UNIVERSITY OF TARTU

Faculty of Science and Technology

Institute of Molecular and Cell Biology

Chair of Developmental Biology

Mosaic analysis of BMP receptors to study intercellular communication during *Drosophila melanogaster* pupal wing development

Bachelor's Thesis

12 EAP

Joosep Karu

Supervisor(s)

Professor Osamu Shimmi, PhD

Associate Professor Tambet Tõnissoo, PhD

Tartu 2025

INFO PAGE

Mosaic analysis of BMP receptors to study intercellular communication during *Drosophila melanogaster* pupal wing development

During the metamorphosis of *Drosophila melanogaster,* complex structures and organs develop from relatively simple clusters of undifferentiated cells. The development of wing veins in the fly involves a variety of morphogens, signalling molecules that form gradients of activity across tissues. One of such morphogens, BMP type ligand, Decapentaplegic (Dpp), is expressed within the developing wing veins and is spatially restricted by its receptor Thickveins (Tkv), thereby maintaining a short-range signal. The precise mechanisms that prevent Dpp from diffusing remain unclear. This study used a heat shock inducible FLP-FRT system to generate a random mosaic of constitutive active Tkv clones, driven by the endogenous enhancer, aiming to observe how mosaic clones affect surrounding cells with wild type Tkv receptors. The results show that a post receptor activation signal initiates the formation of a Tkv barrier surrounding Dpp signal positive cells and that lateral exhibition of Tkv might play a role in limiting the Dpp diffusion range. These findings contribute to a better understanding of how spatial restriction of morphogen signalling is achieved during wing patterning in *Drosophila* development.

Key words: *Drosophila melanogaster*, Decapentaplegic, Thickveins, pupal wing development

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

BMP retseptorite mosaiikanalüüs rakkudevahelise kommunikatsiooni uurimiseks *Drosophila melanogaster*`i nuku tiiva arengus

Äädikakärbse *Drosophila melanogaster*'i metamorfoosi käigus arenevad keerukad struktuurid ja organid suhteliselt lihtsatest, differentseerumata rakkude kogumitest. Tiivasoonte areng on seotud mitmete morfogeenidega - signaalmolekulid, mis moodustavad kudedes aktiivsusgradiente. Üheks morfogeeniks on BMP-tüüpi ligand Decapentaplegic (Dpp), mis ekspresseerub nuku tiivasoontes ning mille levikut piirab tema retseptor Thickveins (Tkv), tagades sellega signaali lühikese ulatuse. Täpsed mehhanismid, mis takistavad Dpp levikut, on siiani ebaselged. Käesolevas uuringus kasutati kuumšoki abil indutseeritavat FLP-FRT süsteemi, et tekitada nuku tiibades juhuslikke konstitutiivselt aktiivse Tkv-ga kloone, eesmärgiga jälgida, kuidas kloonid mõjutavad ümbritsevaid metsiktüüpi Tkv retseptoriga rakke. Tulemused näitavad, et retseptori aktiveerumise järgne signaal algatab Tkv barjääri moodustumise Dpp-positiivsete rakkude ümber ning et Tkv lateraalne esinemine võib mängida rolli Dpp leviku piiramises. Need leiud aitavad paremini mõista, kuidas morfogeenide signalisatsiooni ruumiline piiramine tiibade arengus saavutatakse.

Märksõnad: *Drosophila melanogaster*, Decapentaplegic, Thickveins, nuku tiiva areng

CERCS: B350 Arengubioloogia, loomade kasv, ontogenees, embrüoloogia

TABLE OF CONTENTS

USED ABBREVIATIONS

ACV - anterior crossvein

AEL - After Egg Laying

AP - Anterior-Posterior

APF - after pupa formation

BMP - Bone morphogenic protein

Ci - Cubitus Interruptus

Cv - crossveinless

Cyo - Curly of Oster

DAPI - 4′,6-diamidino-2-phenylindole

Dpp - Decapentaplegic

En - engrailed

FA - formaldehyde

FLP - Flippase

FRT - FLP recognition target

Gbb - Glass bottom boat

GFP - Green fluorescence protein

Hh - Hedgehog

hsFLP - heat shock flippase

LV - longitudinal vein

Mad - Mothers against Dpp

NGS - Normal Goat Serum

pMad - phosphorylated Mad

PBT - PBS with Triton

PBS - Phosphate-buffered saline

PCV - posterior crossvein

Rb568 - Rabbit 568

Rb633 - Rabbit 633

Sog - Short gastrulation

Tkv - Thickveins

Tlr - Tolloid related

TP - thoracic imaginal disc primordia

INTRODUCTION

The Fruit fly, *Drosophila melanogaster,* is a widely used model organism in developmental biology due to its short life cycle, easy maintenance, well characterized genome and the presence of numerous homologues to human genes. Despite over a century of research and extensive accumulated knowledge, new questions continue to emerge, along with the technology to answer them. New methodological approaches remain valuable in uncovering long standing biological questions.

This thesis provides a theoretical summary of the Drosophila life cycle, with a particular focus on wing development during the larval and especially pupal stages. Morphogens play a significant role in both stages, forming gradients that guide tissue patterning. The BMP-type morphogen Decapentaplegic (Dpp) is necessary for the differentiation and formation of the wing veins during the pupal stage. It has been described that Dpp'-s own receptor Thickveins (Tkv) restricts Dpp spread into the intervein area, but the precise mechanism remains unclear.

To better understand how receptors influence the spreading of morphogens, this thesis aims to develop a protocol for the generation of a mosaic pattern of modified Tkv receptors. This system is used to study how Dpp influences wing vein pattern formation. Adult wings are analysed for morphological changes, while pupal wings are immunohistochemically stained to further understand receptor localization. Confocal microscopy is employed to analyse the three-dimensional communication within the developing tissue. Based on these observations, models will be proposed to explain the signalling dynamics and spatial patterns observed in and around the mosaic clones.

The thesis was conducted in the Chair of Developmental Biology at the Institute of Molecular and Cell Biology at the University of Tartu.

1. LITERATURE REVIEW

1.1 *Drosophila melanogaster*

1.1.1 Drosophila melanogaster life cycle

Drosophila melanogaster, hereinafter referred to, simply as "*Drosophila*" or "fruit fly", belongs to the order *Diptera* and the family *Drosophilidae*. It is a holometabolous insect, meaning that it undergoes complete metamorphosis during its pupal stage (Tyler, 2000). Among the main advantages of using *Drosophila* as a model organism in genetic research is its short life cycle. This simplifies observing phenotypic and morphological changes throughout an individual organism's development. The short life cycle also allows for crosses between different genetic lines to made easily and relatively quickly (Ashburner, 1989).

The speed of *Drosophila* development is influenced by ambient temperature. The life cycle from the egg to adult fly takes 9-10 days at 25ºC but slows down considerably to 19 days at 18ºC (Linford et al., 2013).

During mating the female stores sperms from the male in the seminal receptacle (Lefevre & Jonsson, 1962). A female can then lay eggs continuously throughout the day, up to seventy eggs per day. *Drosophila* eggs are around half of a millimetre long and are laid into the food medium with one end sticking out of the food for gas exchange (Ashburner, 1989). After roughly a day at 25ºC the eggs will have completed embryonic development and hatch into the first instar larva stage. The first instar larva feeds on the growth substrate for around 24 hours at which point the larva molts into the slightly larger second instar larval stage. During the second instar larval stage the larva continues feeding for another 24 hours and then undergoes its second molting into the third instar larval stage. During the third stage the larva is the largest, it continues feeding but will begin climbing upward out of the food medium where it searches for a suitable spot for pupariation. About 30 hours after emerging the third instar larva molts into the pupa (Tyler, 2000).

At the beginning of pupariation, the larva becomes stationary, and the outer cuticle transforms into the puparium, a shell that while initially yellowish white and soft get progressively tougher and darker in colour. The puparium also becomes more easily visually separable from the developing fly inside. Within the puparium the fly undergoes complete metamorphosis into the adult fly. During the metamorphosis process most larval tissues are lysed with only a few

exceptions, including the nervous system, fat bodies, excretory system and gonads. The new tissues and organs of the adult fly are grown from sets of undifferentiated cells that have been grown inside the larva called the imaginal discs, imaginal cells and histoblasts. After 3-4 days the adult fly will emerge from the pupal case. Adult males are sexually mature within a few hours; however, females will only reach maturity in 8-12 hours (Tyler, 2000, Hales et al., 2015).

1.1.2 *Drosophila* imaginal discs development

Drosophila imaginal discs are small packets of epithelial cells that divide and grow during the larval stages without undergoing differentiation. During the pupal stage the imaginal discs begin differentiation, forming the external cuticular structures of the adult fly (Beira & Paro, 2016).

Imaginal discs are precursors to most structures in the adult fly's head, thorax, and genitalia. Individual discs are easily identifiable inside the larva from both the larval organs and one another. The discs are also relatively flat, with most cells being a single epithelial layer. Those factors make them valuable tools in studying tissue development by using fixed tissue and live imaging (Tripathi & Irvine, 2022).

Wing imaginal discs are already present in the larva, forming earlier, during embryogenesis (Bate & Arias, 1991). The wing discs originate from the thoracic imaginal disc primordia (TP). During embryonic development, the TP develops distinct regions identifiable by the expression of specific transcription factors with Snail and Vestigial in the future wings (Requena et al., 2017). As development progresses the wing disc primordia become physically separated from the TP, forming of the dorsal side of the TP and adjacent non-TP cells (Cohen et al., 1993). During rapid growth in the larval stage many larval tissues become polyploid, increasing the copies of the genome in a cell, and through this the size of the cells. This makes the cells larger, gives more templates for transcription and increases control over development. The wing imaginal discs, however, remain diploid throughout larval development and increases in size through cell division. During this division the discs grow nearly 1000 times. Parallel to this growth the disc morphs from a simple sac of epithelial cells to a complex organ. Cells in different regions of the disc are assigned distinct cell fates. By the third instar larval stage the imaginal wing disc can be divided into four morphologically identifiable regions: the wing pouch, the proximal wing and wing hinge, the notal region and the peripodial epithelium (Tripathi & Irvine, 2022). These regions are established through complex progressive refinement. Initially the wing is divided into broad regions with transcription factors. First with

Engrailed (En) expressed in the posterior to produce Hedgehog. Hedgehog then serves as a morphogen in the anterior compartment, transcribing *dpp* in a stripe flanking the anterior-posterior axis of the imaginal disc. The wings are then subdivided even further with combinations of additional transcription factors, signalling molecules and their targets. The result is a large collection of gradients of different morphogens with differing shapes throughout the disc (Brier, 2000).

1.1.3 *Drosophila* wing morphogenesis in the pupal stage

During the larval stages the wing disc develops complex patterns and morphologic regions but is still a comparatively simple structure relative to the adult wing. The complex three-dimensional structures of the adult wing develop during the pupal stage metamorphosis. These processes turn respective segments of the wing disc into the adult wing, hinge and notum, the dorsal side of the fly's thorax (Tripathi & Irvine, 2022).

The first stage of pupal morphogenesis is the eversion of the wing disc, which serves as a transition between larval and pupal development. During this the imaginal disc bends in a way that the apical surface of the disc ends facing the outside rather than an internal lumen (Figure 1) (Fristom & Fristom, 1975). To achieve this, the segment that forms the wing blade elongates and folds to create a hollow sack structure (Figure 1, II). The dorsal and ventral surfaces of the two ends are then attached to one another (Brabant et al., 1996). The wing is positioned facing the posterior and begins to elongate (Figure 1, III), during this the wall of the dorsal larval cuticle is ruptured and the wing is eventually pushed out (Figure 1, IV) (Waddington, 1940).

