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Screening of genes vital for the maintenance of apicobasal polarity with Scribble using *Drosophila melanogaster's* wing imaginal discs

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Screening of genes vital for the maintenance of apicobasal polarity with Scribble using *Drosophila melanogaster's* wing imaginal disc

Apicobasal polarity (ABP) is a type of cell polarity unique to epithelial cells and is maintained by three complexes, including the Scribble (Scrib) complex. Recent studies have revealed that the formation of neoplasia due to local loss of Scrib is affected by the dosage of Scrib within the tissue. This provides a hypothesis that the loss of Scrib-derived neoplasia formation is influenced by additional genes that exhibit synergy with *scrib*. To identify the vital genes, a screening was performed using the deficiency lines of the right arm of the third chromosome in *Drosophila* to determine genomic regions containing potential candidate genes. The screening protocol used conditional knockdown of *scrib* using the RNAi mechanism. This study demonstrates the robustness and suitability of the screening protocol for conducting a finer screening of candidate genes throughout the entire *Drosophila* genome.

Keywords: *Drosophila*; wing imaginal disc; ABP; Scribble; gene screening.
CERCS: B350 Development biology, growth (animal), ontogeny, embryology

Apiko-basaalse polaarsuse säilitamiseks koostöös Scribble'iga vajalike geenide sõeluuring kasutades *Drosophila melanogaster'i* tiiva imaginaaldiski

Epiteliaalsetele rakkudele iseloomulikku apiko-basaalset polarisatsiooni (ABP) aitavad säilitada kolm konserveerunud valgukompleksi nende hulgas Scribble (Scrib). Hiljutised uuringud on näidanud, et Scrib funktsiooni lokaalsest kadumisest põhjustatud neoplaasia teke on seotud Scrib kogusega koes. Sellest lähtuvalt püstitati antud bakalaureusetöös hüpotees, et äädikakärbse tiiva imaginaaldiski neoplaasia teke on mõjutatud geenide poolt, mis omavad sünergiat *scrib* geeniga. Välja selgitamaks potentsiaalseid kandidaatgeene, kasutati geneetilist sõeluuringut kasutades deletsioone spetsiifilises kolmanda kromosoomi genoomi osas ning RNAi mehhanismiga *scrib* geeni mahasurumist. Välja sõeluti potentsaalsed piirkonnad, millele tuginedes on võimalik läbi viia fokuseeritud sõeluuring, et identifiteerida geenikandidaate kogu *Drosophila* genoomis.

Märksõnad: *Drosophila*; tiiva imaginaaldisk; ABP; Scribble; geeni sõeluuring.
CERCS: B350 Arengubioloogia, loomade kasv, ontogenees, embrüoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

A-P – anterior-posterior

ABP – apicobasal polarity

AJs – adherence junctions

aPKC – protein kinase C

BDSC – Bloomington Stock Centre

BMP – bone morphogenetic protein

Crb – Crumbs

CyO – Curly of Oster

D-V – dorsal-ventral

Df – deficiency line

Dlg – Disc Large

Dpp – Decapentaplegic

dsRNA – double-stranded RNA

En – Engrailed

GAL4 – galactose-responsive transcriptional activator

$GAL80^{TS}$ – galactose-responsive temperature-sensitive repressor of GAL4

GFP – green fluorescent protein

Hh – Hedgehog

Hu – Humeral

KD – knockdown

Lgl – Lethal giant larvae

LRR – Leucine-rich repeats

MAGUK – membrane-associated guanylate kinase

mRNA – messenger RNA

one-copy RNAi control – Fly genotype (*scrib*RNAi/*scrib*-) that contains one copy of *scrib*RNAi

Par – Partitioning-defective

PBS – phosphate-buffered saline

PBT – buffer based on PBS and non-ionic detergent Tween 20 (Roche®)

PDZ – postsynaptic density protein (PSD95), *Drosophila* disc large tumour suppressor (DlgA), and zonula occludens-1 protein (zo-1)

pns – *pinstripe*

Ptc – Patched

RNAi – interference RNA

RT – room temperature

Scrib – Scribble complex

Sd – Scalloped

siRNA – small interference RNA

SJs – septate junctions

Tb – Tubby

TCJs – tricellular junctions

TJs – tight junctions

two-copy RNAi control – Fly genotype (*scrib*RNAi/+) that contains two copies of *scrib*RNAi

