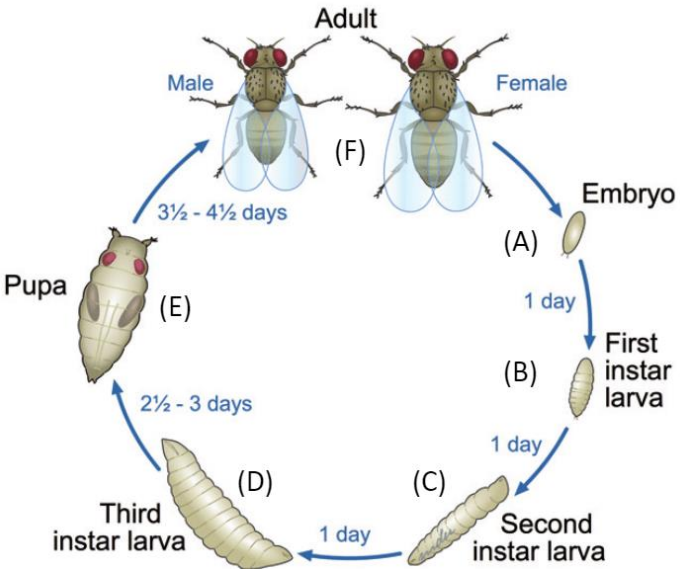

Figure 1. Life cycle of the *Drosophila melanogaster*: Upon fertilization, embryogenesis [(A)] is completed in ~24 hr, followed by three larval stages (termed first [(B)], second [(C)], and third instar [(D)]) with a molting event at each stage transition. The first two instars each last on average 1 day, whereas the third instar typically requires 2 days. Thus, 5 days after fertilization, larval development is complete, and the animals metamorphose within a hard, protective chitinbased pupal case (or puparium) that forms from the outer larval cuticle. Pupariation [(E)] lasts 3-4 days before finally becoming an adult [(F)] . Modified from Ong et al., 2015

1.3 Wing imaginal disc

While many genes have been identified through embryonic mutant screens, the characterization of critical gene function at different developmental stages has substantially benefitted from studies in imaginal discs. These structures are epithelial tissues that develop during the earlier stages of the life cycle in holometabolous insects and ultimately give rise to major adult body parts such as eyes, wings, legs, or genitalia.

Drosophila is a holometabolous insect, meaning that it undergoes complete metamorphosis, practicably upon the pupariation through the differentiation of cells that have formed and divided in the so-called imaginal disc during the larval stages of the life cycle (Tripathi & Irvine, 2022).

Imago is the Latin word for image, and it is used by entomologist to refer to or describe the "image" of the final, developed adult insect body (imaginal body). This disc is a sac-like epithelial structure, giving a disc shape. Given the two arguments, hence its name: Imaginal disc.

The imaginal disc is the forerunner of the organs whose 3D shapes are manifested in the adult stages, like the head, the thorax, and the genitalia (Tripathi & Irvine, 2022; Aldaz & Escudero, 2010), for, again, it's the imaginal disc that consists of a cluster of undifferentiated cells that get specialized and give rise to such organs upon the pupal transformation. Although these cells aren't differentiated before the pupariation, their fate is already assigned during the larval stage.

Of course, it's not solely made up of epithelial cells as there is a small number of neurons and associated glia within the wing disc epithelium, whose functions are avowedly to form sensory organs, sensory bristles in the notum, and definitely to send axons between the epithelium and the basal lamina of the wing disc (Huang et al 1991; Gomez-skarmeta et al 2003; Tripathi & Irvine, 2022).

1.3.1 Wing development stages

1.3.1.1 Embryonic stage

The growth of the wing disc is usually an increase in the number of cells, rather than an increase in the size of the cell. The wing primordia in *Drosophila* originate upon an invagination from the embryonic ectoderm during embryonic development with less number of cells (between 12 and 24) and the disc is merely a two-layered epithelial tissue (Bate & Arias, 1991; Garcia-Bellido & Merriam, 1971; Matamoro-Vidal et al., 2018; Requena et al., 2017). During this stage, the cells aren't differentiated yet, however, regulation by certain genes and morphogens (Wingless and Decapentaplegic) takes place to guide the specification and patterning of the wing disc, leaving the disc with cells that have assigned fates but not differentiated.

1.3.1.2 Larval stage

Upon transitioning into the larval stage, the wing disc starts proceeding the development with 50 primordial cells and, throughout the larval stages up to the pupariation, the wing disc

grows at a rapid rate, increasing from around 50 cells to about 50,000 cells in total in adulthood (Garcia-Bellido & Merriam, 1971; Neto-Silva et al., 2009; Tripathi & Irvine, 2022). In this stage, the cells begin to differentiate in the various cell types, forming, for example, neurons, glia, epithelial cells as well as the muscle precursor cells. Also, larval stage exhibits morphogenetic processes, such as cell shape changes, tissue remodelling, cell rearrangements, that all give the tissue its shape and readiness to differentiate into the final adult wing and notum, which eventually are manifested in the pupal stage.

1.3.1.3 Pupal stage

Upon pupariation, the wing disc begins to undergo a more complex morphogenesis upon a trigger by metamorphosis-stimulant steroid hormone, ecdysone, hereby dictating the beginning of metamorphosis and pupariation (Auerbach, 1935; Waddington, 1939). The phases are referred to as the after pupariation (AP), and are subdivided into three phases: First apposition, inflation, and second apposition (Gui et al., 2019; Matamoro-Vidal et al., 2018).

1.3.1.3.1 First apposition

First apposition takes place right upon pupariation and lasts up to 10 hours. The process is known as eversion, as it exhibits the eversion of the wing layers as it turns inside out, extending the appendages externally (from the body) and revealing the apical surfaces that are on the surface of the fly upon the elongation and flattening of the wing pouch, marking the transformation from a simple, 2D-layer of epithelia into a 3D shape with dorsal and ventral layers of the wing (Aldaz & Escudero, 2010; Fristom & Fristom, 1975; Gui et al., 2019; Tripathi & Irvine, 2022). The attachment of the dorsal and ventral layers is due to the binding of integrin to laminin, and this attachment undergo inflation before the reattachment at the end of the pupal development (Brabant et al., 1996; Sun et al., 2021).

1.3.1.3.2 Inflation stage

Inflation stage proceeds 10 hours AP, and lasts for up to another 10 hours (so, it takes place during the second 10 hours of the whole pupal development period), and during this stage the wings physically separate as a result of the pumping of the hemolymph, and restructuring and reshaping of the wing tissue takes place (Gui et al., 2018, 2019; *The Organization of Drosophila Wing Epithelial Cells after Wing Inflation*, 2012). Most of the cell division takes place in this stage accompanied with changes in cell proliferation and differentiation, regulated by BMP/Dpp signalling pathways. BMP has its role also in maintaining lateral

trafficking to result in a normal tissue growth and vein formation. In this stage, microtubule-based protrusion, known as interplanar bridges, are involved in the separation of two wing epithelia (Tran et al., 2024). They disassemble resulting in the loss of cell-cell contacts between the dorsal and ventral wing layers, a process that is needed for the tissue proliferation between the layers.

1.3.1.3.3 Second apposition

Second apposition occurs 20 hours AP and is the last phase of the pupal development that marks the completion of the morphogenic events. The two wing layers re-appose, forming the final 3D wing shape (Gui et al., 2018). It is found that BMP signalling has it role in the reappositioning through the switch from long-range to short-range signalling, which helps in the final refinement of the wing vein patterning. BMP signalling is also involved in the important vertical communication that is happening between the two layers.

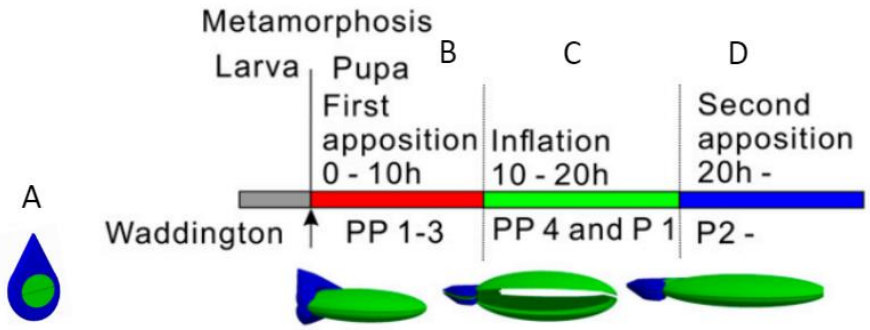

Figure 2. Overview of the wing metamorphosis upon the transitioning from larval to pupal phase. (A) the wing disc is a flat, 2D-layer. (B) *First apposition* (0–10 hours after pupariation): The single-layered wing epithelium everts and forms dorsal and ventral epithelia to become a rudimentary two-layered wing. (C) *Inflation* (10–20 hours after pupariation): The two epithelia physically separate before fusing in the third phase. (D) *Second apposition* (around 20 hours after pupariation): The dorsal and ventral epithelia re-appose and fuse to form the mature wing structure. *Modified form Gui et al., 2019.*

1.3.1.3.4 Adult stage:

In this stage of the life cycle, the adult flies start to eclose from the pupal case and the wings begin to spread out and become usable. The final wing differs between the males and females in terms of size, where the females usually exhibit larger wings. However, the anatomy is same: final adult wing consists of 6 longitudinal veins and 2 cross veins. These veins are

basically ectodermal tubes consisting of thicker cuticle which are secreted by the cells of the veins, rendering the vein a structure that serves as vessels for hemolymph and blood cells (Blair, 2007).