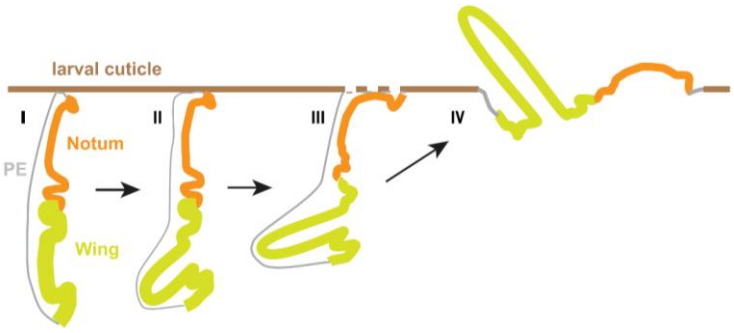

Figure 1. Model of *Drosophila* wing eversion. The future wing highlighted in yellow, notum in orange, peripodial epithelium (PE) in grey and cuticle in brown. (I) imaginal disc is attached to the cuticle by the PE. (II) The PE attaches to the cuticle and the wing pouch begins elongating and flattening. (III) The PE merges with the larval cuticle over the notum, ruptures and allows the wing disc to move through. (IV) the wing disc has everted to outside the larval cuticle. Adapted from (Tripathi & Irvine, 2022).

Following eversion, the next roughly 24 hours of pupal tissue dynamics can be divided into three stages: first apposition, inflation and second apposition (Bridges, 1920). In the first apposition stage from the beginning of pupariation to the 10 hours after puparium formation (APF) the cells of the wing change from a columnar to a cuboidal shape. This leads to the wings becoming much thinner, as the cells decrease in height and increase in base area (Waddington, 1940). During this stage the longitudinal proveins are formed as broad gaps between the dorsal and ventral epithelia (Blair, 2007). During the second, inflation, stage the two epithelia layers separate, the gaps between them are filled with fluid and the gaps between the veins are lost. This process starts at 8 to 9 hours after puparium formation and reaches its greatest extent at 12 hours APF (Waddington, 1939). The separation of the layers occurs because of an increase in pressure in the hemolymph between them. During the inflation stage the layers of the wing grow rapidly in size and shape. This includes cells that were previously prevented from undergoing mitosis. At around 20 hours APF the second apposition stage begins with the two layers re-opposing each other (Brown, 1993, Fristom et al., 1993). Only the cells of the veins do not re-oppose, maintaining a lumen between the two layers for fluid (Blair, 2007). During this stage cell growth slows down significantly and further development focuses more on changes in cell shape and differentiation (Fristom et al., 1993). Contraction of the wing hinge and anchoring of the wing to the pupal cuticle lead to the hinge decreasing and wing increasing in size, granting the adult wing its final shape (Johnson & Milner, 1987).

During pupal wing development, wing veins are formed along the wings. These veins are ectodermal tubes strengthened with a thick cuticle that act as support structures for the wing and as vessels for trachea, the air-filled breathing tubes of insects, nerves, hemolymphs and blood cells (Blair, 2007). The wing veins are orderly and in wild type flies will always follow an almost identical pattern. The veins in *Drosophila* are the five longitudinal veins (LV) running along the wing and the two crossveins running perpendicular to the LVs, these are the anterior crossvein (ACV) and posterior crossvein (PCV) (Waddington, 1940).

1.2 Molecular mechanisms in the developing *Drosophila* wings

1.2.1 *Drosophila* BMP type ligand Decapentaplegic

In the development of tissues and organs it is critical for individual cells to understand their position within the growing field. One of the key components in ensuring this connectivity is morphogens. Morphogens are signalling molecules secreted from a localized source that form a gradient of activity and effect (Schwank & Basler, 2010). In *Drosophila* the morphogen Decapentaplegic (Dpp) is widely used throughout the organism in different signalling functions throughout development (Restrepo et al., 2014). It is one of the best studied pathways in the fly and in the wing regulates several aspects such as wing vein patterning, cellular growth rate, differentiation fate and adhesion. Dpp is a fly homolog of the vertebrate Bone morphogenic proteins (BMPs) and its biological activity is highly conserved between the fly and mammals, leading to its value as a model in development (Lecuit, 1996).

In the "French flag model" of a morphogen the exponential decay of the signal leads to three distinct cell fates: the red with the highest concentration, a separate effect in the white area with a lower concentration and the "default" in the blue zone that does not receive a high enough signal to affect cell fate (Figure 2) (Wolpert, 1968).

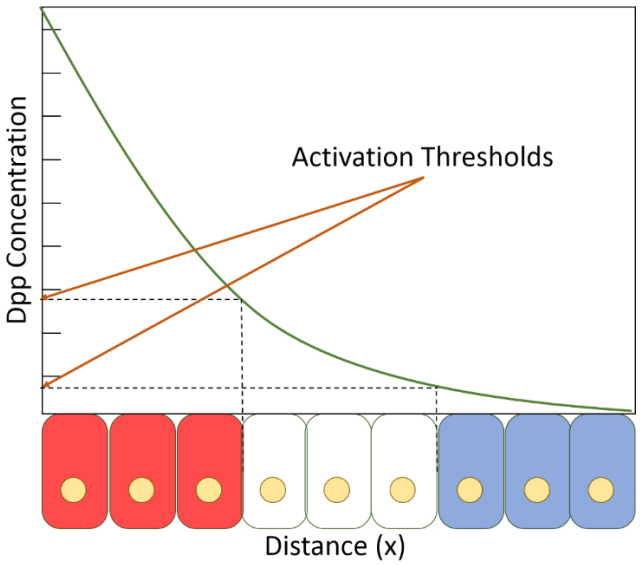

Figure 2 The French Flag model. The Dpp morphogen concentration is in relation to distance from the source, leading to different cell fates. The Dpp gradient decays exponentially, with the closest layer (red) of cells receiving a high concentration above the first threshold and will follow a certain cell fate. Cells coloured in white receive a weaker signal, but still above the second threshold, leading to them undergoing a different cell fate. Blue cells have a very low Dpp signal and develop as "default". Adapted from (Alber, 2018).

The range and activity of Dpp are largely controlled by the expression of its major type 1 receptor Thickveins (Tkv). Dpp binds to Tkv and the type II receptor Punt, forming a ligand-receptor complex which leads to the phosphorylation of Mothers against Dpp (Mad) inside the cell, turning it into phosphorylated Mad (pMad) (Neumann & Cohen, 2005, Affolter & Basler, 2007). pMad accumulates inside the cell, especially into the nucleus and regulates the transcription of various transgenes. Its accumulation can be used as an indicator of Dpp signalling activity by staining it with antibodies recognizing phosphorylated form of Mad and fluorescent molecules (Ashe & Briscoe, 2006). In the imaginal discs Dpp has been shown to downregulate *tkv* transcription. Most likely this repression takes place through Dpp-s downstream transcription factor or target genes. It is unclear how exactly Dpp downregulates the expression of its own receptor (Chen, 2019).

1.2.2 Dpp in the larval wing imaginal discs

During wing development it is crucial to establish distinct regions of tissues which will eventually come to form the compartments of the wing and give it its shape. This process begins with the establishment of morphogen gradients in the larval imaginal discs. Dpp plays a key role in these early stages (Paul, 2013). The disc contains these regions from its inception in the embryo, with one of the first to form being the anterior-posterior compartments. In order to form these, the transcription factor Engrailed (En) is expressed only in the posterior cells (Morata & Lawrence, 1975, Kornberg, 1981). En represses the expression of the transcription factor Ci, which in turn represses Hedgehog (Hh). This chain of molecular interactions results in Hh having its effect only in the areas directly adjacent to the posterior side, forming a thin line of Dpp expression along the anterior-posterior (AP) divide (Figure 3) (Lecuit, 1996).

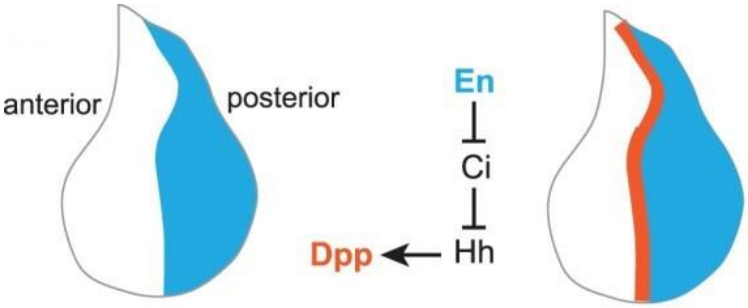

Figure 3. Morphogenic control of Drosophila wing disc anterior-posterior (AP) patterning. Formation of the AP divide in the imaginal wing disc using Engrailed (En)(blue), Cubitus Interruptus (Ci), Hedgehog (Hh), and the downstream formation of the Dpp (orange) line running along the AP divide. Adapted from Tripathi & Irvine 2022.

The most popular and widely accepted explanation for how Dpp spreads through the wing from the central AP line is extracellular diffusion. However, it has been debated to what degree this occurs through free diffusion or is restricted by binding to receptors and glypicans (Yan & Lin, 2009). In the imaginal disc it has been proven that Tkv levels influence the spread of Dpp. In a wildtype disc Tkv is expressed weakly in the centre and strongly along the edges of the disc, almost the opposite when compared to Dpp. When the expression of Tkv is altered artificially to occur either stronger throughout the disc or in random spots more toward the AP boundary it begins to limit the spread of Dpp, which can be visualised by observing Dpp-‘s downstream effects (Tanimoto, 2000). Thus, Tkv is used by the developing wing imaginal disc to regulate Dpp-‘s long-range diffusion into the tissue by forcing it to bind to the receptors instead of moving forward along the organ (Lecuit & Cohen, 1998).

The positioning of the forming morphogenic gradients and the pattern of cell division in the larval imaginal disc do not directly lead to the formation of the wing veins. The positional information of wing primordia appears to be identified in the larval wing imaginal discs through a morphogen-transcriptional network. Cell differentiation, however, does not become visible until the subsequent pupal stage (Celis, 2003).

1.2.3 Dpp in the pupal wing

For the formation of wing veins, a set of highly conserved signalling pathways are required. In the larval stages these signals work to initially define and set into place the future sites of the veins. In the pupal stages, however, the objective of these signalling molecules shifts mainly to maintaining the already existing patterns set into place earlier (Bryant, 1975). The role of Dpp changes considerably in the pupal stage compared to the larval. Seeming in effect to function more as a wing vein determinant than a typical morphogen (Matsuda & Shimmi, 2012). During the pupal stages Dpp stops being expressed along the A-P boundary and instead begins being expressed along the vein primordial cells. Dpp presence in the vein appears to be the primary signal to differentiate it from the intervein. As when Dpp expression is repressed in the wing the veins will not form, and when Dpp is expressed ectopically in the intervein area the whole wing will differentiate into a vein-like structure (Celis, 2003).

Initially the specification of the veins is undertaken by the Epidermal growth factor receptor (Egfr). It is required for it to be expressed continually along the wing veins to maintain vein fate and eventually induce the expression of Dpp (Zecca & Struhl, 2002, Yan et al., 2009). Dpp

is secreted by vein cells as either a homodimer Dpp:Dpp or as a heterodimer with another BMP-type ligand, Glass bottom boat (Gbb) Dpp:Gbb (Ray & Wharton, 2001, Matsuda & Shimmi, 2012).

Crucially Dpp is not produced in the crossveins and must be transported there through diffusion to initiate and maintain the vein cell fate. During the inflation stage Dpp can easily diffuse laterally over long distances and aid in rapid growth of the wing (Figure 4, B, B'). During the second apposition, however, Dpp can no longer maintain long-distance signalling (Figure 4 C, C') (Gui et al., 2019). Between the stages of 18 hours APF to 28 hours APF Dpp is not produced inside the PCV but is detectable there (Ralston & Blair, 2005). After 28 hours Dpp begins being expressed in the PCV itself. In the timeframe when it is not produced but is detected a system of BMP binding proteins facilitates the transport of Dpp out of the LV and into the PCV area. These proteins are Sog, Cv and Tlr. The Sog/Cv complex binds to Dpp:Gbb in the LV and facilitates its transport to the PCV area. In the PCV area Tlr protease cleaves Sog, releasing the Dpp:Gbb heterodimer into the PCV. After which the Dpp:Gbb binds to the Tkv-Punt receptor and promotes vein formation (Matsuda & Shimmi, 2012).