UAS – upstream activating sequence

Wg – Wingless

Wts – Warts kinase

Yki – Yorkie

ZA – zonula adherens

INTRODUCTION

Apicobasal polarity (ABP) is one of the characteristics of epithelial cells and is essential for the localisation of cellular components. Correct alignment of the cellular components ensures correct tissue homeostasis, organ development, and epithelial barrier function (Campanale *et al.*, 2017). Disruption of any ABP-determining complexes will result in the loss of ABP (Jung *et al.*, 2019).

The main complexes responsible for the maintenance of ABP are Partitioning-defective (Par), Crumbs (Crb) and Scribble (Scrib) (Su *et al.*, 2013). Scrib is located in the basolateral apex of epithelial cells and consists of three tumour suppressor genes *scribble* (*scrib*), *disc large* (*dlg*), and *lethal giant larvae* (*lgl*) (Wodarz, 2000). The missense and nonsense mutations in the scrib locus cause different dosages of Scrib which has been shown to play a role in the loss of ABP (Zeitler *et al.*, 2004).

The wing imaginal disc of *Drosophila melanogaster* is an important experimental system for researchers (Handke *et al*., 2014). The differentiation of the wing imaginal disc occurs during embryogenesis and consists of 30 cells but the number exceeds 35 000 cells by the end of the third instar larva stage, which makes it a perfect model to study tissue growth control (Tripathi and Irvine, 2022). The deficiency "kits" were invented to study specific genes in the fly genome (Wright *et al.*, 2010). Deficiency lines (Dfs) are chromosomal deletions of a specific region in the genome, resulting in only one copy of the targeted genes, leading to a haploid state for these genes in the affected fly stock (Cook *et al.*, 2012).

According to prior research, the conditional knock-down (KD) of Scrib in the Patched (Ptc) compartment of the wing imaginal disc has been found to impact neighbouring cells and result in severe neoplasia (Huang *et al.*, 2023). This highlights the importance of junction-mediated cellular communication in the development and dissemination of cancer. In addition, screening of the left arm of the third chromosome was performed to locate the genes involved in tissue homeostasis through intercellular alignment with Scrib (Fischbach, 2022).

The main aims of this thesis are to perform a finer screening of one of the previously identified Dfs, containing the genes involved in the maintenance of ABP with Scrib and continue screening the right arm of the third chromosome.

1. LITERATURE REVIEW

1.1 Overview of *Drosophila melanogaster* as a model

Drosophila melanogaster (hereinafter referred to as *Drosophila)*, also referred to as fruit fly, is a multicellular eukaryotic organism, which has been used as a model organism since early 1900 (Rubin, 1988). It is a very beneficial model system because it contains about 75% of known human disease genes (Pratomo *et al.*, 2022) Moreover, around 60% of *Drosophila* genes have orthologs in mammals. Compared to other model organisms, fruit flies have fewer ethical restrictions involved in the experimental research (Staats *et al*., 2018)

The life cycle of the fruit fly is short compared to other model organisms, as it takes about ten days (at 25ºC) for a fertilized egg to grow into an adult fly (Figure 1). The life cycle of *Drosophila* is divided into two stages: embryonic and postembryonic. Postembryonic development additionally consists of three distinct phases: larval, pupal, and adult stages. The day after fertilization, the embryo will develop into first instar larvae. One day from first instar to second instar larvae, and after another day to third instar larvae. In 2,5-3,5 days third instar larvae will start to pupariate, all the organs will degenerate during the metamorphosis and after an additional 3,5-4,5 days *Drosophila* will restructure into an adult fly shape (reviewed in Ashburner *et al.*, 2005).