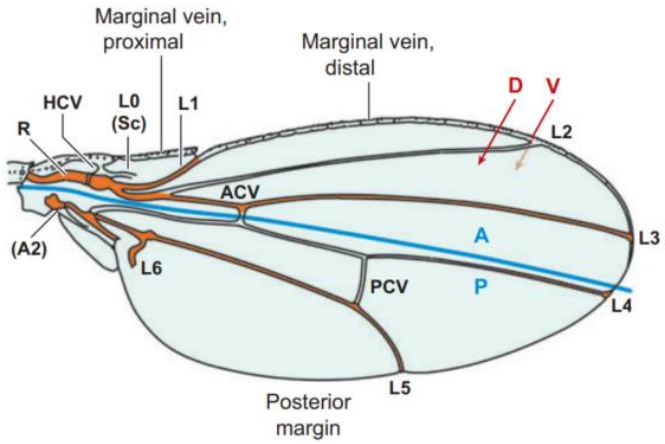

Figure 3. The final, adult wing of *Drosophila*: The blue line is an imaginary line that separates the anterior (A) and posterior (P) compartments. There are five main proximodistally longitudinal veins (L1-L5); two smaller veins (L0 & L6); three crossveins: anterior crossvein (ACV), posterior crossvein (PCV), and humeral crossvein (HCV). *Figure from Blair, 2007.*

1.4 Temperature and growth:

It is of great importance to know, and is a successful practice to consider and control, that the temperature has its effect on the development, be it on the development speed, the number of progenies, the size of the fly, or the organs health (Cavieres et al., 2018; Huang et al., 2020). Generally, the lower the environmental temperature, the longer it takes to achieve adulthood—and vice versa (Kuntz & Eisen, 2014). For example, flies cultured at 18 degrees achieve adulthood at around day 20 after fertilization – which is twice the amount of time it takes compared to when cultured at 25 degrees. This is because metabolic rate decreases at lower temperatures, which suggests that longer development times should be observed at lower temperatures. However, high temperatures doesn't mean a fast development, at least not in a safe and healthy manner (The Relationship between Developmental Temperature and

Longevity in *Drosophila* | Gerontologia | Karger Publishers, n.d.)(Cavieres et al., 2018; Genetics on the Fly: A Primer on the *Drosophila* Model System - PubMed, n.d.; Huang et al., 2020). Shorter development times can be observed at high temperatures, but up to a limit, usually 29Celcius, at which point the heat denaturation of proteins should negatively affect growth and survival rate (S. Y. Chen et al., 2013; Huang et al., 2020).

Temperature can also affect the size. The main way that temperature influences body size is through affecting critical size, which is the developmental stage at which larvae start the hormonal cascade that ends growth and begins metamorphosis. Variations in the impact of temperature on the rates of cell proliferation during trait growth are reflected in the differences in thermal plasticity amongst characteristics; certain traits have lower thermal plasticity in relation to size, while others have higher thermal plasticity in relation to total body size (McDonald et al., 2018; Trotta et al., 2006).

All in all, temperature significantly affects the developmental stage of *Drosophila* melanogaster by influencing the duration of the developmental stages, the time to maturation, and the body size of the adult flies. Lower temperatures increase the amount of time required for D. melanogaster larvae to reach adulthood, while higher temperatures accelerate growth up to a certain limit before negatively affecting growth and survival rate. Additionally, temperature affects the time to maturation of D. melanogaster, with insects growing faster but smaller in size at warmer temperatures and slower but larger in size at cooler temperatures. Finally, temperature affects the developmental stages of D. melanogaster, with temperatures continuously above 29°C being lethal.

1.5 Epithelial cells and animal development

One of the basic units of the animal organ development are the epithelial cells, for they play a key role in the formation of tissue such as the body cavities, tubes, gastrointestinal tract. They also function as a protective barrier of the organs and facilitate the uptake of substances and the transportation of molecules (Z. Chen et al., 2020; Tadeu & Horsley, 2014). These cells undergo differentiation and specialization to form the diverse epithelial organs; moreover, the epithelial cells experience dynamic shape changes that are regulated by the cell-cell communication, which also regulates other processes like cell proliferation, positioning, and apoptosis and which are all essential to the tissue morphogenesis (Gómez-Gálvez et al., 2021; Paluch & Heisenberg, 2009; Tran et al., 2024).

Although there is an understanding and awareness of such phenomena present, the exact mechanisms that accurately regulate such changes aren't fully understood and profiled (Erdemci-Tandogan et al., 2018; Slack, 2008; Slavkin et al., 1984). There are some knowns in the issue: different kinds of cell shape changes include formation of membrane protrusions, meaning that cell shape changes can be the result of dynamic changes in the cell's intrinsic properties like adhesion and cell contractility (Demontis & Dahmann, 2007; Paluch & Heisenberg, 2009; Ramírez-Weber & Kornberg, 1999). However, still, the exact mechanisms need to be depicted to understand the whole development as well as the pathology of some diseases.

1.6 Protrusions importance in cell communication:

Protrusions are essentially dynamic cellular (outward) extensions from the cells surface, and play a role in and enable cell-cell communication, hereby facilitating the cell's interactions and its contribution to organ development (Inaba et al., 2022; Magalhães et al., 2021). They vary in size, shape, and functionality.

They could be tubulin-based microtubules and/or actin-based filaments, such as cytoneme and tunnelling nanotubes (TNTs), which are the constituents of the cell cytoskeleton, and are involved in the trafficking of vesicles and signalling molecules between cells, specifically the transmission of molecules between cells over long distances (Buszczak et al., 2016; Cohen et al., 2010).

1.6.1 Microfilaments and their role in animal development:

Microfilaments are thin protein filaments that are part of the cytoskeleton in eukaryotic cells. They are approximately 5-9 nm in diameter and are made up of two helical, interlaced strands of polymers of actin subunits (globular actin or G-actin, and therefore some scientific papers refer to them as actin filaments) and play crucial roles in various cellular functions (Jiang et al., 2021; Vogl & Guttman, 2018). Each actin subunit has a binding site for adenosine triphosphate (ATP), which is hydrolyzed during polymerization. Microfilaments are polarized, with a fast-growing barbed end (+) and a slow-growing pointed end (-) (Davidson & Wood, 2016).

Microfilaments serve to provide structural support and maintain the shape of the cell, where they form a tough, flexible framework that helps the cell resist deformation and maintain its integrity; they also play a crucial role in cell motility and changes in cell shape in response to stimuli, where, by the virtue of their rapid polymerization and depolymerization, they form

various cell surface projections, such as filopodia, lamellipodia, and stereocilia, which are important for cell migration; and, an important function also, is their involvement in cell division by forming the contractile ring that constricts the cell during cell division, physically separating the two daughter cells (Aryal, 2022; Davidson & Wood, 2016; Vogl & Guttman, 2018). Microfilaments also play a role in endocytosis and exocytosis, which are processes involved in the uptake and release of materials from the cell, respectively. They contribute to the movement and positioning of vesicles and organelles within the cell, facilitating the transport of materials.

The role of microfilaments in the development of *Drosophila* and it's wing is significant and crucial for various processes: is significant, particularly in processes such as cytoplasmic streaming, cell movement, and oocyte maturation, cytoskeleton formation, cell shape determination, and tissue morphogenesis (Guild et al., 2005; Gutzeit, 1986; Neto-Silva et al., 2009; Sullivan & Theurkauf, 1995).

Microfilaments play a significant role in cytoplasmic streaming, a process observed during the last phase of oogenesis in *Drosophila*, where the distribution of F-actin filaments in nurse cells has been studied, revealing thick bundles of microfilaments that span the nurse cell cytoplasm and contribute to cytoplasmic streaming by pressure flow (Gutzeit, 1986). The presence of microfilaments spatial distribution is also observed in the early phases of gastrulation(Callaini, 1989); and, during the oocyte maturation and fertilization of *Drosophila*, where they regulate processes such as homologous chromosome separation, spindle anchorage, and polarity establishment. In addition to the maturation, microfilaments also play a role in oocyte polarity and development and are considered important for localizing determinants that influence body-plan specification in *Drosophila* oocyte.

Furthermore, actin filament bundles play a significant role in shaping cellular extensions, such as wing hairs, into different forms during *Drosophila* wing development and it is thought that this regulation and shaping of actin filament cross-linking and in a bundle shape is essential for the morphogenesis of wing structures in *Drosophila* (Guild et al., 2005). In addition, actin dynamics in the wing of *Drosophila* play a role in planar polarization and hair outgrowth, highlighting the importance of microfilaments in shaping the wing structures.

Microfilaments do interact with microtubules and intermediate filaments to coordinate all of the aforementioned, and other, various processes during *Drosophila* wing development -- nuclear movements, hair morphogenesis, cell mechanics, tissue growth, and contractility--

which are all regulated by actin-binding proteins and adhesion molecules to ensure the proper development and shaping of the wing (Guild et al., 2005; Gutzeit, 1986; N. Kumar et al., 2022; Sullivan & Theurkauf, 1995).

1.6.2 Microtubules:

Microtubules are hollow, cylindrical structures that are part of the cytoskeleton in eukaryotic cells. They play crucial roles in various cellular processes, including cell division, intracellular transport, and cell motility. Their structure consists of α-tubulin and β-tubulin heterodimers that polymerize to form protofilaments, and these constitute the distinct polarity of the microtubule, with a plus end (β-tubulin exposed) and a minus end (α-tubulin exposed) (González et al., 1998; Krishnan et al., 2022). They have different purposes in cells, such their role in intracellular transport such as the transport of various organelles, vesicles, and molecules within wing cells (González et al., 1998) and also contribute to cell polarity in *Drosophila* wing development . They are essential for the elongation and shaping of the highly polarized *Drosophila* bristle cells.