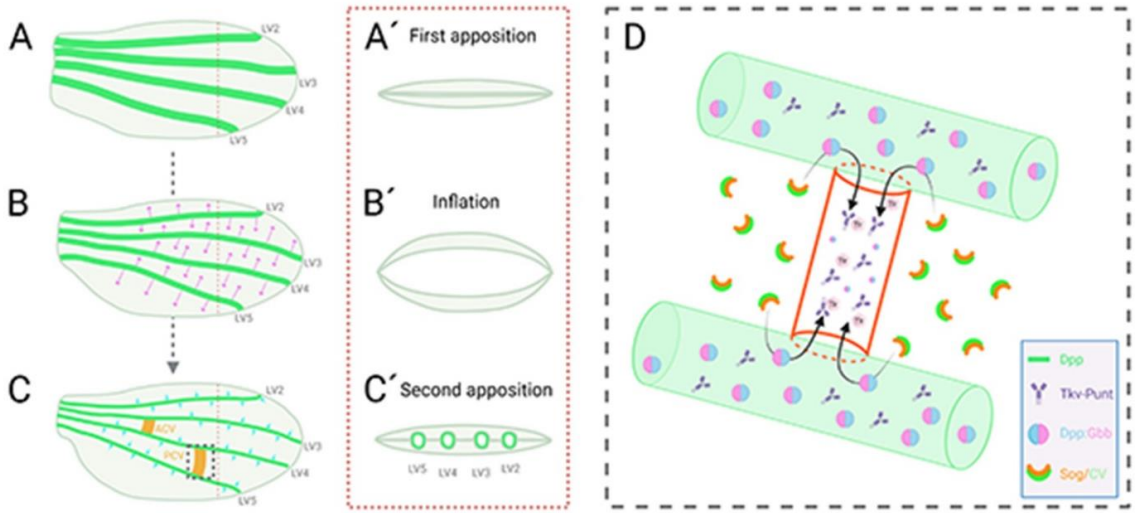

Figure 4. Posterior crossvein development of the *Drosophila* pupal wing. (A, A') During the first apposition stage the Dpp (green) is expressed along the longitudinal veins. (B, B') During the inflation stage the cell layers separate and Dpp diffuses laterally and forms a long-range signal (magenta arrows). (C, C') in the second apposition stage the cell layers realign and Dpp maintains a short-range signal (blue arrows). During this stage the crossveins (yellow) become visible. (D) A mechanism of Sog/Cv facilitated transport of Dpp is needed to form the crossvein while long range transport is not present. Dpp:Gbb forms a complex with the Sog/Cv, is transported out of the vein and broken down in the PCV area to release the Dpp. Adapted from (Antson et al., 2022).

The type I receptor Thickveins plays a crucial role in proper vein formation. Like its effects in the imaginal discs it serves to limit the diffusion range of Dpp. In flies whose *tkv* expression is

lowered the LV grow much thicker than in wildtypes. Giving the gene its name “thickveins”. In contrast if it is overexpressed it leads to the loss of Dpp in the crossvein area. During pupal development *tkv* is expressed at much higher levels in the cells immediately adjacent to the LV, functionally trapping Dpp inside the longitudinal veins until taken out by the Sog/Cv pathway (Ralston & Blair, 2005).

1.3 FLP-FRT recombination system

The FLP-FRT system is a genetic tool isolated from the yeast *Saccharomyces cerevisiae* genomes which is used in nature by the cell to manipulate specific portions of its own DNA to correct missegregation events (Schlake & Bode, 1994, Zhu & Sadowski, 1995). The system has two components: the recombinase flippase (FLP) protein, a member of the integrase family of site-specific recombinases, and FLP recognition target (FRT) sites. The FRT sites consist of three 13-bp elements which, when flanking a section of DNA act as binding markers for FLP. Upon binding to the FRT sites FLP induces a bend in the FRT site, cleaves either the top or bottom strand of DNA and induces its removal from that position in the genome. The system can be used for numerous genomic purposes including insertion, excision, inversion and translocation of the chosen fragment of DNA. In excision the segment of DNA flanked by FRT sites is removed from the genome completely when FLP is either expressed inside the cell or introduced to it from the surrounding environment (Figure 5) (Zhu & Sadowski, 1995).

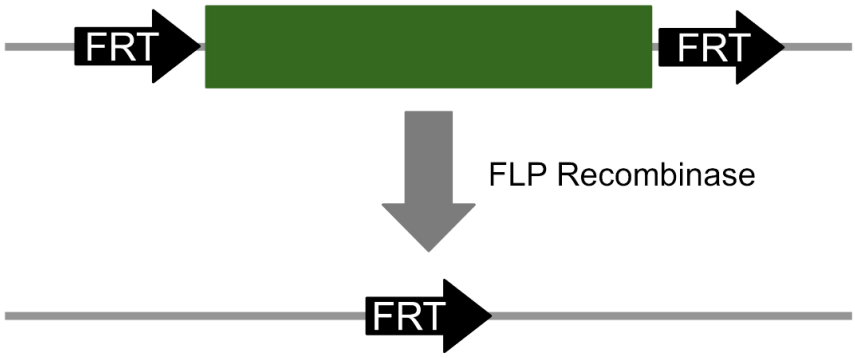

Figure 5. Model of the FLP-FRT systems usage in excising a segment of DNA. The desired segment of DNA between two FRT sites (green) is removed from the genome after FLP recombinase is applied. Both ends are reattached, and the total length of DNA continues being used by the cell normally. Adapted from (Muratoglu, 2018).

The FLP-FRT system has been shown to be highly efficient in *Drosophila* and gives a less leaky expression when compared to alternatives such as the UAS-Cre system. Compared to RNAi

based methods it has the critical advantage that cells affected by the FLP-FRT lose their gene function completely and there is no risk of off-target effects. Because of this it is a valuable method for cell-specific gene inactivation in the fruit fly (Frickenhaus et al., 2015).

Heat shock proteins are expressed and produced as a response to environmental stress from high temperatures. In *Drosophila* and various other *Diptera* species these proteins are naturally occurring in the fly's genome. If the FLP protein expression is combined with and put under the control of a heat shock promoter, then the expression of FLP and thus excision of the target DNA sequence can be artificially induced and controlled simply by exposing the flies to high temperatures. This, however, only takes significant effect if the fly is in the process of cell division and thus is most effective in the larval and early pupal stages (Astakhova et al., 2015).

2. EXPERIMENTAL PART

2.1 Aims of thesis

The primary purpose of the thesis is to better understand the mechanisms by which receptors influence the regulation of morphogen signalling, and through this the formation of tissue morphogenesis. Two aims were chosen to achieve this:

1) To generate a mosaic of altered Tkv receptors in the organism which remains under the control of the endogenous *tkv* enhancer, allowing for controlled mosaic clone formation.
2) To elucidate receptor placement and patterning on mosaic wings using immunohistochemical analyzes and characterize 3D communication in wing cells both horizontally between individual cells and vertically between the cell layers with sufficient quality to distinguish different cell types.

2.2 Materials and methods

2.2.1 Fly stocks

To generate flies with the desired mosaic forming genotypes, two different fly stock were crossed with each other. The first stock: *w; tkv:GFP>>Q199D-mCherry / Cyo* (obtained from Takuya Akiyama (Akiyama et al., 2018)).

- TkvGFP is a wildtype Tkv receptor tagged with green fluorescent protein.
- TkvQ199D-mCherry (TkvQD-mCherry) is a constitutively active mutant of Tkv, which does not require the presence of Dpp to begin phosphorylation of Mad inside the cell. It is tagged with the fluorescent protein mCherry.

The Cyo, Curly of Oster, balancer is easily visually detectable due to the wings curving backwards in shape. Over time however this balancer can be lost and only the *tkvGFP>>tkvQD-mCherry* alleles remain.

Males of this non-curly phenotype were picked up guaranteeing homozygous *tkvGFP>>tkvQD-mCherry* flies. These flies were then crossed with virgin female flies collected from a *yw ; hsFLP[70] / Dp(1;Y)y ; Tm2 / Tm6Sb* stock (obtained from Bloomington Drosophila Resource Center, #6419). This cross would guarantee that 100% of the offspring would be heterozygotes with one copy of both the fluorescent protein tagged *tkv* gene and the heat shock controlled *FLP (hsFLP)* gene.

The gene array is structured in the following order: the natural *tkv* enhancer; *tkvGFP*, flanked by FRT sites; *tkvQD-mCherry* (Figure 6). This structure will ensure that without a heat shock *tkvGFP* will be expressed throughout the fly in wildtype levels. However, when a heat shock is applied a randomly varying number of cells throughout the larva will begin expressing FLP and have the *tkvGFP* excised, putting the t*kvQD-mCherry* under the control of the enhancer. These clones of *tkvQD* would continue dividing while maintaining the new *tkvQD* genotype forming spots of varying size throughout the organism.

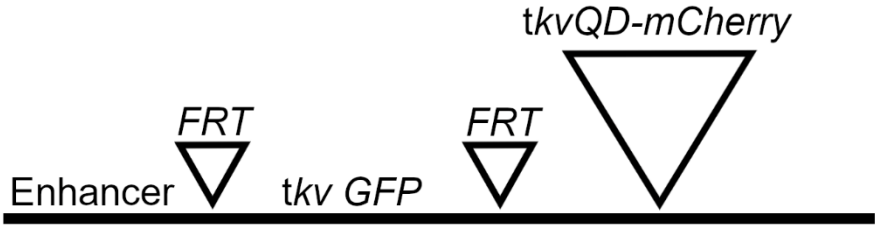

Figure 6. Gene array used to generate TkvQD-mCherry clones. The system is under the control of the natural *tkv* enhancer, followed by TkvGFP flanked by flippase recognition sites, and lastly TkvQD-mCherry. during heat shocking TkvGFP is excised and TkvQD-mCherry starts being expressed.

2.2.2 Immunohistochemical staining protocol

Upon the male and female flies being introduced to a fresh food vial the time was marked. The flies were then raised at 25ºC for either 72, 96 or 120 hours to test at which stage of development the heat shock would produce the best patters of mosaic for analysis of the effect of individual spots (Figure 7).

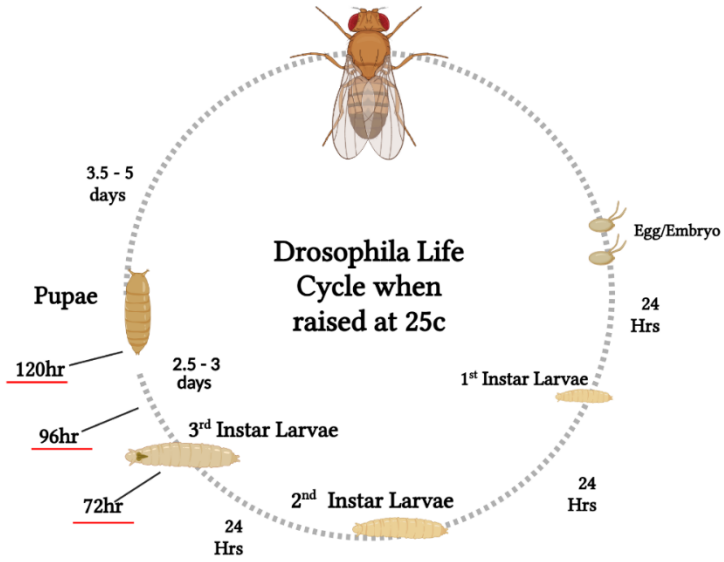

Figure 7. *Drosophila* life cycle with 72, 96 and 120 hours after egg laying marked. Created using Biorender.com.

Too dense of a pattern of small mosaic spots would make reaching conclusions on their effects on neighbouring cells impossible. After being grown at 25ºC for the appropriate time the larva would be heat shocked at 36ºC for exactly 20 minutes in an incubator (Orbital Incubator SI50

by Stuart Scientific). The vial with the larvae would then be returned to the regular incubator at 25ºC. The larva would then be monitored daily to catch the exact day that pupa would form. Due to the heat shocking the normally predictable time frame of the fly's life cycle was altered, with the larval stage being considerably longer than in non- heat shocked flies. This was expected due to Tkv being present throughout the fly, not only in the wings and its disturbance in vital organs could lead to mortality or in this case a longer period of development.