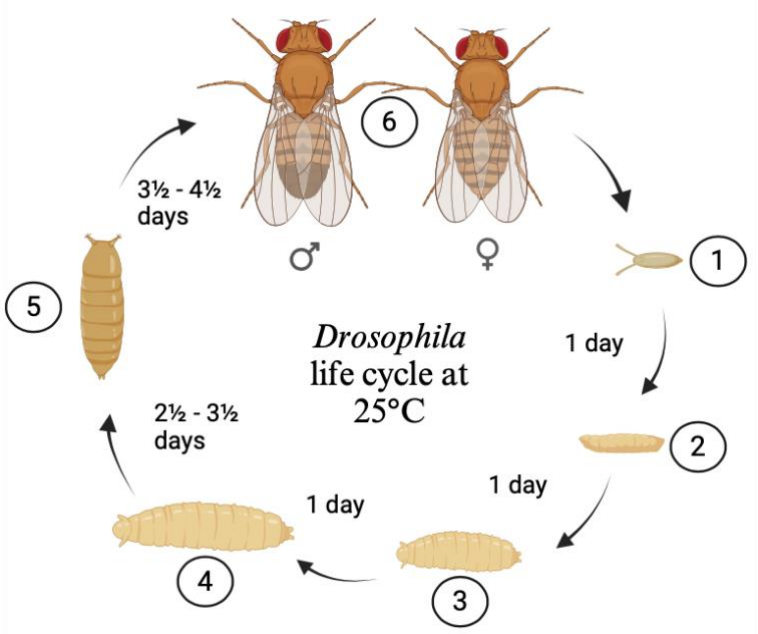


Figure 1. The life cycle of *Drosophila melanogaster* at 25ºC. Six stages of *Drosophila* life cycle (1-6) include: embryonic stage (from fertilized egg to hatching larva) (1) and postembryonic stages:

first (2), second (3), and third instar (4) larval stages, pupal stage (5), and adult stage (6). Figure created with https://biorender.com.

Fruit fly is often used to study genetics due to its simple genetic structure. The genome is packed into four pairs of chromosomes. The first pair is sex chromosomes XX (female) or XY (male). The second and third chromosome pairs are large autosomal chromosomes. Lastly, there is a fourth autosomal chromosome pair, which contains only 5% of the genes. Because of the low genetic content, the fourth chromosome is not widely studied (Adams *et al.*, 2000; Rand, 2010).
Maintenance of *Drosophila* is relatively cheap because it does not require sophisticated equipment. The diet consists of agar, yeast, corn and soy flour, sugars, and antibiotics to prevent bacterial and fungal growth (Staats *et al.*, 2018). These flies can be housed in 20-50mL vials, allowing several genotypes to fit into a small space (Rand, 2010).

1.2 Fly genetics and molecular tools

1.2.1 Balancer chromosomes

Balancer chromosomes are rearranged chromosomes frequently used in *Drosophila* genetics to maintain deleterious mutations in the population (Miller *et al.*, 2020). They were first discovered almost 100 years ago and are commonly used for the genotypic determination of flies based on their phenotype, maintaining stocks, and complex crosses (Miller *et al.*, 2019). Balancer chromosomes play a vital role in homozygous lethal or sterile mutations from being lost from a population. Additionally, they carry recessive lethal or sterile mutations that prevent them from becoming homozygous in stock. Moreover, the balancer mutations help to prevent the separation of multiple alleles on the same chromosome during the meiotic recombination (Miller *et al.*, 2018). These balancer mutations feature two essential components - recessive deleterious mutations, in which a recessive lethal or sterile mutation can be maintained in the population by combining it with another gene in the homologous chromosome, and inversion breakpoints, which prevent meiotic recombination from occurring between the deleterious mutations (Casso *et al.*, 1999).
During an experiment, it is crucial to differentiate homozygous and heterozygous progenies, the balancer mutations have a dominant marker, which causes a distinguished phenotype (Table 1). Dominant marker makes it possible to contrast the flies with the desired mutation from the flies without it (Miller *et al.*, 2018).

Table 1. Examples of dominant and recessive markers used in this study.