Microtubules are nucleated and organized by Microtubule-Organizing Centers (MTOCs), such as the centrosome found in the center of many animal cells or the basal bodies of cilia and flagella (Hess & Ross, 2017; Knossow et al., 2020). The elongation can occur at both the (+) and (-) ends, but it is significantly more rapid at the (+) end.

The primary MTOCs in *Drosophila* are the centrosomes and are responsible for organizing the mitotic spindle and for the accurate chromosome segregation during cell division providing structural support, cell shape, polarity, and intracellular highways for vesicular transport (Tillery et al., 2018) .

The centrosome is composed of two centrioles surrounded by pericentriolar material (PCM) (Gottardo et al., 2015; Tillery et al., 2018). The PCM contains various proteins, of which is the most essential one --γ-tubulin ring complex (γ-TuRC), which is crucial for microtubule nucleation and organization. Other centrosomal proteins, such as ninein and patronin, also do play essential roles in regulating microtubule nucleation and anchoring (Sanchez & Feldman, 2017).

The γ-TuRC within the centrosome nucleates an array of microtubules in interphase, extending their (+)-ends radially into the cytoplasm towards the cell periphery(Tillery et al.,

2018; Vinopal et al., 2023). This radial array of microtubules serves as tracks for motor proteins to transport cargoes, such as vesicles, within the cell.

1.7 Signalling pathways:

Different pathways regulate the crucial processes of cell proliferation, cell differentiation, cell apoptosis, and gene expression that eventually lead to a successful and normal adult wing. These can also interact with one another to form signaling cascade relevant to the intended processes.

1.7.1 BMP/Dpp pathway:

The BMP/Dpp pathway is a highly conserved and essential pathway required for the patterning and growth of the cells, as it is involved in the regulation of cell differentiation and the relevant morphogenetic processes (Raftery & Umulis, 2012). It is relevant to the TGF- β

Superfamily in mammals, which also has its role in several processes related to organ formation, such as cell fate determination, tissue patterning, and cell differentiation (Hamaratoglu et al., 2014).

BMP involves the roles of certain ligands, like the key component Dpp in *Drosophila*, which is analogous to BMP2/4 in mammals, in addition to relevant receptors to which they bind to, such as the Thickveins (Tkv) and Punt, which are involved in the regulation of the wing development by forming concentration gradients that induce several cell types in diverse spatiotemporal manner that contribute to the formation of adult wing veins (Hamaratoglu et al., 2014; Raftery & Umulis, 2012). Tkv and Punt play a crucial role in facilitating the role of Dpp, where they form a complex, inducing signaling event, such as the phosphorylation of intracellular protein (Mad) resulting in its activation and translocation into the nucleus for the targeting of certain gene expression.

These factors and other BMP inhibitors form a gradient along the dorsal midline of the embryo where their role is to specify different cell fates. The cells respond to the signals based on the levels of the Dpp, and according to this concentrations target genes will be signaled to be actiaved for the specification of the cell fats along the dorsal-ventral axis (Greenfeld et al., 2021; Yan & Wang, 2021).

Therefore, BMP signaling is crucial for the control of the cell fate specification and proliferation. This spatiotemporal control is dependent on certain proteins like the Pent, Ltl,

Dally, and HSPG, which act in a transcriptional feedback-regulated manner, rendering cells actively and accordingly respond to the this morphogen gradient needed for a controlled, accurate patterning (Akiyama et al., 2022; Raftery & Umulis, 2012).

BMP regulation of the wing development isn't just independent of other pathways, as it also is needed for the interaction with other signaling pathways, like Notch, Wnt, and EGF pathways for the establishment of a signaling cascade responsible for the tight and precise regulation of several processes such as apoptosis, differentiation and cell migration (Matsuda & Affolter, 2023; Wang et al., 2016).

1.7.2 Notch signaling:

Notch signaling is another contributor to the wing development through the regulation of successful and proper patterning and growth of wing tissue and veins, as it regulates certain genes (at the dorsal/ventral boundary) that are relevant to the control of cell fate and tissue patterning (Baonza & Garcia-Bellido, 2000; Go et al., 1998; Hing et al., 1994). Notch signalling is thought to be a crucial contributor to normal development of the wing bristles and the wing margin. Important ligands in Notch signaling are the Delta and Serrate, and any defect in the ligands, or the pathway in general, is associated with ectopic wing margins (Baonza & Garcia-Bellido, 2000). These two ligands have an indirect effect on wing development, as in they initially bind to Notch receptor, where from then the regulation of all the important processes become effective: controlling development of undifferentiated cells in wing imaginal disc; regulating the lateral inhibition and induction between neighboring cells, processes that lead to the inhibition and induction of certain genes to depending on when should certain cells adopt certain fates, phenomena that is thought to contribute to the formation of wing margin; and the induction of mitotic activity (Bocci et al., 2020; Y. Chen et al., 2023; Go et al., 1998).

1.7.3 Hippo pathway:

Hippo pathway contribution is mainly to regulation of the size of the wing and its homeostasis also by controlling the common processes of cell proliferation, apoptosis, and differentiation (Halder & Johnson, 2011; Irvine & Harvey, 2015).

Kinases Hippo (Hpo) and Warts (Wts) as well as the adaptor protein Salvador (Sav) and a transcriptional co-activator Yorkie (Yki) are the main components of the pathway that experience phosphorylation, inhibition, and translocations accordingly to control the cell proliferation relevant genes. For example, overexpression of Yki is related to tumor

formation; Sav and Wts interact with Hpo, forming a signaling pathway where phosphorylation and inhibition of Yki takes place, hereby rendering Hoppo pathway a tumor suppressor one, where conclusively its inactivation develops and progresses cancers (Gou et al., 2018; Halder & Johnson, 2011; Mirzoyan et al., 2019). Defect in these components has also lead to abnormalities in the growth of the wing imaginal disc and the structures they give rise to (Halder & Johnson, 2011). Speaking of the wing imaginal disc and cancers, Hippo signaling mainly deals with the production of the number of cells, controlling the cell proliferation, as well as inducing the apoptosis of any cells found in excess. On that note, mutations in this pathway results in an uncontrolled cell proliferation, where cells can freely and quickly increase beyond the normality, even achieving resistance to any apoptotic signals (Gou et al., 2018; Halder & Johnson, 2011).

The Hippo pathway kinase cassette and yki/YAP are highly conserved between *Drosophila* and humans; Hpo for *drosophila*, MST for humans; Sav/SAV1; Wts/LATS; Mats/MOB (Y. Chen et al., 2020; Fu et al., 2022)

1.7.4 Hedgehog pathway:

As in the case with the aforementioned pathways, Hedgehog also has its role in the regulation of the cell survival and proliferation, and all these pathways can interact to achieve a successful and normal growth through the control on balance of cell proliferation and apoptosis, rendering it a great regulator of the patterning and general wing development (Blair & Ralston, 1997; Lu et al., 2017). Hedgehog (Hh), its receptor Patched (Ptc), and a transcription factor Cubitus interruptus (Ci) constitute the main components of the pathway and are responsible for the regulation of gene expression and the specification of cell fates, notably along the anterior-posterior axis of the developing wing (Blair & Ralston, 1997; Brook, 2000; Hatori & Kornberg, 2020; Nahmad & Stathopoulos, 2009).

The homologs for Hh in vertebrates are Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehod (Dhh), and Ptch1 and Ptch2 are the vertebrates homolog of *Drosophila*'s Ptch (Liu et al., 2011; Zhou et al., 2021).

Hh with its morphogen-like functions and Dpp with its gradient formation are shown to interact with each other to pattern the central region of the wing by the activation of certain

transcription factors, implying the importance of the two pathways in controlling the cell fates and growth (Crozatier et al., 2002; Vervoort, 2000).

1.7.5 Wnt signaling pathway:

Another highly conserved signal transduction cascade that is involved the embryonic development and tissue generations. Multiple processes has been shown to involve the successful Wnt signal: it is needed for cell fate determination, maintaining neural patterning as well as cell polarity and migration (Komiya & Habas, 2008; Sheikh et al., 2014). It is basically a complex of secrete lipoproteins/glycoproteins that induce signaling upon the binding onto cell surface receptors (Palomer et al., 2019; Sheikh et al., 2014).

The pathway is divided into two major groups. One group is called the canonical pathway that involves β-catenin-dependent whose proper stabilization and nuclear translocation is used for the regulation of specific gene expressions that regulate cell fate specification and differentiation; while, the other group, called the non-canonical pathway, which is also subdivided into Planar Cell Polarity and the Wnt/Ca2+ pathway, regulates the cell motility, polarity, and calcium signaling, without the utilization of β-catenin.

1.8 Genetic tool-related concepts:

1.8.1 Balancer chromosomes:

Dealing with a mutated organism entails the assurance of the successful and continuous propagation of the mutation throughout several generations. As a diploid organism, *Drosophila* melanogaster is susceptible to loss of mutation due to the inevitable genetic processes: be it due to genetic drift, due to selection against homozygous mutants that could be lethal, or due to recombination between the mutated and the wild-type chromosome (Fry & Nuzhdin, 2003; Terzian et al., 2007)

If two flies carrying a mutation mate, the product progenies would be heterozygous, or homozygous (not carrying the mutation). This means that at some point down the generation line, flies might end up produced without the mutation and eventually the mutation would be lost.