Upon the flies emerging from the food and preparing for pupariation the vial was set aside for closer monitoring. The larvae would have to be collected and set aside as soon as they entered the pupal stage, to guarantee that that the flies being analysed and photographed were in as similar a developmental stage as possible. To achieve this, "white pupa", the earliest and a visually identifiable stage of pupariation, would be collected and transferred to a new vial every hour. These groups of collected white pupa would be marked with the time they were collected. The next day, either 26 or 28 hours after the white pupa were collected, preparing would continue. Pupa were grown for 26-28 hours due to two reasons: first the wing being as close to the final adult wing as possible, and second that the wing is easier to dissect the more the fly develops. It is feasible to successfully collect 100% of the wings from a pupa grown for 28 hours, but the success rate gets considerably lower at earlier development stages. At 18 hours APF the wings are less rigid and are covered much more tightly by the wing cuticle, making it harder to remove. Because the cuticle must be removed for clear imaging many wings are lost due to physical damaging during dissection in earlier stages.

After being grown at 25ºC for 26-28 hours the flies would be fixed in 3,7% formaldehyde. To do this the front head part of the pupal case was removed under a stereomicroscope (Nikon SMZ745) using forceps. A small hole was made using the forceps into the neck area, just behind the eye to allow the formaldehyde (FA) to better penetrate the tissue. For the fixing a solution of Phosphate-buffered saline (PBS) was prepared. This would then be mixed with Triton X-100 (by Fisher Bioreagents) to form a 0,3% PBS with Triton (PBT). Triton was added to permeabilize the membranes in the pupa and allow for the FA to diffuse more easily throughout the tissues. Finally, FA was added to this to form a 3,7% formaldehyde PBT solution. The pupa would be submerged in the FA solution over two nights at 4ºC. This guarantees that the tissues inside the pupa cease development.

After fixing the pupa would be washed with PBT to remove excess FA, stopping the fixation, making working with the pupa safer and removing any pieces of food, pupal case or

other debris that might have been attached to the flies. The second dissection would then be carried out: first the pupa would be submerged in a small drop of PBS on a soft silicone pad. Then, under a stereomicroscope (Nikon SMZ745), the entirety of the pupal case would be removed. The pupal wings could then be taken out and put into a smaller separated drop of PBS on a concave glass slide. After all the wings had been collected the cuticle would be removed from the wings. After the removal of the cuticle the wing would be taken to a second concave with a drop of PBS on the slide to further clean it of any pieces of cuticle or wing hinge that might have stuck to it. Finally, the clean wings would be transferred to a clean drop of PBT on a third concave slide. The wings could then be pipetted to an Eppendorf in which a mixture of PBT with 10% Normal Goat Serum (NGS) had been prepared and mixed to block the wings. During blocking NGS binds uniformly to antigens throughout the tissue, saturating them. This prevents the primary antibody used later for staining from binding non-specifically and causing background noise. The wings would be kept in the blocking solution overnight at 4ºC.

The next day in the morning the blocking solution would be removed, being careful to keep some in the Eppendorf to prevent also pipetting up the wings. Next the primary antibody solution was added to the wings. This consisted of an anti pMad antibody (P-Smad Rabbit mAb by Cell Signalling Technology) at 1:500 concentration, 5% NGS and PBT. The primary antibodies were added in the morning to ensure that they can be left on for longer than the secondary antibodies. The wings with the primary antibodies added would be left overnight at 4ºC. In the evening of the next day the primary antibodies would be removed, after which the wings would be washed three times with PBT with each instance of washing being left for 15 minutes to remove the primary antibodies. After the washing, secondary antibodies could be added. The secondary antibodies consisted of: for anti-pMad either Rabbit 568 or 633 (Goat anti-Rabbit IgG Cross-Adsorbed Secondary Antibody Alexa Fluor 568/633 by Thermo Fisher Scientific) at 1:200 and 4′,6-diamidino-2-phenylindole (DAPI) (by Thermo Fisher Scientific) at 1:1000. Anti-pMad and DAPI were added together. More fluorescent regions couldn't be reliably used due the already present GFP and mCherry in the genome.

The secondary antibodies would be left on overnight at 4ºC. In the morning the wings would again be washed three times each for 15 minutes with PBT. After washing the wings would be mounted with Vectashield (by Vector Laboratories) on a glass slide and then sealed to prevent evaporation and contamination. Initial images were taken using an epifluorescence microscope (Olympus BX51, with an Olympus DP71 camera) to confirm that the fluorescent staining had worked, after which confocal microscopy was used.

Two slides of wings were prepared for microscoping. Due to emission maximum overlap three different fluorescent colours were chosen to be used on either slide. DAPI was used on both slides to make locating the wings easier under the microscope with its strong signal but was not used during later microscopy due to overlapping and conflicting with the GFP fluorescence. The two slides prepared were (Table 1):

1) Stained with anti-pMad and Rabbit 633 (Rb633). Using Rb633 allowed visualizing both TkvGFP, TkvQD-mCherry and their pMad product. Further referred to as experiment one.
2) stained with anti-pMad and Rabbit 568nm (Rb568). Due to mCherry overlapping with Rb568 it could not be observed on this slide. Further referred to as experiment two.

Table 1. heat shock timing, fixing time after pupa formation and fluorescent colours visible on slides in the thesis. By author.

			fluorescent colours			
	heat shock time AEL	time after pupa formation	1	2	3	4
1. Exp. 1	96 h	28 h	pMad Rb 633 1:500	DAPI 1:1000	TkvGFP	TkvQD-mCherry
2. Exp. 2	96 h	26 h	pMad Rb 568 1:500	DAPI 1:1000	TkvGFP	

Flies were heat shocked 4 days, roughly 96 hours, after egg laying (AEL), and fixed 28 or 26 hours after pupa formation.

2.2.3 Microscopy

Slides were first inspected using the Olympus BX51 fluorescence microscope with 20x and 40x objectives and photographed with a Olympus DP71 camera. To see the effect clones might have on the 3D structure of the bilayer wing the slides were analysed using an Olympus FV1000, and Zeiss LSM900 confocal microscopes, with 60x objectives. Imaris viewer 10.2.0 by Oxford Instrumentals was used to process and analyse the confocal z-stack scans. On high-definition scans DAPI would bleed-through to TkvGFP, because of which it was not included in further confocal z-stack imaging. On average each scan included around 30-40 stacks.

2.3 Results

2.3.1 Establishing a protocol for the mosaic analysis of Tkv

I first attempted to establish optimal conditions to generate a mosaic of TkvQD suitable for addressing my aims. Initial tests with setting up the mosaic stock and heat shocking proved successful. Although the first groups of hatching pupae appeared to be ones without flip-out, the later ones clearly had the mosaic wing patterns.

To gauge the general effects of creating TkvQD clones on the fly's survival rates, a test was set up to compare different heat shocking times. Three independent crosses were set up for the genotype *hsFLP/+; tkvGFP>>tkvQ199D-mCherry/+* flies. These crosses were heat shocked for 20 minutes at 72, 96 or 120 hours after egg laying. The resulting mosaic clone phenotypes vary depending on the timing of the heat shock. The adult flies were examined and mosaic forming flies were counted based on the visual detection of spots visually similar to veins but located in the intervein area (Figure 8). The number of mosaic wing possessing flies were counted and measured as a percentage of all flies that hatched (Table 2). The results show that larvae heat shocked at 72 hours after egg laying (AEL) did not produce any visually identifiable clone mosaic flies, suggesting that mosaic flies are lethal. The greatest amount of mosaic clones was in the 96-hour heat shocking time, at an average of ≈25% and a slightly lesser amount, ≈15% at 120 hours (Table 2). Based on initial visual assessment of the patterning on adult wings, 96-hour AEL heat shock larvae were selected for adult wing imaging.

Table 2. Percentages of flies with mosaic wing phenotypes at differing heat shocking time points. Flies were inspected and counted based on visual detection of irregularities in the intervein area. N = 50 (72h), 51 (96h) and 148 (120h).

	72h	96h	120h
% of mosaic clone phenotype flies	0%	24,70%	14,90%

2.3.2 Adult wing phenotypes with TkvQD mosaic clones

The wings of the observed adult flies possessed easily identifiable TkvQD mosaic clones, which appeared visually distinct from one another in patterning (Figure 8). Two general types of clones were noted. On type A clones, many small dot-like cluster of darker cells had formed (Figure 8, A). In type B the TkvQD mosaic clones were larger and had formed vein-like structures in the intervein area, however were fewer in number throughout the wing (Figure 8, B). Occasionally, these structures would meet up with the real veins, causing them to bend, branch off or develop gaps (figure 8, B, white arrowhead). On a few wings both phenotypes were present but on most one dominated.

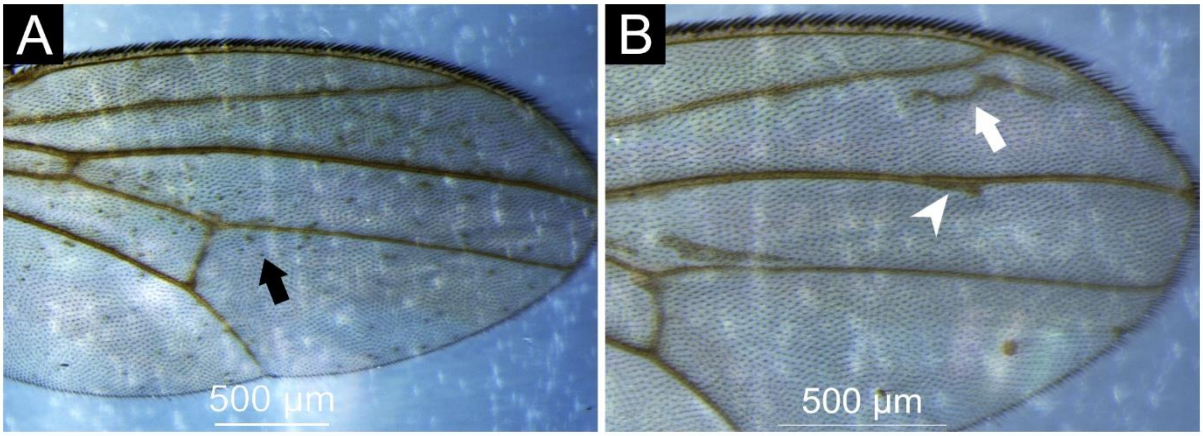

Figure 8. TkvQD mosaic clones' morphology in the adult wing. (A) small dot like clones throughout the intervein area, one highlighted with a black arrow. (B) large, vein-like structures in the intervein area (highlighted with a white arrow), sometimes merging with the wing vein or causing offshoots from it (highlighted with a white arrowhead). Scale bars: 500 µm.

Visual loss of the wing vein appeared much more significant on the wings with clusters of small clones rather than those with large regions (Figure 9). On some wings large type B clones were positioned over the crossvein but did not appear to disrupt its formation significantly (Figure 9, A, B). In comparison, even relatively small and distant dots of clones appeared to cause large parts of the PCV to disappear (Figure 9, C, D). This loss of PCV wasn't, however, complete, with areas of the PCV distant to the clones seeming to form normally, though slightly weakened and more transparent (Figure 9, C, D).

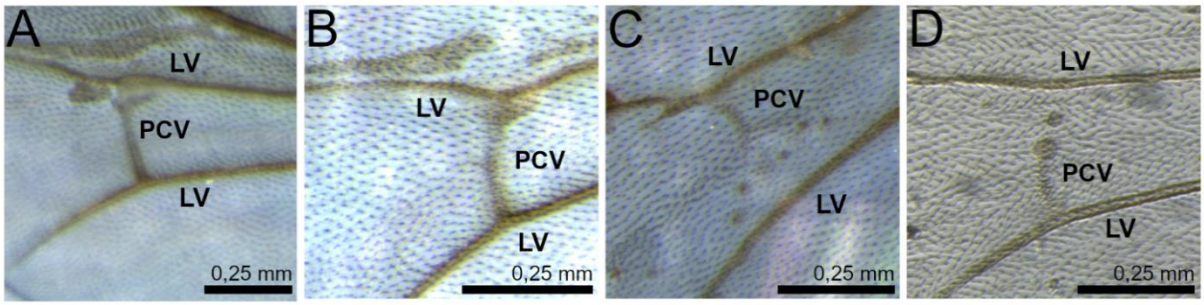

Figure 9. Vein- and dot-like TkvQD mosaic clones overlapping with the posterior crossvein (PCV). (A, B) bigger, singular clones overlapping with the PCV that do not seem to negatively influence its formation. Slight increase in transparency noticeable near the clones. (C, D) Smaller dot shaped clones on or near the PCV cause its complete loss in areas near the clones. On C the PCV is lost further away from the clones while on D the clones disappear directly after the clone. Labels for the PCV and longitudinal veins (LV) added to the figures for clarity. Scale bars: 250 µm.