Marker abbreviation and name	Phenotype
cu (cutoid/curled)	Smooth abdomen
Cy (Curly)	Curly wings
Dr (Drop)	Small, drop-shaped eyes
Hu (Humeral)	Thicker thorax bristles
Sb (Stubble)	Short abdominal and blunt bristles
Tb (Tubby)	Flattened body during pupal stage
w (white)	White eye colour
w+ (mini white)	Yellow eyes
y+ (yellow)	Yellow body colour

Drosophila markers can change the phenotype of the fly's body colour and shape, eye colour and shape, wing size and structure, and bristle length and structure. The dominant or recessive nature of a marker can be determined by the first letter of the symbol (capital letter - dominant; small letter - recessive). Obtained from Bloomington Stock Centre (https://bdsc.indiana.edu/) and FlyBase (https://flybase.org/).

In this study, the most used markers for the experiments were Curly of Oster (CyO) and Tm6Tb. CyO is a second chromosome balancer which contains the Cy marker. This makes the fly wings bend upwards from the top, so the wings look curled (Figure 2A). This phenotypic change makes it easy to recognise the flies which carry the mutant gene (Ward, 1923). The Tm6Tb is a third chromosome balancer which consists of Tb and Hu markers. This enables to distinguish the larvae and pupae, which carry the mutation because the Tb marker makes the larvae and pupae shorter and chubbier than the wild type (WT) (Figure 2B). Tb marker is not recognisable on the adult fly, but the Hu marker makes the bristles on the dorsal thorax thicker and there are extra bristles compared to the WT (Figure 2C) (Canales Coutiño *et al.*, 2021).

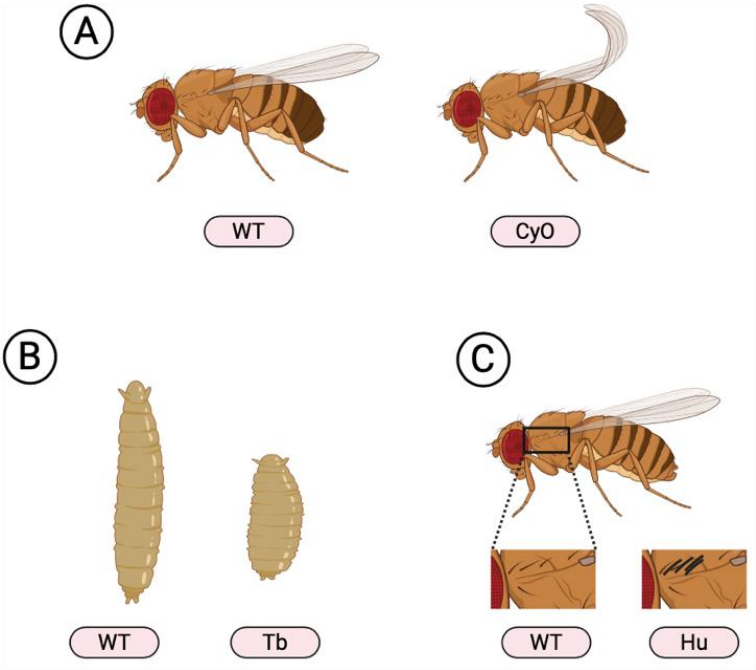


Figure 2. Phenotypic differences of CyO and Tm6Tb compared to the wild type flies. (A) The wings of the wild type (WT) are straight but the Cy marker makes the wings of CyO curly. (B) Third instar larva of the WT is long and skinny compared to the Tm6Tb (Tb) larva, which is short and chubby due to the Tubby marker. (C) The wild type has fewer bristles on the dorsal thorax than the Tm6Tb (Hu) fly, which contains the Humeral marker. Figure created with https://biorender.com.

1.2.2 RNA interference

RNA interference is a gene-silencing method that leads to the degradation of homologous messenger RNA (mRNA). RNA interference (RNAi) is triggered by double-stranded RNA (dsRNA) (reviewed in Tomari and Zamore, 2005). RNA silencing was first observed in animals by Guo and Kemphues, who used antisense RNA on *Caenorhabditis elegans* (Guo and Kemphues, 1995). RNAi is activated by the Dicer enzyme (Figure 3A) which cuts the dsRNA into 21-27 nucleotides long small interfering RNAs (siRNAs). siRNAs incorporate into RNA effector nuclease complex (RISC), which recognizes and destroys the targeted mRNAs (Figure 3E) (Hannon, 2002). The discovery of RNAi systems presents endless possibilities to explore the genetics of *Drosophila* development, physiology, and pathology both *in vitro* and *in vivo* (Dietzl *et al.*, 2007).