Balancer chromosomes are extremely powerful and essential part of the fly toolbox and are used to successfully propagate the mutation throughout the generations by preventing

recombination (Miller, Cook, Yeganeh Kazemi, et al., 2016). *Drosophila* melanogaster karyotype consists of four chromosomes: the two sex chromosomes X and Y, and three autosomes which consist of two large metacentric chromosomes (chromosome 2 and 3) as well as small, dot chromosome 4 (Celniker & Rubin, 2003; Hartmann & Sekelsky, 2017; Larsson et al., 2001; Lemeunier & Aulard, 2000 - pg: 137).

The way balancer chromosomes prevent mutation loss is by the virtue of their features: First, they are designed to carry recessive lethal mutations that affect the fitness of homozygous flies (flies that carry two balancer chromosomes), hereby eliminating homozygotes that do not carry the desired mutation; Second, they contain inversions the prevent the occurrence of the meiotic recombination; thirdly, they carry an observable, dominant marker which can be used to identify the heterozygous progenies that carry the balancers (Hentges & Justice, 2004; Miller, Cook, Arvanitakis, et al., 2016; Miller et al., 2018; Zheng et al., 1999).

Balancer chromosomes can be found on all the aforementioned chromosomes of the *Drosophila* melanogaster. Examples of the frequently used balancer chromosomes and their phenotypical effect is found in the table.

1.8.2 RNA interference (RNAi):

RNA Interference (RNAi) is a conserved cellular mechanism that utilizes RNA-guided degradation of messenger RNA (mRNA) transcripts, but not the gene itself, to silence gene expression hence it is a powerful tool for studying loss of gene function effect on the phenotype of the organism, including *Drosophila*, allowing for the identification of molecular mechanisms that regulate biological processes that shape the final body of the *Drosophila* and its wing (Heigwer et al., 2018; Kao & Megraw, 2004; Kavi et al., 2005).

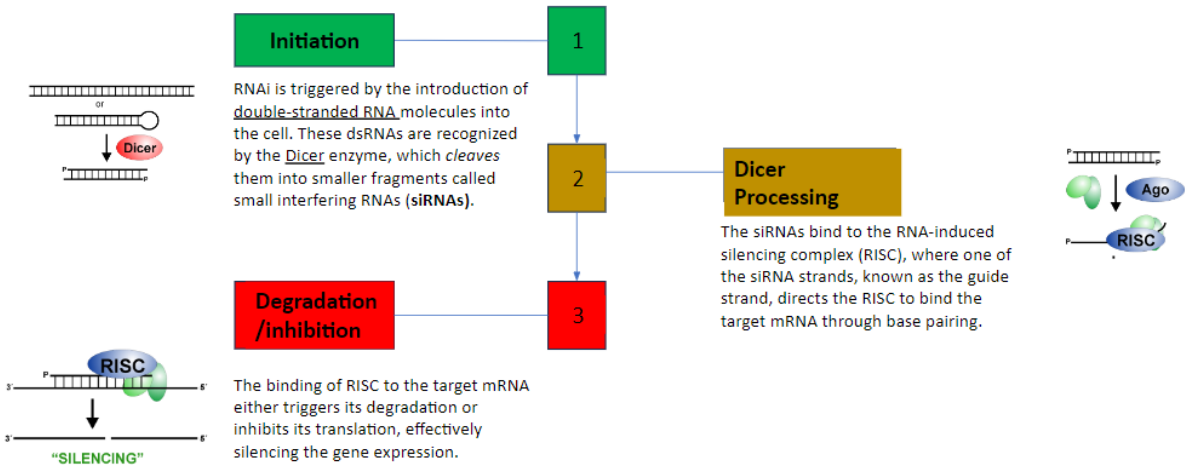

Figure 4. Mechanism of RNAi: The double-stranded RNA (dsRNA) is cleaved into ~19bp singlestranded (siRNA) which integrate into the RISC complex. The siRNA is directed to its homologous mRNA to which it binds and is eventually degraded.

1.8.3 UAS/GAL4/GAL80 ts system

To conduct gene expression control in a tissue-specific and temporal manner, researchers utilize the UAS/GAL4/GAL80 ts system, and is the most used method for this matter in *Drosophila* (Barwell et al., 2017).

It consists of three main components: the UAS, GAL4 driver, and GAL80 inhibitor.

The UAS is, as the name suggests, a specific sequence upstream of the intended gene's promoter region, typically within the promoter sequence and is used to as a binding site for the transcription activator proteins to bound to it, controlling when to activate specific genes (S. Li et al., 2022). The length of the sequence can vary depending on the organism; in *drosophila*, UAS can be as short as 17 base pairs up to 400, or even 1000 base pairs (Golenberg et al., 2009; S. Li et al., 2022; Sonnenfeld, 2009).

GAL4 is a yeast transcriptional activator and can bind to UAS, to express downstream target genes, process regulated by a tissue-specific promoter to induce the transcription of the target gene in the tissue-specific manner, while GAL80 is used as an inhibitor of GAL4 activity, where binding to the activation domain of GAL4 disallows its interaction with the transcriptional machinery, thereby repressing gene expression (Barwell et al., 2017). This can help conduct the temporal control whereby controlling the GAL80 activity means having control on the temporal activity of GAL4, eventually activating/repressing the gene of interest whenever intended.

The gene-specific temporal control can be achieved through the utilization of a temperature-sensitive GAL80, known as. More on this in the Materials and Methods section, under Temperature shifting subsection.

2 The aim of the thesis:

Pupal wing serves as a powerful tool to study the mechanisms and genes the regulate all the cellular and biological processes that lead to organ formation. The wing transitions from a 2D to 3D epithelia and has shown to have similar signaling pathways and genetic involvement to

other mammalian species, rendering it a useful tool to profile the genes underlying the processes.

In this project, the aim was to:

- Determine genetic control and understand what role they might play in the signaling pathways and how they contribute to the organogenesis.

RNAi-mediated knockdown of candidate genes in vivo, utilizing the GAL4/UAS/GAL80ts system for time and tissue-specific screening, was practiced to understand the role of the genes through the observation of their effects on phenotype.

3 Experimental part

3.1 Materials and methods

3.1.1 Materials:

In our acquisition are two stocks: The UAS stocks and the GAL4.

The UAS stock consists of multiple subpopulations with each containing a candidate gene to be targeted during the GAL4/UAS/GAL80ts system process. The genes were obtained from Stock Centers and were chosen upon the acquisition of information from literature regarding their probable expression during the development of the fly.

3.1.1.1 Driver lines:

The second stock (the GAL4) has two subpopulations consisting of tissue-specific promoters, otherwise known as drivers, which control the expression of genes spatially in the GAL4/UAS/GAL80ts system. The two drivers are nubbin (nub-Gal4/Gal80) and apterous (ap-Gal4/Gal80).

Nubbin driver drives the gene expression in the whole wing, i.e. the dorsal and the ventral side of the fly's wing (Ng et al.,1995). Apterous drives the gene expression in the dorsal part of the wing disc, and later the dorsal layer of the adult fly's wing (Bejarano et al., 2008; Milán & Cohen, 2003).

Candidate genes used in this study, along with their stock number are provided in Table 1, below.

atat	62331
Blos2	45835
CG13018	58091
CG3270	52887
CG3803	42948
cos	44472
Cox10	51864
Csl4	82985
Dis3	67919
Dysbindin	67316
Eb1	26599
KHC	35770
Kif19A	58193
Klc	42597
klp64D	40945
Klp67A	62383
Klp98A	39037
ND-13A	51860
ND-23	51797
ND-42	34526
ND-B12	61321
ND-B17	36695
Neb	80389
NudE	38954
Pldn	67884
Ringer	38287
Rrp45	58202
Rrp6	42604
Scox	55179
Sicily	55332
Ttc19	64958
UAS-HDA	51181
unc-104	58083

Table 1. Candidate genes and their stock number in Blooming *drosophila* center

3.1.2 Methods:

3.1.2.1 Crossing:

Each fly line that contains the candidate gene under the UAS control was crossed with each of the driver line. An arbitrary but constant number of flies were selected for the crossing: twenty virgin female adults from each of the driver lines nub-Gal4/Gal80ts & ap-Gal4/Gal80ts mated with ten male adult flies from the UAS line and put in vials. Throughout the crossing period, the flies were kept in the incubator under the appropriate temperature of 25 degrees Celsius to produce the progenies and get the final adult progenies, which is achievable in ten days.

3.1.2.2 Temperature shifting:

Before the completion of the life cycle i.e. before the flies become adults, specifically at the third instar larval stage, all the larvae from each vial are collected and separated into new vials. These vials would be shifted into an incubator under 29 degree Celsius and kept there for a certain period of time, usually 16 hours (however, sometimes might take up to 19 hours). During this time, the larvae grow and become pupa. Upon the pupariation, the vials were again taken out of the 29-degree Celsius incubator from which white pupae were collected and separated and eventually put in new vials back in the 29 degree Celsius incubator for three to four days, before becoming adults and proceeding onto the next steps of the workflow.