2.3.3 Immunofluorescent analysis of wing discs with TkvQD mosaic clones

The wings showed clear disturbances in both the general cellular and Tkv patterns (Figure 10). Although the time after egg laying of the heat shocks was the same in both experiments, 96 hours, the patterns of TkvQD clone formation were quite different: wings from experiment one possessed on average much fewer and smaller TkvQD clones (Figure 10, A-C). On wing A no TkvQD clones had developed on the wing, result in it imitating the normal distribution of Tkv (Figure 10, A). In comparison the wings of experiment two had a majority of the intervein area covered in TkvQD clones of varying sizes (Figure 10, D-F).

Due to the mCherry signal being weak it could not be observed precisely before confocal imaging. Clones were visible by observing the areas with loss of TkvGFP signal. The altered patterns of TkvGFP distribution showed very clearly that the TkvQD clones generated by heat shocking had, as expected, grown and formed relatively large areas throughout mostly the intervein areas (Figure 10, B-F).

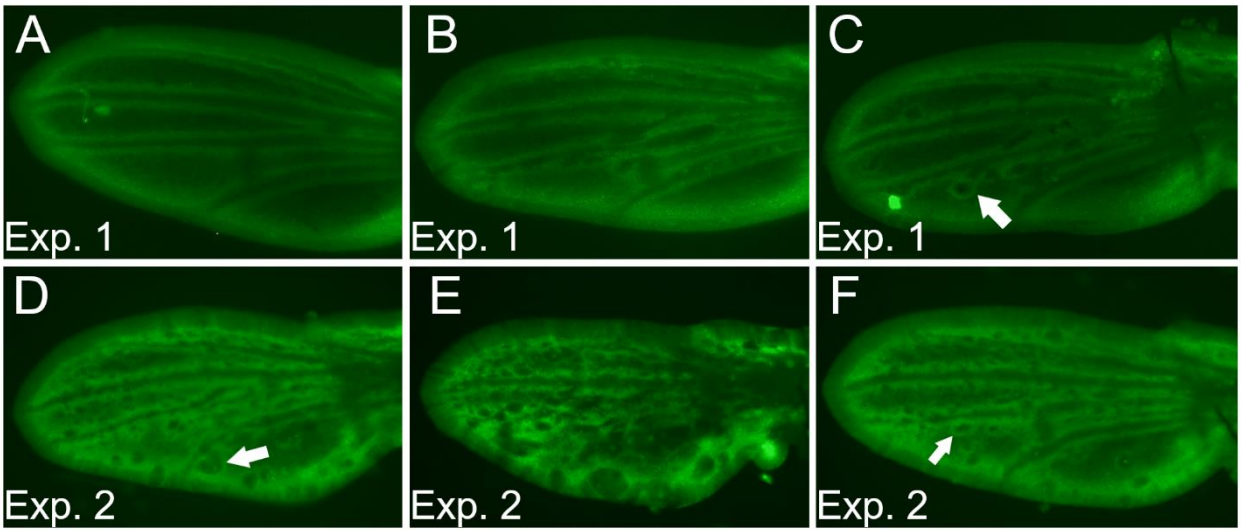

Figure 10. TkvGFP epifluorescence images showing differing intensities of TkvQD clone formation on two different slides of wings. TkvQD – mCherry Clones are visible by observing the areas with loss of TkvGFP (green) signal, examples indicated by arrowheads. (A) Wing with a minimal number of clone formations, showing the TkvGFP patterning when not disturbed. Stronger TkvGFP signal around the wing veins. (B, C) smaller and fewer clones. The effects of individual clones on the intervein area are more easily observable. (D, E, F) Dense mosaic of smaller TkvQD clones. The density of clones makes observing their individual effect difficult. Wing shape seems to be affected.

It was observed that TkvGFP formed highly elevated signal strength areas along the edges of the newly formed TkvQD clones in the intervein area (Figure 11). This looks similar to the TkvGFP flanking regions around the veins (Figure 11). This elevated area of Tkv around wing veins and clones will further be referred to as the Tkv barrier. These barrier areas were roughly the same width as the ones surrounding the veins and a relatively similar signal strength.

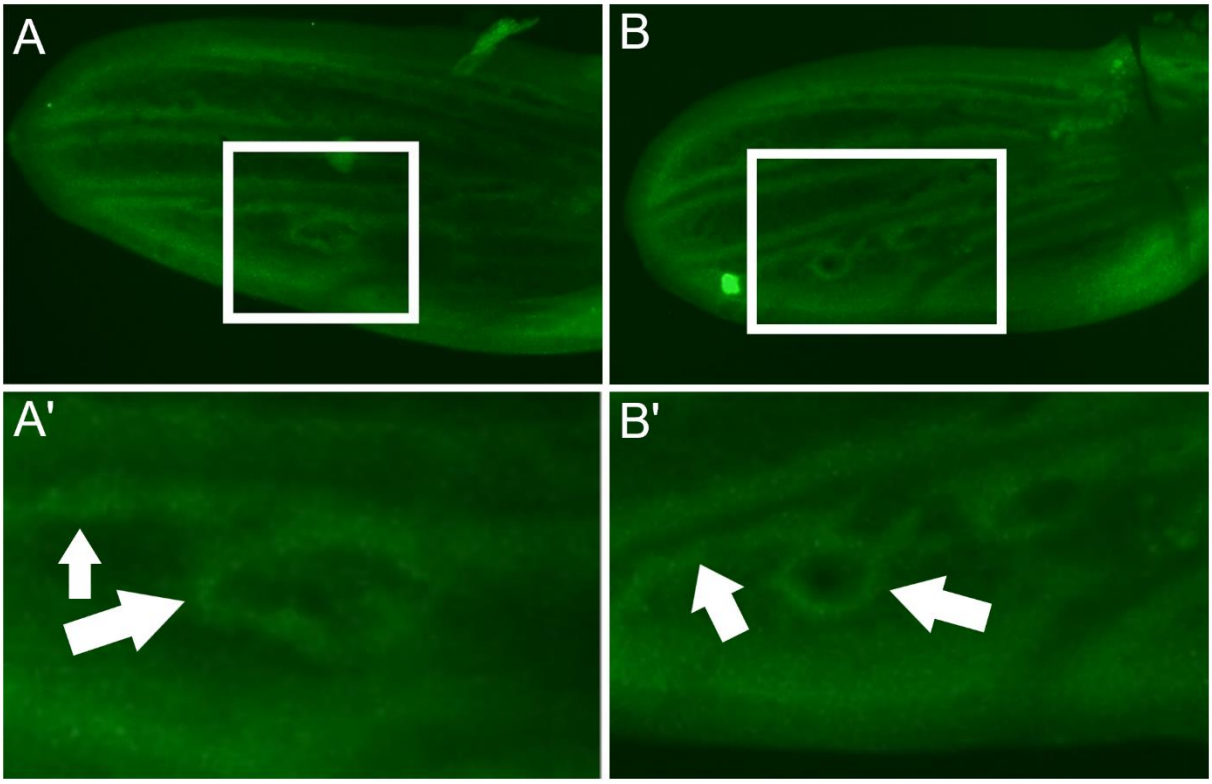

Figure 11. Examples of a Tkv Barrier around small circular TkvQD clones in the intervein area. TkvQD clones visualized by gaps in the TkvGFP (green) signal. Arrows indicating the TkvGFP barrier area around the wing vein and around the clone on both A' and B'. (A, A') a small, irregularly shaped clone, the TkvGFP barrier around the clone has roughly the same signal strength as the nearby vein Tkv barrier. (B, B') Two small circular clones with a clear surrounding Tkv barrier.

2.3.4 Analysis of mosaic TkvQD clones using confocal microscopy

Following confirmation that the fluorescent staining was visible, the wings were analysed with confocal microscopes. Z stack scans were taken of analysed wings.

Confocal imaging confirmed that TkvGFP and TkvQD-mCherry clones are complementary (Figure 12). Individual clones took a wide variety of shapes even on a singular wing. Noticeable again were two general types of clones, matching the ones seen in adult wings: Type A: roughly circular or square shaped clones, usually located farther away from the wing

veins (Figure 12, A). Type B: long, linear clones following the edge of the wing veins (Figure 12, B). these will be referred to as either circular or linear clones for simplicity. Linear clones appeared to be larger than circular ones and would on some wings stretch almost half of the wing's length along one of the veins.

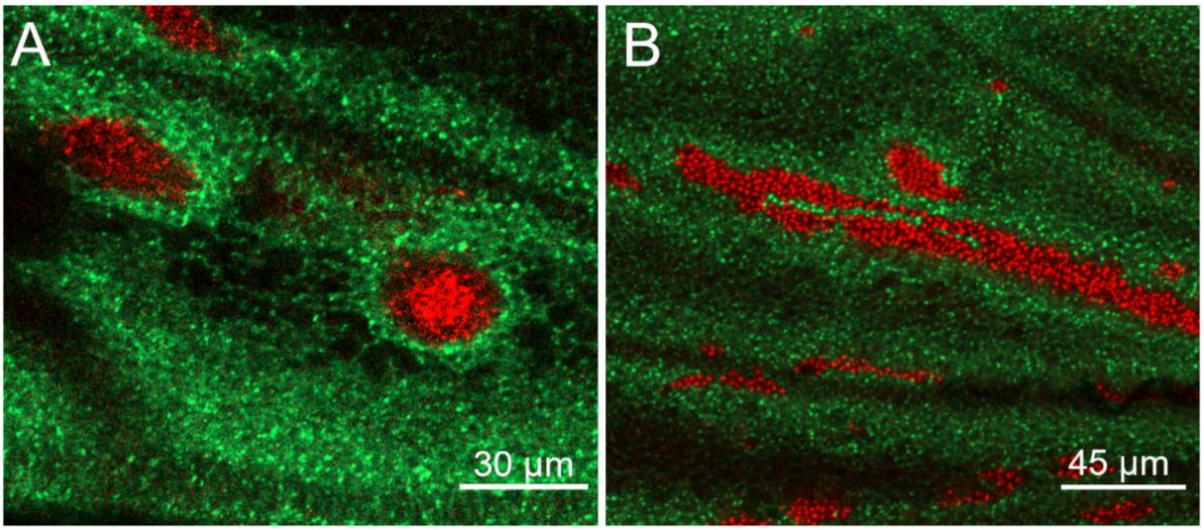

Figure 12. TkvQD- mCherry clone formation in the intervein area. (A) Smaller circular TkvQD clones (red). Neighbouring wild type TkvGFP (green) cells directly surrounding the clones possess significantly more of the receptor than regular intervein cells, with a sharp cut between barrier and regular intervein cells. (B) linear shaped TkvQD clones along the wing veins. Small linear clones are a few cells wide but more than ten long. Large linear clones span large parts of the wing and are comparable in width to a wing vein. Scale bars: (A) 30µm and (B) 45µm.

2.3.5 Changes in wing planar structure and concaving

The Dpp-Tkv signal serves as a determinant of wing vein development through inducing cell shape changes. Therefore, I wondered whether TkvQD clones may sufficiently act as wing vein tissues and begin to form vein-like structures such as the basal lumen, or if clones formed in two-layered epithelia at the identical position may be needed. Singular clones on one epithelial layer with wild type cells (no TkvQD clones) on the opposite side showed little to no structural changes. However, if two TkvQD clones had formed on the exact same position, but opposite sides of the wing it appeared to deform: the apical cell layer would bend inwards, towards the other cell plane and form a concave structure (Figure 13). No outwards concaving or initial formations of interior lumens were noted. This is further referred to as apical constricton.