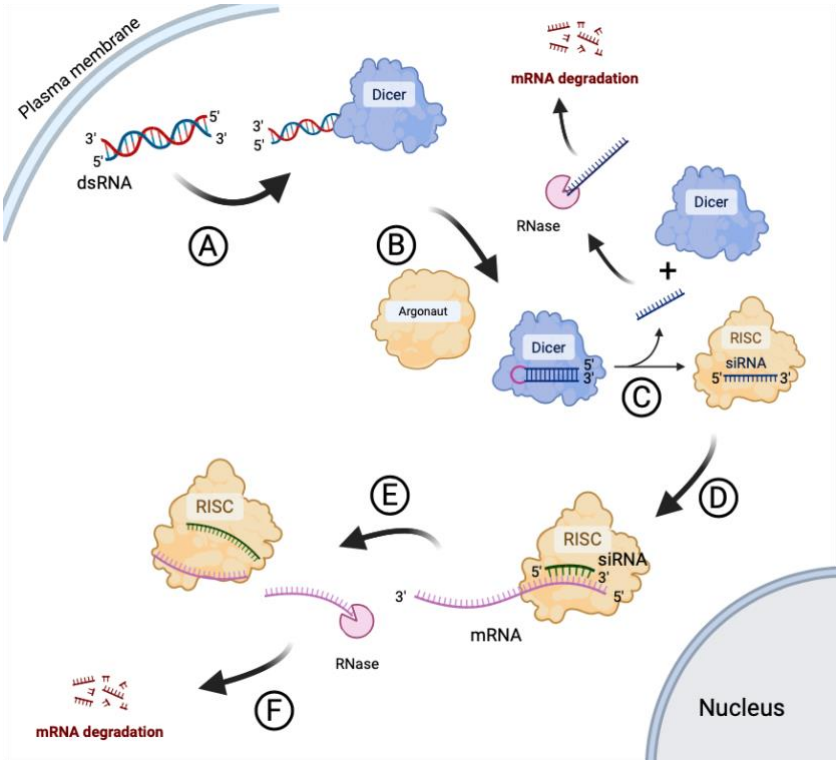


Figure 3. The mechanism of RNA interference. The double-stranded RNA (dsRNA) is recognized by Dicer enzymes which bind to it (A) and cleave it into smaller units. Argonaut protein then binds to the dsRNA/Dicer complex (B) resulting in the formation of an RNA effector nuclease complex (RISC) complex (C), a single-stranded molecule of small interfering RNA (siRNA) which gets degraded by RNase, and a free Dicer. The RISC complex containing a 3'-5' siRNA molecule recognizes the mRNA with a complementary sequence (D), binds and catalyzes the cleavage of messenger RNA (mRNA) (E) with its further degradation (F). Figure created with https://biorender.com.

1.2.3 UAS/GAL4/GAL80^{TS} system

Drosophila researchers frequently use the UAS/GAL4/GAL80^{TS} system for spatio-temporal manipulations. It is used to determine the role of genes and pathways regulating the tissue and/or cell type of interest, either directly or indirectly affecting separate tissue (Brand and Perrimon, 1993).

The galactose-responsive transcriptional activator (GAL4) gene, which is active in yeast, is randomly inserted into the *Drosophila* genome. The expression of GAL4 is controlled by many different genomic enhancers and promoters. GAL4 protein binds to the upstream activating sequence (UAS) binding site and activates the transcription of the effector gene downstream (Fischer *et al.*, 1988). The UAS/GAL4 system is excellent for controlling gene expression in a

specific region but it does not allow experimentation with specific periods in which the expression of a transgene is required for a given phenotype in a specific tissue. To address this limitation, there is a temperature-sensitive version of the GAL80 protein (GAL80^{TS}). Under normal conditions, GAL80^{TS} functions as a repressor of GAL4 in yeast. But in both yeast and *Drosophila*, GAL80^{TS} regulates GAL4 activity in a temperature-sensitive manner (Figure 4 18°C vs 29°C) and makes it possible to create conditional knockdown (KD) conditions (McGuire *et al*., 2003).