This step of temperature shifting is performed as per the functioning of the UAS/Gal4/Gal80ts system: Each of these transcription regulators' activity is affected by the temperature: GAL4 activity is enhanced at higher temperature (29 degrees) and is diminished at lower temperatures (around 18 deg); while, in contrast, GAL80 activity is enhanced at lower temperature and is diminished at higher temperature (29 degrees) (Ewen-Campen et al., n.d.; Kogenaru et al., 2024; McGuire et al., 2003; Qiao et al., 2018). At lower temperature (18 degrees), GAL80 is active, meaning that it is bound to the Gal4 resulting in repressing the activity of Gal4, which means no activation of the UAS. To activate the UAS, Gal4 needs to be active, and this is achieved only after the GAL80 activity reduction which is achieved at higher temperature (29 degrees), resulting in its dissociation (Bosch et al., 2016; Ewen-Campen et al., n.d.; McGuire et al., 2003; Rozich et al., 2023). This is how gene-specific expression is controlled at specific stages of the life cycle of the *Drosophila* Melanogaster.

Since puparation stage is when the wing imaginal disc start to develop (cells differentiation and other mechanisms begin), it is important to perform temperature shifting right at the third instar larval stage (before pupariating) – where the *drosophila* melanogaster are white larvae and which will later become white prepupae—which is basically the indicator of the beginning of metamorphosis (Keroles et al., 2014; Ward et al., 2021).

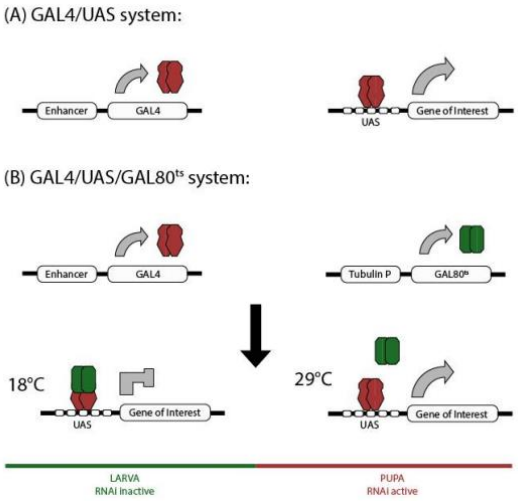

Figure 5. Gal4/UAS/Gal80ts system. (A) Gal4/UAS system. (B) Gal4/UAS/Gal80ts system. Gal4 (red), Gal80ts (green). At 18ºC, Gal80ts is active and is bound to GAL4, preventing it from its activation role, resulting in RNAi not being expressed. However, when at 29ºC, Gal80ts activity is reduced and starts to dissociate from Gal4, allowing it to regain its active function and RNAi will be expressed. (from Classen et al., 2008).

3.1.2.3 Mounting:

Once the progenies became adults, they were collected into Eppendorf tubes with 96% ethanol for tissue fixation. For statistical analysis, ten flies were collected from each gender and put into an Eppendorf tube. After that, they got washed with Phosphate-buffered saline (PBS) to eliminate residues. Then, the wings got dissected using the forceps. In this phase, one wing from each fly from each of the twenty flies (ten males and ten females) was carefully separated, by pulling the hinge part of the wing using the forceps, and then put into a PBS drop on a silicone pad. One PBS drop for each gender. Microscopic glass slides were used to put the wings on. Two thin lines (10-12ul) of 75% glycerol (diluted with MQ) were pipetted on the glass slide, one line for each ten-wings-set of each gender. The wings were taken from the PBS drops on the silicon pad and put on the glycerol line on the glass slide, covered by a glass, and then taken for imaging. It is important during this step to slowly and carefully put the cover glass without rush to avoid the formation of bubbles, which will affect the imaging step later.

3.1.2.4 Imaging and quantification:

Microscopy (4x magnification) was used for the analysis of the wing. Later, after the images were gathered, they were quantified in ImageJ software for statistical analysis. The average

area and length of the wings of the flies were calculated and compared with those of the control flies.

3.2 Results and discussion:

Wing development undergoes metamorphosis upon transition from the larval into the pupal stage. Transitioning into a final, adult shape in 3D form includes cell shape adaptation to these changes, and obviously must be regulated by certain signalling pathways and cellular

processes (Gómez-Gálvez et al., 2021). Considering that, it sets as a great tool to study organogenesis, and understand the genetic control and how the signalling pathways as well as other cellular mechanisms result in the final shape.

In this project, candidate genes were chosen to study their control in the process. These genes were subjected to temporal knockdown using RNAi. The effect of their knockdown on the phenotype of the wing was studied. This consists of effect on size and length changes, as well as formation of the bristles and veins.

Genes were analysed based on their effect on phenotypes and their predicted functions. Flybase.org and literature reviewing were the sources and approaches used to understand the genes and why did they cause the change in phenotype.

The phenotypes of the adult wings were checked for the following features: Wing's shape area (using ImageJ), and abnormalities in the veins and bristles (microscopy). Out of all the screened genotypes, only few (7) showed phenotypic differences when compared to the control (Fig. 6 B-H). The rest showed great similarities in terms of vein and bristles formation, but with differences in the lengths and area.

The size variations presented are for the nub-Gal4 line of both sexes, while the phenotypes are presented for the nub-Gal4 female flies. Apt-Gal4 lines were chosen to be screened only when the same gene showed a phenotype in nub-Gal4 lines. However, no apt-Gal4 line showed a phenotype. The reason apt-Gal4 was used was to test if a change in one layer would either rescue or cause similar, or even more sever changes, in the other layer. In the case of this project, none of the genes that caused a phenotype in nub-Gal4 lines has shown a phenotype in the apt-Gal4 lines.

In terms of sexes, the phenotypes were witnessed only in the females, but not in males. This could mean that there are certain genes considered more important for the wing development of a specific sex, and these genes might be expressed at a higher levels in one sex compared to the other, as seen by a study that addressed the genetic contributions to the wing development in a sexually dimorphic manner (Carreira et al., 2011), which could be the reason behind the manifestation of phenotypes in female wings than in male wings. In the case of this thesis, these genes are *Blos2, ND-B17, Klp64D, Unc-104, Dysbindin, Ringer, CG3720.* Moreover, another reason to such pattern might be that due to having only one X-chromosome, males witness a higher upregulation in X-chromosome-linked gene expression compared to female wings, otherwise known as dosage compensation, and is carried by a

complex of proteins known as the male-specific-lethal (MSL) complex, which allows for the equal expression of X-linked genes between females and males (Gorman & Baker, 1994; Lucchesi & Kuroda, 2015). The availability of the buffering functionality of MSL in males and not in females could be the reason behind the successful regulation of genes that compensate for the phenotypes, due to the balance that might help mitigate or prevent the manifestation of phenotypes in males after a knockdown through dosage compensation and epigenetic regulations, (Pierre et al., 2008; Valsecchi et al., 2018). However, this doesn't mean that phenotypic changes will never occur in males; there could be some genes that counter the functionality of the MSL and do cause a phenotype, as we can tell from the bar graphs (Fig. 7) that the genes did at least result in a change in the size of the male wings. For now, only the mentioned genes have been shown to be prevented by the MSL complex from causing any changes in terms of veins and bristles formation.

In the bar graphs (Fig. 7&7) we see that even though phenotypes in terms of vein and bristle formations are witnessed in females only, size variations in both sexes had occurred. In general, size variations that occurred didn't have one effect; namely, either caused an increase only, or a decrease only, but rather both effects are seen. The increase in wing size was caused by most of the genes in both sexes. Only few caused a reduction in size. Interestingly, the genes that caused phenotypes in females had the same effect on both sexes in terms of size. For example, *CG3270, Dysbindin, Blos2, Ringer,* and *Unc-104* all resulted in the same effect and in both sexes—reduction in wing size, and as for *ND-B17* and *Klp64D* they both resulted in an increase in the wing size. However, we don't see that pattern in the gene knockdowns that didn't show phenotype: we can see that, for example, *cos* gene knockdown resulted in an increase in the female size but a decrease in male size; *NudE* and *Klp98A* produced a smaller wing size in females compared to control, while in males they produced flies with bigger wings than the controls. All these difference in the pattern of the sizes and in the non-equal effect of the genes can be also linked to the sexual dimorphism that is evident in *Drosophila melanogaster* or any epistatic effect. Normally, the size of female wings is bigger than the male, and as seen in Fig. 9, no matter what phenotype was seen or how sizes changed, the phenomena is still valid—female wings kept the "reputation" of having bigger wings than the males. We can conclude from these bar graphs that there is a definite relationship between gene expressions and sex-specific hormones, and probably, sex-specific pathways, where a specific sex can be more affected by changes in cellular processes, like cell proliferation and apoptosis, than the other sex. However, we still need to investigate the connection between

such genes and how do they result in sex-specific phenotype that is not identical across the board. For example, Hippo pathway is the central and the most regulator of the wing size, though, of course, Hippo doesn't work independently of other signalling pathways, which also contribute to the wing development. On that note, it would be a good idea to understand the relationship between specific genes and their effect on Hippo pathway, and eventually the effect of both the sex-specific genes and the Hippo pathway on the final wing development. More specifically, how does each gene alter the activity of the Yorkie activator of the Hippo pathway is a good question to start addressing, as phosphorylation and cytoplasmic localization of the Yorkie activator play a huge role in the expression and suppression of Hippo target gene that regulate wing size, cell proliferation, and apoptosis.

In the end, we didn't witness another phenomenon where a gene knockdown results in male flies having bigger wings than their females counterpart, as that then would have called for another approach to understand the differences in the mechanisms by which pathways interact with sex-specific hormones and regulators to determine wing sizes. So far, all what we need to understand is how do these genes have different effects on both sexes considering their effect on the signalling pathways, beyond the usual concepts of sexual dimorphism and the MSL complex—a deeper insight must be achieved beyond the perspective of evolution and sexual selection pressure.