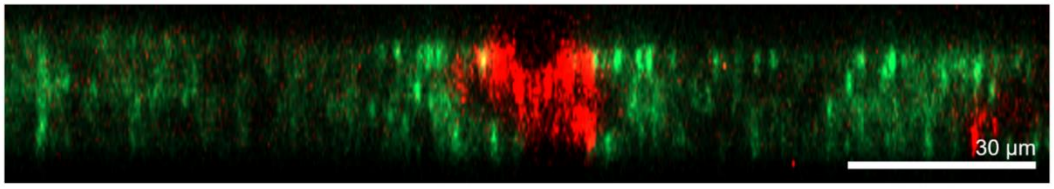

Figure 13. Confocal image of a cross section of the two layered pupal wing with two opposing TkvQD- mCherry clones. Clone cells marked in red and the surrounding TkvGFP in green. The clones do not form a vein-like basal lumen structure. Both clones are concaved inwards, towards one another. The concaving is much more noticeable on the upper clone, almost forming a "U" shape. Image taken using the Zeiss LSM900 confocal microscope. Scale bar: 30µm.

The same TkvQD clone site was then imaged using the Olympus FV1000 confocal microscope in higher quality to see the Tkv localization in individual cells. In overlapping clones with apical concaving TkvQD clones showed considerable widening towards the basal side of the cell layer (Figure 14 A). The apical side of the clones were considerably less wide that basal side, with surrounding TkvGFP cells moving in and forming an overlapping area (Figure 14, B, C).

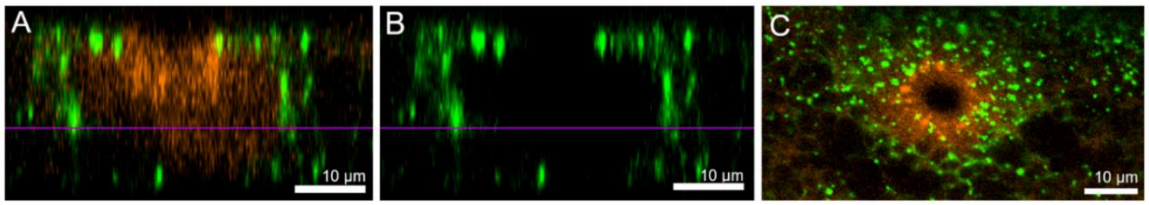

Figure 14. Cross sections (A, B) and apical compartment top view (C), showing basal expansion of overlapping clones. (A) TkvGFP (green) seen strongly expressed over the top of a TkvQD – mCherry (orange) area. Middle of the wing is highlighted with purple. The basal compartment of clone cells is expanded and is present underneath the apical compartment of adjacent TkvGFP cells. (B) Gaps lacking TkvGFP, in which only TkvQD is present can be seen on both the top and bottom side. Middle of the wing marked with purple. (C) top view of the of the top clone from A, B. TkvGFP cell receptors seen overlapping with the lower TkvQD-mCherry ones, covering them almost entirely, except for the small region of clone cell apical sides in the middle. Images taken using the Olympus FV1000 confocal microscope.

2.3.6 Laterally positioned TkvGFP surrounding the TkvQD-mCherry clones

It was noticed that on cross sections of circular clones the elevated flanking TkvGFP signal would also appear to be much stronger along the lateral, not just on the apical and basal sides (figure 15, A, B).

To confirm whether this was unique to the clones or a general mechanism: wing veins were also investigated for this pattern. On analysis the same positioning of TkvGFP was observed on the wing veins, with cells adjacent to the vein expressing TkvGFP along their lateral sides (figure 15, C).

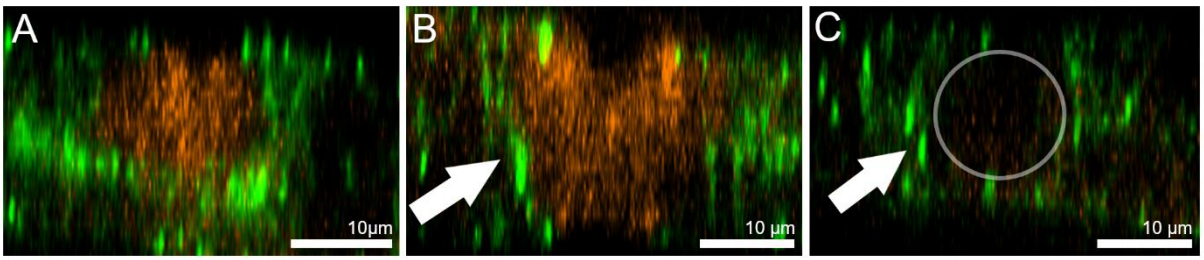

Figure 15. Laterally located TkvGFP surrounding TkvQD clones and the wing vein. (A) one wing layer clone showing strong lateral TkvGFP (green) localization directly adjacent to TkvQD clone (orange) cells. (B) two clones opposing on both sides of the wing, strong TkvGFP signal along the edge of the clones, indicated with an arrow. (C) cross section of a longitudinal wing vein showing high TkvGFP surrounding the vein, including a strong signal around the lateral side of adjacent cells. Flanking lateral TkvGFP signal highlighted with an arrow. Rough position of the Longitudinal vein shown using the grey circle. Images taken using the Olympus FV1000 confocal microscope.

2.3.7 Uniform signal level of TkvQD-mCherry inside cells of the clone and surrounding area

It was observed that inside of TkvQD-mCherry clones the TkvQD receptor signal was detectable not only at the membranes of the cells but also at a weaker level inside the cell (Figure 16 B). The signal was strongest at the apical side of the cells and proceeded to form a gradient towards the basal side. Lateral placement of TkvQD akin to that of TkvGFP in Figure (15, B) were not noted during analysis. In addition, a weaker signal of TkvQD-mCherry was detectable around the wing, not only inside clearly defined clone sites (figure 16, A). This accumulation was most noticeable around the edges of the wings and visually formed a honeycomb-like signal. The TkvQD signal appeared to be stronger on the wing layer with a clone present (figure 16, B).

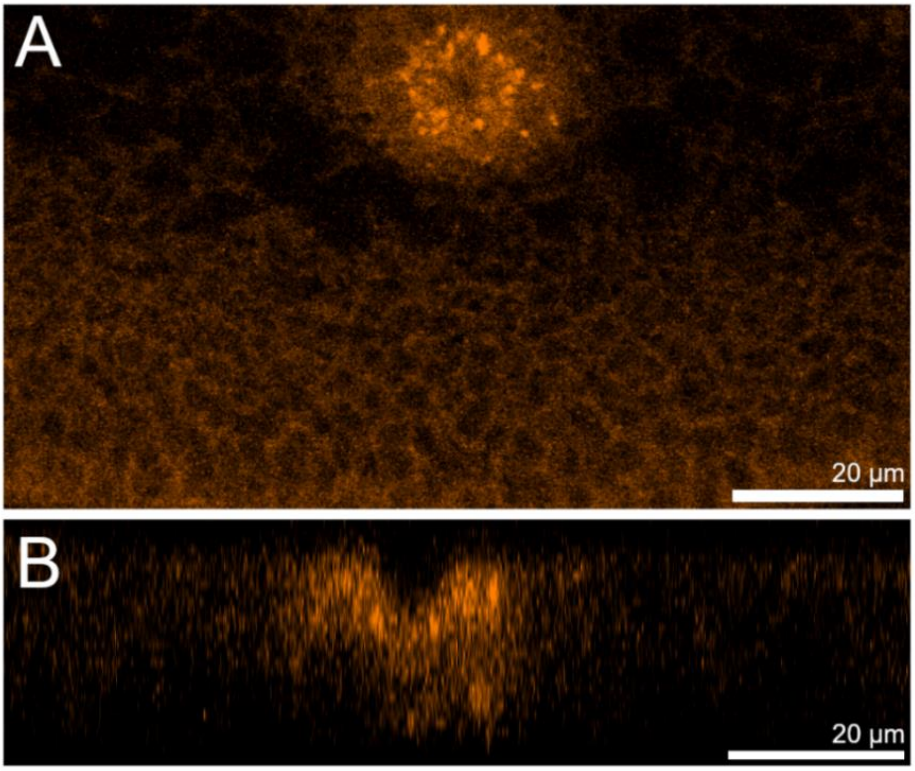

Figure 16. Top view and cross section of a TkvQD clone showing a positive signal outside the clone areas. (A) a honeycomb pattern of TkvQD is seen in the lower side, closer to the edge of the wing. The immediate area around the clone has a very weak signal, similar to the regular intervein area. (B) cross section of the clone from image A, the signal of TkvQD appears to be stronger on the layer of the clone.

2.4 Discussion

2.4.1 Issues with the heat shocking protocol

In this thesis project, I first established a protocol for mosaic analysis of TkvQD expression the pupal wing. There are two issues to be discussed.

First, the timing of the heat shock is critical. I compared three different timings: 72h, 96h, 120h after egg laying, and found that no mosaic flies were present at 72h. I assume that mosaic flies heat shocked at 72h were lethal. Since 72h AEL is at the early 3rd instar larval stage, cell proliferation is very active, leading to larger clone sizes. Using a heat shock promoter to express Flp, mosaic clones can be generated throughout the body. Ectopic BMP signalling is known to be toxic in some contexts, and generating larger clones could be lethal (Buratovich & Bryant, 1995). Heat shock at 96h still allows proliferation to reach substantial clone sizes, which might not be too harmful for the fly's survival. In contrast, heat shocking at 120h is close to the end of the 3rd instar stage, where proliferation ceased. In addition, proliferation occurs in the pupal wing only a few times, therefore, clones might be generated but the size might be too small to detect in my adult wing screening.

Second, my results provide two different types of clones, termed in this study as type A, circular and type B, linear clones (Figures 8-10). Although the precise mechanisms underlying the formation of these different shapes remain to be addressed, we may consider the following mechanisms. If the clone cells are generated on the proximal side of the larval wing imaginal discs, proliferation may actively extend into the distal side, resulting in long thinner clones (type B). In contrast, if the clone cells are on the distal side, the number of proliferations might be more limited and mitotic orientation could be more random, resulting in more round shaped clones (type A).

2.4.2 Loss of the crossvein from morphogen competition

Previous studies revealed that ectopic expression of TkvQD in the pupal wing sufficiently disrupted PCV formation through the loss of pMad signal when tkvQD was overexpressed using the UAS-Gal4 system (Toddie-Moore, 2022, Gui, 2016). Therefore, it has been proposed that competition mechanisms for BMP signaling may play a critical role in wing vein patterning. To further understand this, I employed FLP-FRT mediated tkvQD mosaic clone analysis, with

tkvQD expression under the control of the endogenous *tkv* enhancer. Compared to previous overexpression of TkvQD (Toddie-Moore et al., 2022), PCV formation appears to be varied. Although partial loss of PCV or ectopic vein phenotypes around PCV are observed when TkvQD clones are generated, PCV formation remains robust in most mosaic analysis of TkvQD expression. Therefore, the previous competition model has not been fully supported. Further experiments will be needed to confirm this hypothesis.

2.4.3 Lateral localization of Tkv receptor and a Tkv barrier formation model

One of the unique aspects of Dpp signaling during wing development is that signaling ranges are adapted in a context dependent manner: long-range in the larval wing imaginal discs and inflation stage of the pupal wing, and short-range at the second-apposition stage of the pupal wing (Lecuit, 1996, Nellen, 1996). Although *tkv* transcription has been proposed to complement Dpp signaling to sustain Dpp diffusibility in both the wing imaginal disc and pupal wing, how Tkv contributes to both long- and short-range signaling remains unclear (Firth et al.,2010). This study provided subcellular resolution of Tkv protein distributions under various conditions including flanking TkvQD clones and vein progenitors and their flanking.

My results showed that TkvGFP proteins not only localize at apical and basal compartments but also laterally when cells are adjacent to the TkvQD clones (Figure 15). This might provide an answer as to how Dpp transitions from long-range to short range signaling throughout development. A high concentration of lateral Tkv placement could effectively block out the horizontal transport of Dpp and lock it into the wing veins. If most of Dpp diffusion in the wing veins occurs through lateral diffusion, then this could reliably block the Dpp molecules from reaching further than a few cells into the barrier area. Based on my results, I propose the Tkv barrier model.