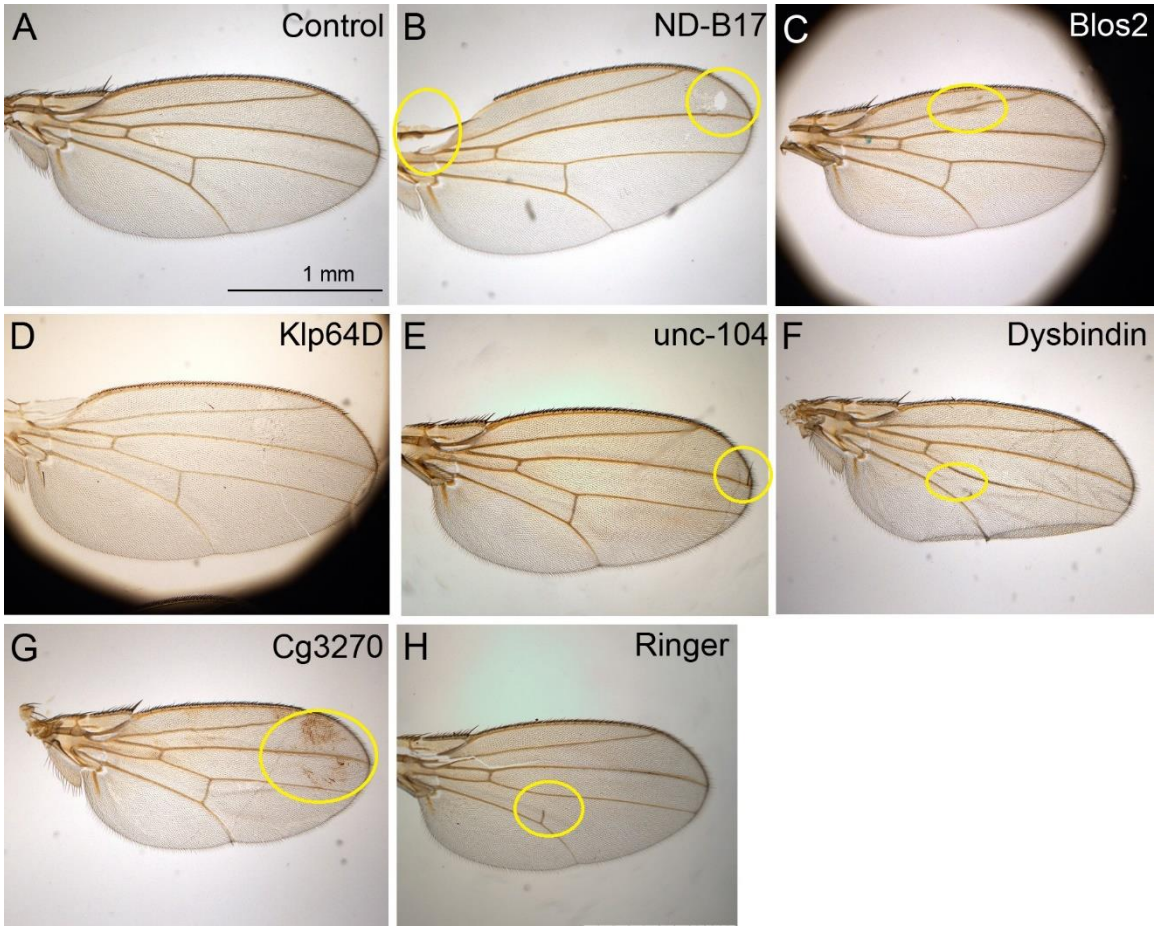

Figure 6. Phenotypes of nub-gal4 adult female wings. (A) The wild-type nub-Gal4 was used as a control. (B) Phenotype of ND-B17 knockdown. (C) *Blos2* knockdown phenotype. (D) Klp64D knockdown phenotype. (E) *Unc-104* knockdown phenotype. (F) Dysbindin knockdown phenotype. (G) cg3270 knockdown phenotype. (H) Ringer knockdown phenotype. Yellow shapes show the areas of the wing that exhibit difference compared to the control. (D) lacks shape guidance because the change is witnessed in the whole wing (light vein color). Scalebar set to 1mm.

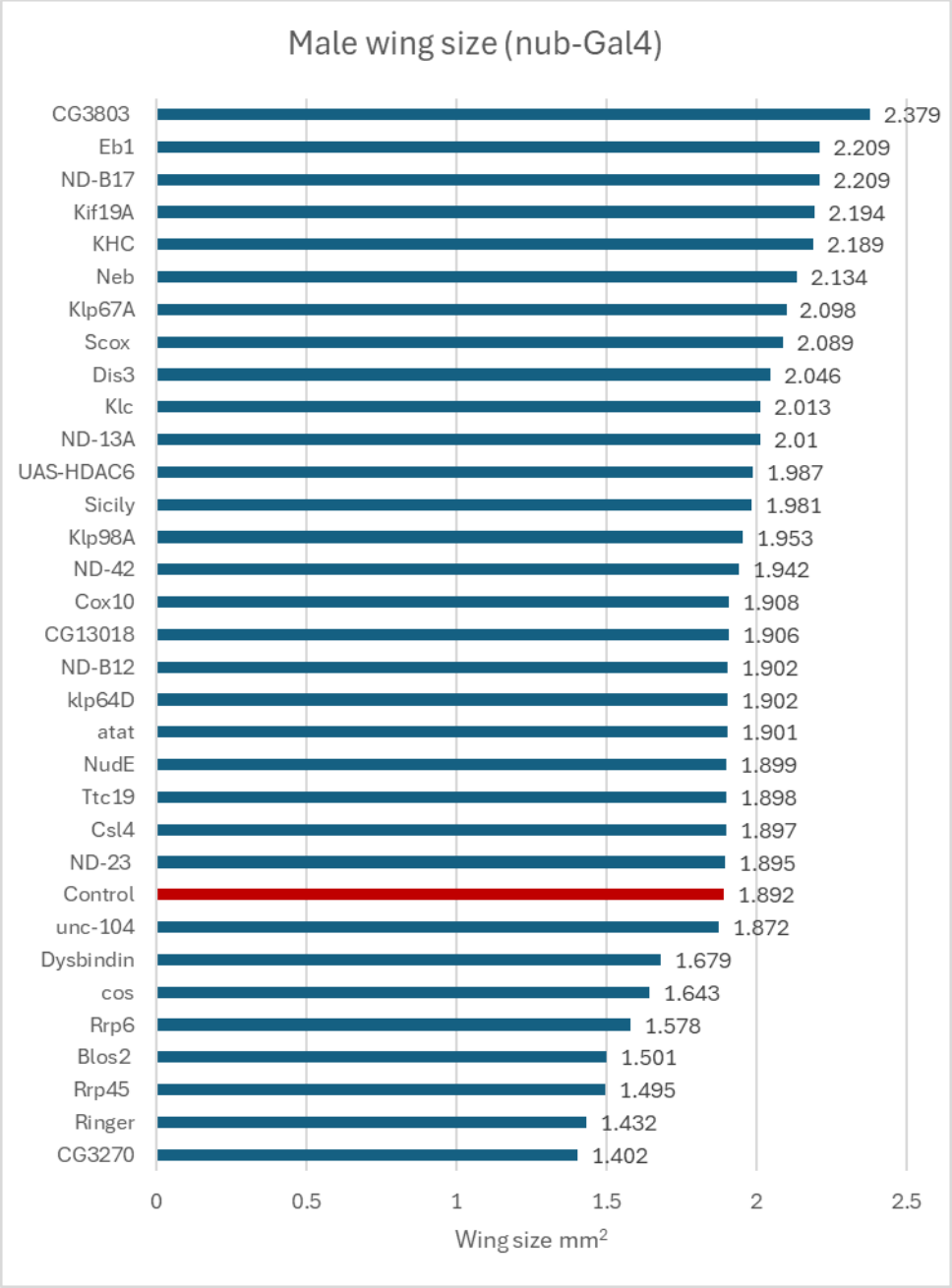

Figure 7. Wing area of adult males (nub-Gal4). Red line is the control. Values put in an ascending order only for the ease of visualisation and comparison. N=10.

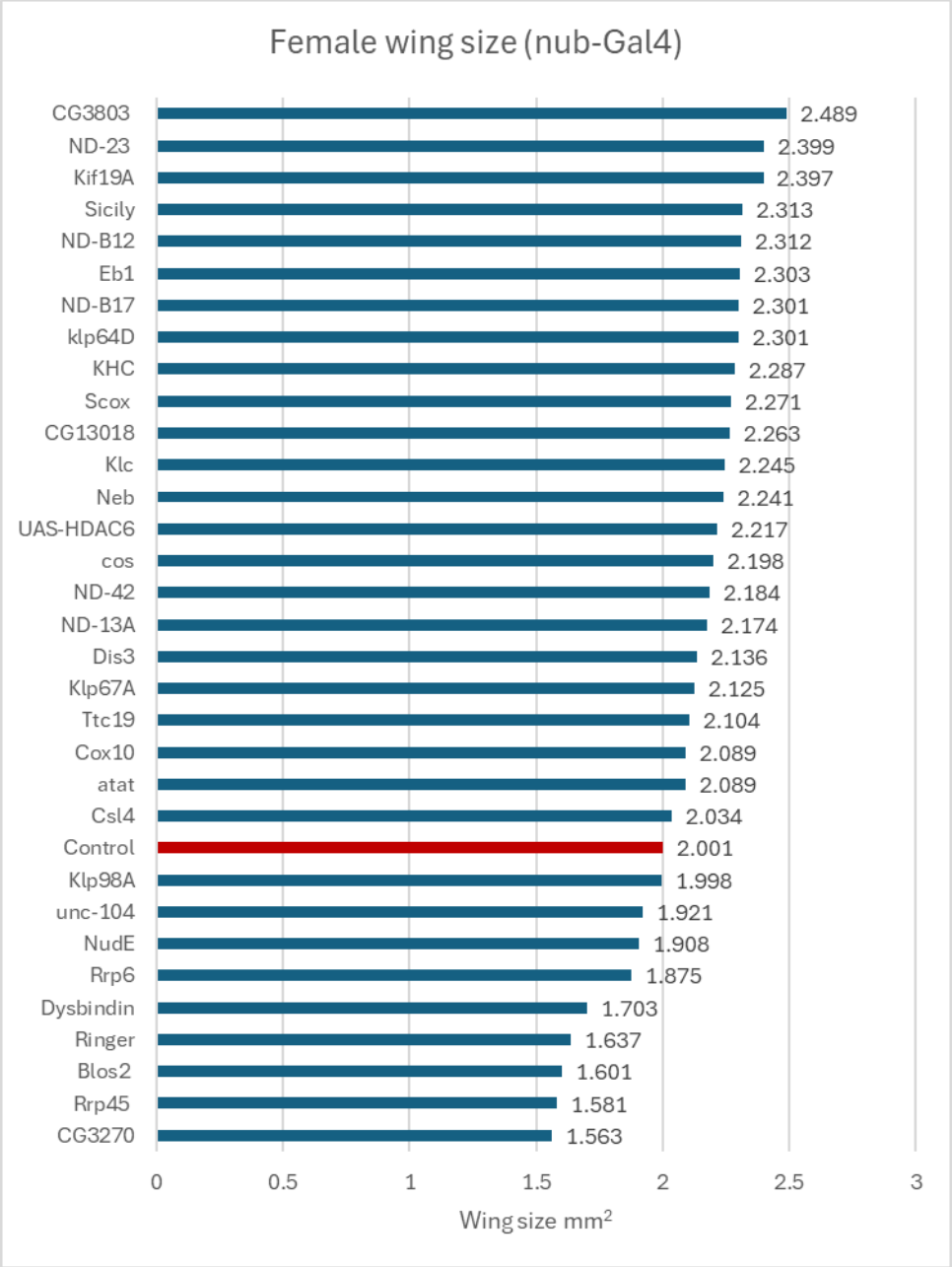

Figure 8. Wing area of adult females (nub-Gal4). Red line is the control. Values put in an ascending order only for the ease of visualisation and comparison. N=10.

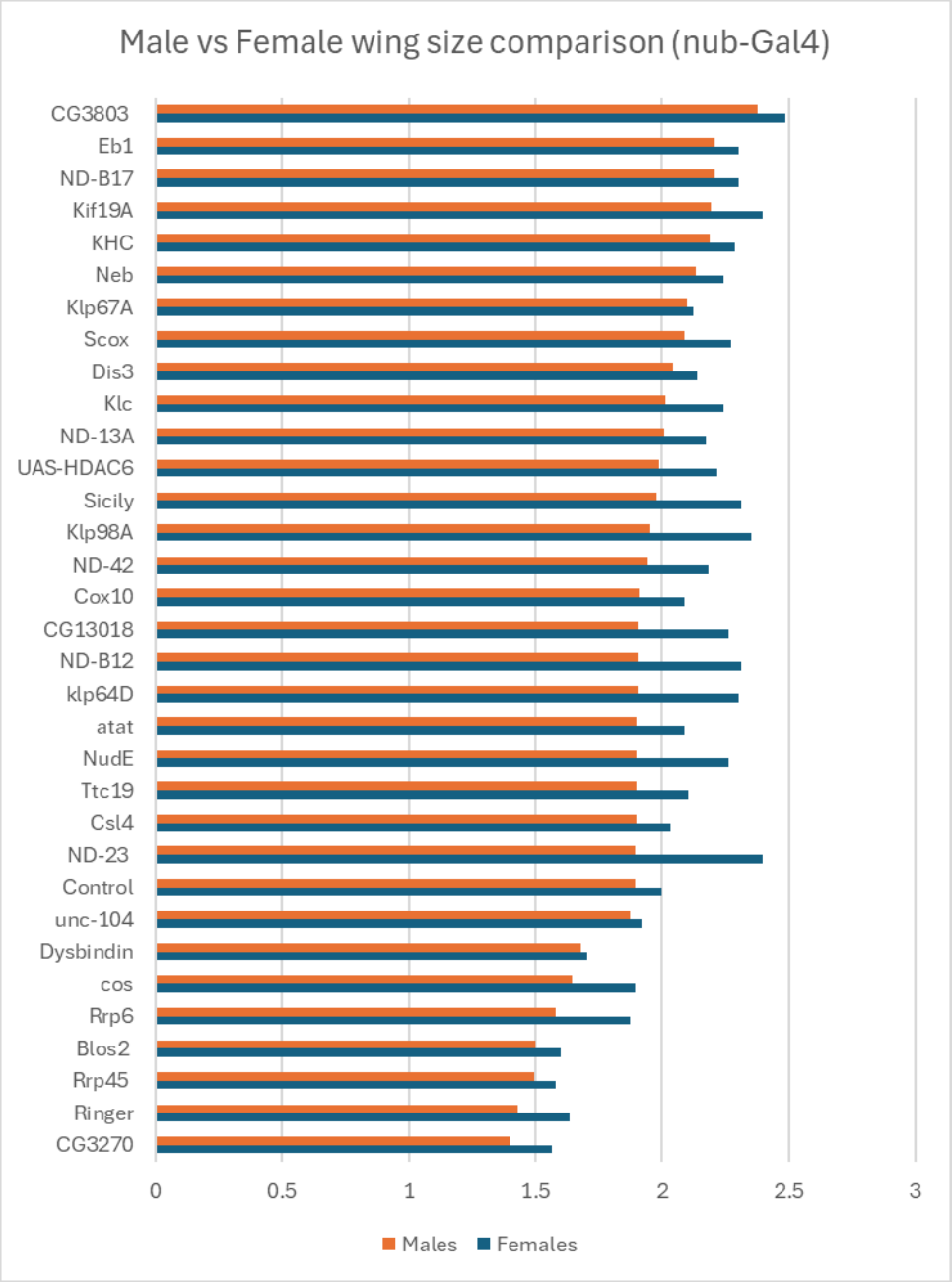

Figure 9. Comparison of wing sizes of adult females with adult males.

3.2.1 *Unc-104* knockdown and the formation of ectopic bristle at distal L3:

The *Unc-104* gene encodes a kinesin-like protein that is essential for axonal transport and synaptic function (De Celis, 1998; *Unc-104*, n.d.; Vagnoni & Bullock, 2018). Vesicle-mediated transport along microtubule also utilize this gene (Barkus et al., 2008; Pack-Chung et al., 2007).

Results show that the loss of this gene's function has caused the formation of an ectopic bristle at distal vein L3. Even though the bristle has developed at L3, what is needed to be understood isn't the specific location of the bristle and why it occurred at L3 instead of the other veins, but what's important is to speculate on what might cause the formation of ectopic bristles anywhere on the wing in general.

Vesicle trafficking is a crucial regulator of the proper extension and development of bristles (Stewart et al., 2001), and vesicles-mediated transport along microtubule also utilize the *Unc-104* gene (Barkus et al., 2008; Pack-Chung et al., 2007).

It seems that abnormalities in bristle development are inevitable after the knockdown of the gene. Moreover, the *Unc-104* gene in *Drosophila* encodes a kinesin-3 motor protein that plays a crucial role in axonal transport, particularly in the transport of synaptic vesicles from the cell body to the synapse. Defects in synaptic junctions can also impair membrane trafficking and, consequently, the proper extension and development of sensory bristles. Additionally, alterations in synaptic junctions can impact signaling pathways like Wingless (Wg) and components of the Notch signaling pathway, which are crucial for wing development. Wingless (Wg) is a secreted morphogen that forms a gradient to pattern the wing. Vesicle-mediated transport is proposed to be important for Wg gradient formation. Defects in Wg secretion and transport can disrupt the Wg gradient, altering wing patterning and structure (Strigini & Cohen, 2000). Finally, the ectopic bristle formation could be as a result of the disruption of the formation of mature boutons, which are sites neurotransmitter releases that allow for synaptic junctions (Pack-Chung et al., 2007; Yang et al., 2022). Considering that, the knockdown contributed to the locomotion and synaptic transmission failure due to a failure in the transportation of synaptic vesicle protein to the synapse.

These could imply that *Unc-104* is extremely essential for the proper synaptic functioning and the axonal transport, and whose knockdown can impair synapses, vesicle transportation, and neural connections, eventually resulting in phenotype alterations, which in this case is the ectopic bristle formation. The functions of microtubules are linked to normal axonal transport and synaptic functioning in neurons; Also, microtubules provide the structural support

necessary for axonal transport, ensuring the efficient movement of cargoes to and from the synapse, hence, disruptions in microtubule dynamics can impact axonal transport, leading to defects in synaptic functioning and potentially contributing to neurodegenerative diseases and other neurological disorders (Guedes-Dias & Holzbaur, 2019; Guillaud et al., 2020; J. Kumar et al., 2010; J. Li et al., 2017; L.-B. Li et al., 2016; Niwa et al., 2016; Zhang et al., 2017).