The Tkv barrier forms during the wing development to prevent Dpp from dispersing too far from the vein area. In the case of a constitutively active clone the Dpp gradient should remain completely unaffected. The only change from a wild type scenario should be in the cells perception of the amount of Dpp in their environment. During the mosaic experiment, however, a clearly noticeable buffer zone of high Tkv area formed around the TkvQD clones.

Based on this, the Tkv barrier does not appear to form as a response to a progressively weaker Dpp signal. Instead, some other, post receptor activation signal traveling from inside the TkvQD cell to adjacent wild type behaviour cells could induce the formation of the Tkv

barrier. This internal signal may induce the production of laterally placed Tkv in bordering cells which was noted in the barrier areas. This would create a feedback pattern in which: a cell that receives a strong Dpp signal sends out a strong internal signal to adjacent cells, which in turn begin exhibiting a strong lateral Tkv signal. This strong lateral Tkv signal would block more of Dpp passing through, limiting its range. The lateral Tkv expressing cell that receives more Dpp would then possess a stronger internal signal and send that signal to its neighbours, continuing the pattern with more lateral Tkv expression in the next line of cells.

Applying this model to the mosaic flies generated in the heat shocking experiments would explain the formation of a TkvGFP barrier surrounding TkvQD-mCherry cells clusters. (Figure 17). The model was further also applied to a wing vein (figure 18)

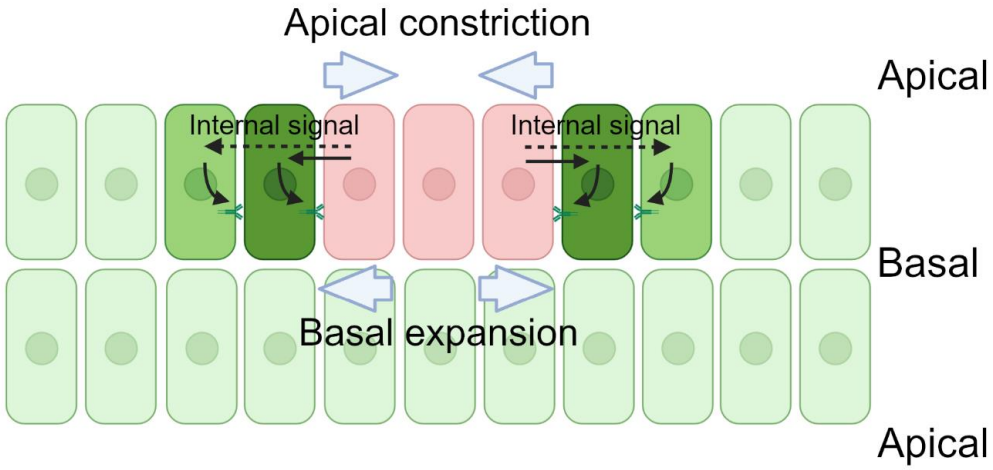

Figure 17. Internal signal model applied to a TkvQD clone scenario. Both epithelial layers of the wing are displayed, with distinguishable areas simplified into individual cells. Internal signal movement showed using smaller black and dotted arrows, induction of lateral Tkv expression showed with curved small arrows. Apical and Basal sides of both cell layers marked on the right. Although this figure shows a one-sided clone, potential apical constriction and basal expansion directions are marked for clarity using large blue arrows. TkvQD-mCherry (red) cells in the top middle possess a strong internal signal. This internal signal is transported horizontally to adjacent TkvGFP cells (dark green). There the internal signal induces expression of laterally placed TkvGFP. The signal from the clone cells diffuses over a short distance (dotted arrow) and also influences the next row (lighter green) to express laterally placed TkvGFP, although at a lower level. The nearby intervein cells (light green) remain unaffected. As this happens in a two sided clone the apical side of the clone cells constricts, forcing the basal side to expand, creating the concave structure seen in circular clones. Figure created using biorender.com.

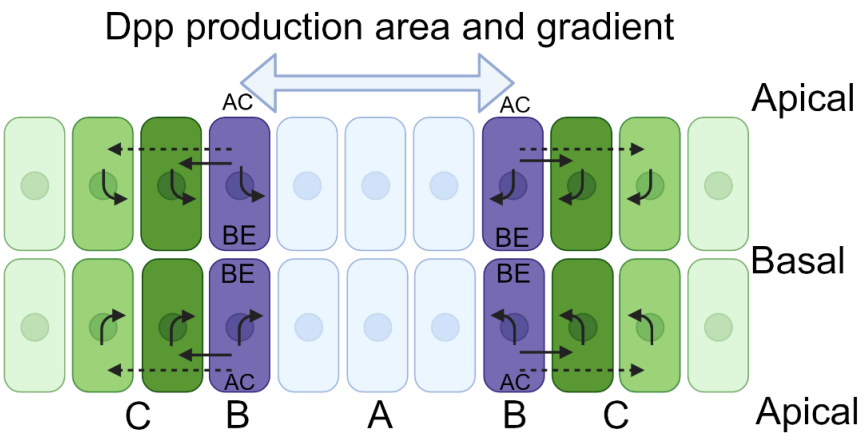

Figure 18. Internal signal model applied to the wing veins. Morphological regions have been reduced to individual cells for simplicity. Internal signal movement showed using smaller black and dotted arrows, induction of lateral Tkv expression showed with curved small arrows. The central wing vein (light blue, A) Dpp producing cells create a gradient of Dpp weakening away from the wing vein. The intervein cells directly adjacent to the vein cells (purple, B) possess Tkv and begin forming the interior signal. This signal induces the expression and placement of Tkv along the lateral side of the cell. The lateral Tkv receptors limit the amount of Dpp that can diffuse to the next cell layer. The internal signal also diffuses to adjacent cells but does not have an effect on vein Dpp producing cells due to the negative feedback mechanism on Tkv transcription. On the next layer of intervein cells (dark green, C) however this signal begins inducing lateral Tkv placement. This causes the dark green layer to absorb more Dpp, which once again limits the amount of Dpp that can diffuse to the next (lighter green) layer. This model could explain how Dpp is kept contained inside the wing vein although its production negatively affects Tkv transcription. As this is happening apical constriction (AC) takes place at the cells where Tkv receptors meet Dpp, most prominently at the purple and dark green layers. This causes the outer sides to concave inwards on both sides, while the basal expansion (BE) takes place in the interior sides. This could help to pull apart the Dpp producing cells and form the wing vein lumen. Figure made using Biorender.com.

To confirm if this model is accurate, the larval imaginal discs could be inspected at different points of development to see if the lateral placement of Tkv does coincide with the mobility of Dpp in the wing.

2.4.4 Theory for basal lumen formation assisted by peripheral apical constriction

One of the interesting observations in this thesis project was that ectopic expression of TkvQD between two-layered epithelia at identical positions is insufficient for basal lumen formation (Figure 13). Although individual cells expressing TkvQD appear to show vein-like structures with apically constricted and basally expanded architectures, these are not enough for basal lumen formation. It has been shown that conserved paracrine factors including Dpp, EGFR and Notch, which are all required for wing vein development, are mutually regulated (Sotillos & Celis, 2005, Martin et al., 2017). Thus, in addition to conserved signalling pathways, I assume that intercellular communications supported by cellular mechanisms may play an important role in basal lumen formation. Apical constriction has been linked to the development of wing veins in the posterior crossvein, this takes place after Dpp is trafficked in using the Sog/Cv complex (Toddie-Moore et al., 2022). It is unclear however how apical constriction leads to the formation of a basal side lumen. My working model is that if this apical constriction took place, instead of in the centre of the wing vein, at its edges, then the constricting of their apical sides could aid in pulling apart the two layers of vein cells (Figure 19).

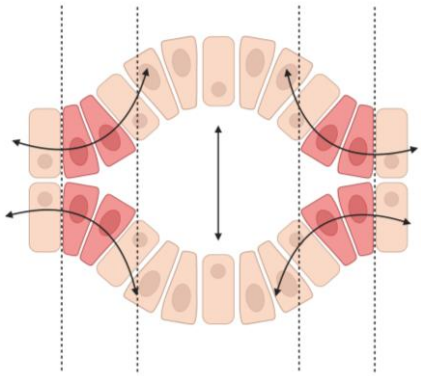

Figure 19. Model for the wing vein basal lumen formation assisted by peripheral apical constriction. Cells highlighted in red and bordered by dotted lines could, by undergoing apical constriction, aid in pulling the cellular layer apart, forming the wing vein lumen on the basal sides. The directions in which individual concaving cell regions in the wing layer would be bending are highlighted with curved arrows. The direction of the separation of the two layers and forming of the interior lumen is highlighted with the vertical arrow. Created using Biorender.com.

SUMMARY

This thesis presents the development and validation of a new method to study receptor related cellular interactions. Flies for the experiment were generated via a heat shock controlled FLP-FRT system. Upon heat shock activation, this system excises a GFP-tagged wild-type Thickveins (Tkv) receptor and replaces it with a mCherry-tagged constitutively active Tkv receptor, all under the control of the endogenous *tkv* enhancer. Adult wings from these flies were then observed for morphological changes, while pupal wings were dissected, fixed and immunohistochemically stained to analyse receptor localization.

This method successfully generated mosaic clones resembling vein like cells within the intervein areas. The shape and distribution of these clones varied depending on the timing of heat shock, allowing for controlled manipulation of clone patterning. Confocal microscope analysis showed that cells surrounding the clones displayed elevated receptor levels, mimicking the natural Tkv barrier observed around wing veins. This suggests that the formation of this barrier is regulated intracellularly and is not directly dependant on Dpp diffusion. 3D confocal scans revealed that Tkv in the adjacent barrier forming cells was largely localized laterally, potentially explaining how Dpp signalling is spatially regulated during wing development. Deformations and apical constriction were noticed in overlapping clones, consistent with the observations in PCV formation.

Two models were proposed to explain the observed phenomena. The first suggests that a post receptor activation signal propagates to neighbouring cells, inducing lateral Tkv localization and forming a localized barrier that limits Dpp spreading. The second model proposes that apical constriction along the edges of wing veins contributes to internal lumen formation.

These finding suggest that the heat shock inducible FLP-FRT system, combined with a mutated protein under the control of endogenous enhancer, is a promising tool for investigating morphogen regulation and receptor dynamics. This approach holds potential for broader applications in developmental biology, both in *Drosophila* and potentially other model organisms.

RESÜMEE

BMP retseptorite mosaiikanalüüs rakkudevahelise kommunikatsiooni uurimiseks *Drosophila melanogaster*`i nuku tiiva arengus

Joosep Karu

Drosophila melanogaster, ehk äädikakärbes, on laialdaselt kasutatud mudelorganism arengubioloogias. Uute meetodite ja tööriistade arendamine on vajalik, et leida vastuseid üha kompleksematele küsimustele, mis on üle aastakümnete kerkinud. Siiamaani ei ole selge, kuidas retseptor-morfogeen interaktsoonid võivad mõjutada üksteise asetusi, tootmist ja liikuvust. Hetkel kasutusel olevate meetoditega on raske analüüsida just neid väikesel skaalal toimuvaid individuaalsete rakkude vahelisi signaale, millel on suured efektid täiskasvanud kärbse morfoloogiale. Nendest morfogeenidest üks uuritumaid on Decapentaplegic (Dpp) ja selle retseptor Thickveins (Tkv).

Käesoleva bakalaureusetöö raames seati kaks eesmärki. Esiteks luua protokoll, mille alusel genereerida mosaiikse Tkv retseptori fenotüübiga äädikakärbseid. Selleks ristati äädikakärbsed, kelles kuumašoki kontrolli all oleva FLP-FRT mehhanismiga saab juhuslikus koguses rakkude genoomist välja lõigata normaalse funktsiooniga ning rohelise fluorestseeruva valguga märgistatud Tkv retseptorid (*tkvGFP*). Selle asemel hakkab rakk sama loodusliku geeni võimendaja abil tootma konstitutiivselt aktiivset mCherry-ga märgistatud Tkv retseptorit (*tkvQD*). Teiseks eesmärgiks oli uurida saadud TkvQD kloonide mõju ümbritsevatele rakkudele ja nende Tkv retseptorite asetusele ning muutustele klooni enda rakkude struktuuris, et paremini mõista rakkudevahelist kommunikatsiooni.

Bakalaureusetöö tulemusena loodi kuumašokile alluvad kärbsed, kelle tiibadel olid detekteeritavad erinevate kujude ja suurustega defektid. Kasutades epifluorestsents ja konfokaalmikroskoope leiti, et TkvQD kloonide ümber moodustus tugeva TkvGFP signaaliga ala. See signaal mimikeeris tiivasooni ümbritsevaid Tkv alasid. Kõrgresolutsioonilistel konfokaalmikroskoopia piltidel märgati, et kloone ümbritsevates metsiktüüpi TkvGFP rakkudes oli retseptori signaal detekteeritav lisaks apikaalsele rakumembraanidele ka lateraalselt. Pakuti välja teooria, kus rakkude sees, pärast retseptori aktivatsiooni, tekkinud signaal annab ümbritsevatele rakkudele edasi käsu Tkv taseme tõstmiseks rakkude lateraalses osas, seeläbi limiteerides Dpp levimist. Lisaks leiti, et olukordades, kus kaks klooni omavahel eri tiiva pooltel (kahekihiline tiivadisk koosneb dorsaalsest ja ventraalsest epiteelist) kattusid, tekkis deformatsioon. Pakuti välja teooria, kus mõlemal pool tiivasoont asuvate rakkude apikaalse osa pitsitamine võiks aidata tiivasoone arengus kaasa tiivasoone valendiku kujunemisele.

USED LITERATURE

Affolter, M., & Basler, K. (2007). The Decapentaplegic morphogen gradient: from pattern formation to growth regulation. *Nature*.

Akiyama, T., & Gibson, M. C. (2015). Decapentaplegic and growth control in the developing Drosophila wing. *nature*, 375-378.

Akiyama, T., User, S. D., & Gibson, M. C. (2018). Somatic clones heterozygous for recessive disease alleles of BMPR1A exhibit unexpected phenotypes in Drosophila. *eLife*, 7:e35258.

Alber, D. (2018, March 9). *Decapentaplegic*. Retrieved from Wikipedia: https://en.wikipedia.org/wiki/Decapentaplegic#/media/File:FrenchFlag.png

Antson, H. (2021). Dynamics of interplanar bridges and tissue morphogenesis in Drosophila melanogaster pupal wing. Tartu: Tartu ülikool.

Antson, H., Tõnissoo, T., & Shimmi, O. (2022). The developing wing crossvein of Drosophila. *Fly*, 118-127.

Ashburner, M. (1989). *Drosophila: A Laboratory Handbook.* Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Ashe, H. L., & Briscoe, J. (2006). The interpretation of morphogen gradients. *Development*.

Astakhova, L. N., & al., e. (2015). Activity of Heat Shock Genes' Promoters in Thermally Contrasting Animal Species. *PLoS One*, 10(2):e0115536.

Bate, M., & Arias, A. M. (1991). The embryonic origin of imaginal discs in Drosophila. *Development*, 755-61.

Beira, J. V., & Paro, R. (2016). The legacy of Drosophila imaginal discs. *Chromosoma*, 573-592.

Blair, S. S. (2007). Wing Vein Patterning in Drosophila and the analysis of intercellular signaling. *Annual Review of Cell and Developmental Biology*, 293-319.

Brabant, M. F., Bunch, T., & Brower, D. (1996). Distinct spatial and temporal functions for PS integrins during Drosophila wing morphogenesis. *Development*, 122(10):3307-17.

Bridges, C. B. (1920). The Mutant Crossveinless in Drosophila Melanogaster. *Genetics*, 660-663.

Brier, E. (2000). Drawing lines in the Drosophila wing: initiation of wing vein development. *Genetics and development*, 393-398.

Brown, N. H. (1993). Integrins hold Drosophila together. *Bioessays*, 383-390.

Bryant, P. (1975). Pattern formation in the imaginal wing disc of Drosophila melanogaster: fate map, regeneration and duplication. *J Exp Zool.*

Buratovich, M. A., & Bryant, P. J. (1995). Duplication of l(2)gd imaginal discs in Drosophila is mediated by ectopic expression of wg and dpp. *Dev Biol.*

Celis, J. F. (2003). Pattern formation in the Drosophila wing: The development of the veins. *Bioessays*, 443-51.

Chen, Z. (2019). The formation of the Thickveins (Tkv) gradient in Drosophila wing discs: A theoretical study. *Journal of Theoretical Biology*, 25-41.

Chen, Z., & Zou, Y. (2019). Anterior-posterior patterning of Drosophila wing discs I: A baseline mathematical model. *Mathematical biosciences*, 13-27.

Cohen, b., Simcox, A. A., & Cohen, S. M. (1993). Allocation of the thoracic imaginal primordia in the Drosophila embryo. *Development*, 597-608.

Firth, L. C., Bhattacharya, A., & Baker, N. E. (2010). Cell cycle arrest by a gradient of Dpp signaling during Drosophila eye development. *BMC Dev Biol.*

Frickenhaus, M., Wagner, M. M., Catinozzi, M., & Storkebaum, E. (2015). Highly efficient cell-type-specific gene inactivation reveals a key function for the Drosophila FUS homolog cabeza in neurons. *Sci Rep*, 5:9107.

Fristom, D., & Fristom, J. (1975). The mechanism of evagination of imaginal discs of Drosophila melanogaster. 1. General considerations. *Developmental Biology*, 1-23.

Fristom, D., Wilcox, M., & Frisrom, J. (1993). The distribution of PS integrins, laminin A and F-actin during key stages in Drosophila wing development. *Development*, 509-523.

Gui, J. (2019). Coupling between dynamic 3D tissue architecture and BMP morphogen signaling during Drosophila wing morphogenesis. *Proceedings of the National Academy of Sciences*, 4352-4361.

Gui, J., Yunxian, H., & Osamu, S. (2016). Scribbled Optimizes BMP Signaling through Its Receptor Internalization to the Rab5 Endosome and Promote Robust Epithelial Morphogenesis. *PLoS Genet*.

Hales, K., Korey, C., Larracuente, A., & Roberts, D. (2015). Genetics on the Fly: A Primer on the Drosophila Model System. *Genetics*, 815-42.

Johnson, S. A., & Milner, M. J. (1987). The final stages of wing development in Drosophila melanogaster. *Tissue and cell*, 505-513.

Kornberg, T. (1981). Engrailed: a gene controlling compartment and segment formation in Drosophila. *Proc Natl Acad Sci U S A*.

Lecuit, T. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. *Nature*.

Lecuit, T., & Cohen, S. M. (1998). Dpp receptor levels contribute to shaping the Dpp morphogen gradient in the Drosophila wing imaginal disc. *Development*, 4901-4907.

Lefevre, G., & Jonsson, U. B. (1962). SPERM TRANSFER, STORAGE, DISPLACEMENT, AND UTILIZATION IN DROSOPHILA MELANOGASTER. *Genetics*, 1719-1736.

Linford, N. J., Bilgir, C., Ro, J., & Pletcher, S. D. (2013). Measurement of Lifespan in Drosophila melanogaster. *Journal of Visualized Experiments*, (71):50068.

Martin, M., Ostale, C. M., & Celis, J. F. (2017). Patterning of the Drosophila L2 vein is driven by regulatory interactions between region-specific transcription factors expressed in response to Dpp signalling . *Development*.

Matsuda, S., & Shimmi, O. (2012). Directional transport and active retention of Dpp/BMP create wing vein patterns in Drosophila. *Developmental Biology*, 153-162.

Morata, G., & Lawrence, P. A. (1975). Control of compartment development by the engrailed gene in Drosophila. *Nature*.

Muratoglu, M. (2018, 10 2). *Flp recombinase*. Retrieved from Registry of Standard Biological Parts: https://parts.igem.org/Part:BBa_K2560271

Nellen, D., Burke, G., Struhl, G., & Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. *Cell*.

Neumann, C., & Cohen, S. (2005). Problems and paradigms: Morphogens and pattern formation. *Bioessays*.

Paul, L. (2013). Dpp-induced Egfr signaling triggers postembryonic wing development in Drosophila. *Proc Natl Acad Sci U S A.*

Ralston, A., & Blair, S. S. (2005). Long-range Dpp signaling is regulated to restrict BMP signaling to a crossvein competent zone. *Developmental biology*, 187-200.

Ray, R. P., & Wharton, K. A. (2001). Context-dependent relationships between the BMPs gbb and dpp during development of the Drosophila wing imaginal disk. *Development*.

Requena, D., Alvarez, J. A., Gabilondo, H., Loker, R., Mann, R. S., & Estella, C. (2017). Origins and Specification of the Drosophila Wing. *Curr Biol.*, 3826-3836.

Restrepo, S., Zartman, J. J., & Basler, K. (2014). Coordination of patterning and growth by the morphogen DPP. *Current Biology*, 245-255.

Schlake, T., & Bode, J. (1994). Use of Mutated FLP Recognition Target (FRT) Sites for the Exchange of Expression Cassettes at Defined Chromosomal Loci. *Biochemistry*.

Schwank, G., & Basler, K. (2010). Regulation of Organ Growth by Morphogen Gradients. *Cold Spring Harbor perspectives in Biology*.

Sotillos, S., & Celis, J. F. (2005). Interactions between the Notch, EGFR, and decapentaplegic signaling pathways regulate vein differentiation during Drosophila pupal wing development. *Dev Dyn.*

Tanimoto, H. (2000). Hedgehog creates a gradient of DPP activity in Drosophila wing imaginal discs. *Mol Cell*.

Toddie-Moore, D. J., Montanati, M., Tran, N. V., Brik, E. M., Antson, H., Salazar-Ciudad, I., & Shimmi, O. (2022). Mechano-chemical feedback mediated competition for BMP signalling leads to pattern formation. *Developmental Biology*.

Tripathi, B. K., & Irvine, K. D. (2022). The wing imaginal disc. *Genetics*, 220 (4).

Tyler, M. S. (2000). *Developmental biology, A guide for experimental study.* Sunderland: Sinauer associates.

Waddington, C. H. (1939). Preliminary Notes on the Development of the Wings in Normal and Mutant Strains of Drosophila. *Genetics*, 299-307.

Waddington, C. H. (1940). The genetic control of wing development in drosophila. *Journal of Genetics*, 78.

Wolpert, L. (1968). he French Flag Problem: A Contribution to the Discussion on Pattern Deve Lopment and Regulation. *Towards a Theoretical Biology*.

Yan, D., & Lin, X. (2009). Shaping morphogen gradients by proteoglycans. *Cold Spring Harb Perspect Biol.*

Zhu, X.-D., & Sadowski, P. D. (1995). Cleavage-dependent Ligation by the FLP Recombinase. *Journal of Biological Chemistry*, 23044-23054.

Lihtlitsents lõputöö reprodutseerimiseks ja üldsusele kättesaadavaks tegemiseks

Mina, Joosep Karu ,

1. annan Tartu Ülikoolile tasuta loa (lihtlitsentsi) minu loodud teose

Mosaic analysis of BMP receptors to study intercellular communication during Drosophila pupal wing development

mille juhendaja(d) on Professor Osamu Shimmi, PhD ,
Professor Tambet Tõnissoo, PhD

reprodutseerimiseks eesmärgiga seda säilitada, sealhulgas lisada Tartu Ülikooli digitaalarhiivi kuni autoriõiguse kehtivuse lõppemiseni;

2. annan Tartu Ülikoolile loa teha punktis 1 nimetatud teos üldsusele kättesaadavaks Tartu Ülikooli veebikeskkonna, sealhulgas digitaalarhiivi kaudu Creative Commonsi litsentsiga CC BY NC ND 4.0, mis lubab autorile viidates teost reprodutseerida, levitada ja üldsusele suunata ning keelab luua tuletatud teost ja kasutada teost ärieesmärgil, kuni autoriõiguse kehtivuse lõppemiseni;

3. olen teadlik, et punktides 1 ja 2 nimetatud õigused jäävad alles ka autorile;

4. kinnitan, et lihtlitsentsi andmisega ei riku ma teiste isikute intellektuaalomandi ega isikuandmete kaitse õigusaktidest tulenevaid õigusi.

Joosep Karu

26.05.25