3.2.2 *Cg3270* knockdown and the formation of tumor:

Cg3270 knockdown has caused the wing to exhibit certain reddish/brownish colors, which can be considered as a melanotic tumor that result from a malfunctioning or due to a leakage of hemocytes from veins (Kiger et al., 2001). *CG3270* predicted role is in mitochondrial respiratory chain complex I assembly. The result can imply that this gene is crucial for the regulation of certain pathways that are involved in cell proliferation and apoptosis. Since it has its role in Complex I regulation, then defects in this Complex I can be the reason behind the formation of tumors (Cho et al., 2012). Loss of function of Complex I has been associated with a decrease in oxygen levels and a leakage in electrons, which can cause the production of radicals and other reactive oxygen species (ROS), which can also result from lower ATP levels that are caused by defects in Complex I activity (Scialò et al., 2020; Thelen et al., 2020). These events can lead to impairment in the apoptosis of cells and alteration in mitochondrial functionality, which can in turn lead to activation of relevant oncogenic signaling cascades.

The mechanisms by which such events occur and how they are causing the formation of tumor needs to be understood. Live imaging can be used to observe the defects in apoptosis and understand the pathways involved in such tumor formation, also techniques that allow for cell number counting can be useful.

What is concluded now is that *cg3270* does impact the activity of Complex I and the general mitochondrial assembly processes and its functionalities, and consequently giving rise to events that eventually lead to tumor formation.

3.2.3 *ND-B17* knockdown and the loss of bristles:

The nd-b17 gene in *Drosophila* melanogaster encodes the NADH dehydrogenase (ubiquinone) B17 subunit, which is part of the mitochondrial respiratory chain complex I-- is involved in mitochondrial electron transport-- which is essential for energy production in the form of ATP as it is an important aspect of the proper formation and maintenance of bristles.

Considering that, mitochondrial dysfunction is also linked to loss of bristles. Also, since microtubules do have their role in the organization of cellular structures which in this case is the bristle, microtubules are involved in the elongation of the bristles. Moreover, the Notch pathway is involved in the cell fate determination and the upregulation of vestigial gene during development (Tien et al., 2008). Given these, it is seen that another consequence of the dysfunctional mitochondria knockdown in ND-B17 results in less involvement of microtubules in bristle elongation and disrupts the progression of the Notch pathway and its interaction with vestigial gene.

This could also explain the size reduction seen in the adult wings of both sexes, as knockdown in this gene disrupted the dynamic reorganizations of microtubule and actin filaments which are essential in the cell length homeostasis and planar cell polarity, implying a role in Wg signalling pathway in size regulation.

3.2.4 *Ringer* knockdown and partial PCV:

Ringer gene is involved in the maintenance of ionic balance and osmotic pressure in the salivary glands, as well as in the microtubule bundle formation and polymerization (George et al., 2019; Mino et al., 2016; Takashima & Murakami, 2001). The observed phenotype after its knockdown is a partial PCV. This probably has to do with a defect in the ion channels, since the case of this phenotype was caused by a gene that regulates such processes.

According to literature, the defect in ion balance has been shown to result in partial posterior crossveinless phenotype in the wing of *Drosophila* by disrupting the signaling pathways involved in wing development (George et al., 2019). Since it's shown that multiple ion channels play a crucial role in regulating cell signaling and cell fate specification during wing morphogenesis, then definitely one cause of such phenotype is a defect in the relevant ion channels. For example, disruptions in ion channels can impact the distribution of signaling molecules like Dpp (Decapentaplegic). Dpp release into the developing wing fly is crucial for

vein formation. It is shown that an ion channel, the inwardly rectifying potassium (Irk), has a role in the release of Dpp into the wing. Hence, it could be that this gene has it role in regulating such channels for the proper dsitrubution of Dpp levels in the cells, which in turn regulate the cell proliferation and vein formation. Defects in these channels might lead to an abnormal release of the Dpp and, since the cells respond to the concentration of Dpp, the abnormal release could result in an abnormal levels of Dpp, which can negatively impact the downstream transcriptional responses (mentioned earlier in the section of BMP, page 18) and hence develop abnormalities in venation.

Therefore, a defect in ion balance, caused by disruptions in ion channels, can lead to aberrant signaling cascades and morphogen gradients, ultimately resulting in a partial posterior crossveinless phenotype in the wing of *Drosophila*. Finally, there are some connections between these ion channels microtubule polymerization, which is crucial for the maintenance of cell polarity and venation. As seen form the phenotype, this gene could have a role in regulating microtubule polymerization through the maintenance of proper channeling of the ions. This validates that microtubule polymerization, Dpp levels, and proper ion channeling do have essential roles in venation and bristle formation.

3.2.5 *Dysbindin* knockdown and absence of PCV:

Dysbindin is important for homeostatic synaptic plasticity and for membrane trafficking (Wentzel et al., 2018).

Here we see that *Dysbindin* knockdown introduces disruption in such membrane trafficking which will consequently disrupt the spatial distribution of the *Dpp* ligand and its regulatos (like GTPase Rab5), and hence affecting the normal long-range BMP signaling gradient, eventually causing defects in the signaling and patterning processes during the development of the wing. Once again, for proper PCV development, certain amounts of *Dpp* ligands are involved in a *Dpp* trafficking mechanism, as this ligand is directionally trafficked form the longitudinal veins into the PCV region in order to signal for its patterning and differentiation (Matsuda & Shimmi, 2012). Any defects in membrane trafficking negatively impact Dpp distribution and its directional transport and has other consequences caused in BMP signaling pathway (Belenkaya et al., 2004; Matsuda & Shimmi, 2012). This highlights the importance of trafficking of *Dpp,* and the *BMP/Dpp,* general role in the proper formation of veins.

The absence of PCV could also be due to abnormalities that might occur in the cytoskeletal organization. F-actin and integrins do contribute to the formation of veins, in general, and PCV, in particular. These proteins also must be normally localized for proper vein formation. *Dysbindin* knockdown does highlight the importance of membrane trafficking of crucial ligands and proteins, as well as the cytoskeletal organization, and any defects occurring will affect the signaling pathways (BMP/Dpp) contribution to the final, normal wing development.

Such events and their contribution to final vein formation require normal synaptic plasticity for neuron transmission (Frank, 2014). PCV cells interact with motor neurons for vein development, therefore it is crucial to maintain such communication for proper response to environmental changes.

We see that since *Dysbindin* does play a role in homeostatic plasticity, and loss of its function has produced a wing lacking PCV. Further research needed to be done to understand the relationship between homeostatic plasticity and vein formation.

Dysbindin is required to be also studied in terms of what membrane-trafficking- related regulators and ligands that get affected by its knockdown, and how do they affect the signaling pathways that are related to the wing formation.

3.2.6 *Klp64D* knockdown and incomplete vein differentiation:

Klp64D encodes the Kinesin-II motor complex, which is involved in Wingless (Wg) signalling pathway for the development of the wing through the regulation of intracellular trafficking of Armadillo (Arm) , an essential protein in the transduction of the Wingless pathway (*Armadillo*, n.d.; Vuong et al., 2014).

It is seen that knockdown of *klp64D* does disrupt the functionality of Arm in vein differentiation. Arm in the Wg signaling functions as a transcriptional co-activator of vein development-related genes in the nucleus, making it an essential signal transducer across the cell membrane, and its interaction with klp64D is necessary for its localization and general intracellular trafficking of its normal levels, findings that are consistent with and evident in study by (Vuong et al., 2020) and (Vuong et al., 2014).

3.2.7 *Blos2* knockdown and ectopic vein formation:

Blos2 is involved in the biogenesis of lysosome-related organelles complex 1, subunit 2, and is believed to regulate gamma-tubulin binding and encode a protein that co-localizes with gamma tubulin.

The phenotype caused by the knockdown of *Blos2* is a formation of an ectopic vein (at L2). Gamma tubulin is essential for the nucleation of microtubules, which is essential for vein patterning (Gunawardane et al., 2000). This result can further highlight the importance of microtubule nucleation in vein formation, and the role of gamma-tubulin that serves as template for this microtubule nucleation. There is not much literature to which the consistency of the results of this thesis can be done, however, we can infer that a defect in gamma-tubulin can cause abnormalities in microtubule organisation, and this can result in abnormalities in vein patterning probably due to mislocalization of signalling molecules or relevant structural components.

Summary:

The study aims to determine the genetic control affected by certain candidate genes over the mechanisms that govern cell shape changes, cell communication, proliferation and organ formation,

specifically in this case of the *Drosophila* wing. By using the *Drosophila* wing and employing conditional RNAi knockdown via the GAL4/UAS/GAL80ts system, the screening of genes of interest has been conducted in a tissue-specific and stage-specific manner. Results reveal that seven candidate genes, *Blos2*, ND-B17, Klp64D, *Unc-104*, Ringer, Dysbindin, and CG3270, play important roles in wing morphogenesis and vein formation. Their knockdown resulted in noticeable changes in the wing when they were knocked down in the whole wing tissue. These observations indicate that the regulation of ion channels and ionic balance, proper microtubule organization, sufficient energy production along with proper membrane trafficking and normal synaptic junctions are crucial for the normal progression of the signalling pathways that play a role in vein formation, bristle formation, cell differentiation, and cell proliferation.

Interestingly, most wing phenotypes via conditional RNAi knocked down resemble the loss-of-function phenotypes of conserved signalling pathways, such as Notch, Wg/Wnt and BMP signals. Taken together, these findings reveal that candidate genes, previously categorized in cellular mechanisms, play a role in regulating conserved signalling pathways during *Drosophila* wing development. Genetic interactions will be further investigated using fly genetics and image analysis to provide molecular details, leading to novel insights into organogenesis during animal development.

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