At lower temperatures from 18°C to 21°C GAL80^{TS} binds to and inhibits the transcriptional activator GAL4, preventing the gene expression of the effector gene downstream. At higher temperatures from 27°C to 29°C GAL80^{TS} becomes inactivated and dissociates from GAL4 allowing the gene expression of the gene of interest (Figure 4) (McGuire *et al.*, 2003).

To accurately determine the phenotypic change, it is necessary to introduce a specific reporter that can precisely locate the protein of interest. Neither GAL4 nor GAL80^{TS} can be observed independently. Green fluorescent protein (GFP) is the most utilised reporter gene, which is expressed downstream of the UAS (Goupil *et al.*, 2022).

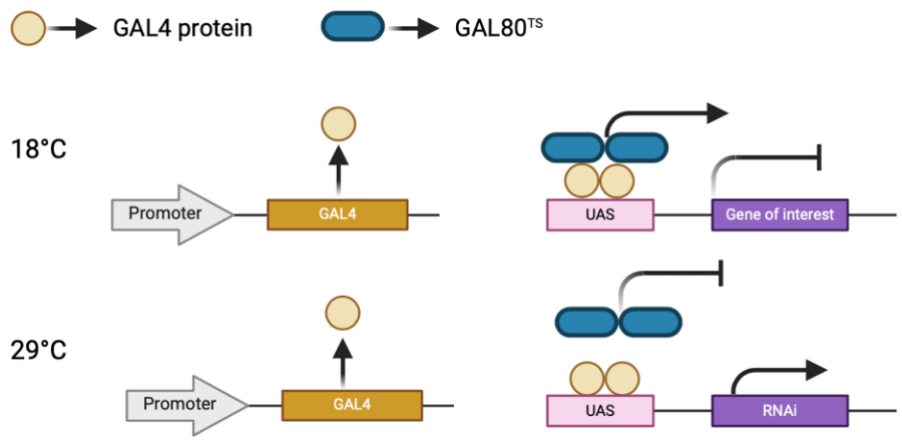


Figure 4. The mechanism of the UAS/GAL4/GAL80^{TS} system. GAL4 protein is a transcriptional activator that binds to UAS to activate the transcription of the gene downstream. GAL80^{TS} is a temperature-sensitive protein that can bind to GAL4 protein and inhibit transcription activation. At 18 °C GAL80^{TS} acts as a repressor to GAL4 and the gene expression is inhibited. At 29°C GAL80^{TS} is inactivated and it will dissociate from GAL4, which activates the gene expression of the gene of interest. Figure created with https://biorender.com.

1.3 Epithelial cells

Epithelial cells are the building blocks of primary tissue found throughout the body originating from ectoderm, mesoderm, and endoderm. They cover the surfaces of the body which are exposed to the external environment. These cells serve different functions and develop differently based on their location (Tepass *et al.*, 2001). The main function is to defend the body from microbes, environmental toxins, and mechanical stress. Plasma membranes of epithelial cells consist of specialized domains serving distinct roles in cell structure and function. These primary domains include the apical surface, facing the external environment, and the basal region, in contact with the interstitial space of the body (Larsen *et al.*, 2020).

Over 80% of malignant tumours in humans are known to originate from epithelial cells (Kajita and Fujita, 2015). As a result, over the past decades, researchers have increasingly used three-dimensional epithelial cell cultures to study pathological features of tumours. In addition, research has shown that loss of cell polarity and loss of cell-cell adhesion are important first signs of early-stage cancers (Eritja *et al.*, 2013).

1.3.1 Apicobasal polarity

Apicobasal polarity (ABP) is a type of cell polarity that is specific to epithelial cells (Flores-Benitez and Knust, 2016). It is crucial for homeostasis and normal tissue functioning throughout the lifetime, and it is additionally important during early development for correct organ growth (Campanale *et al.*, 2017). The main complexes responsible for establishing and maintaining ABP in epithelial cells are Scribble (Scrib), Partitioning-defective (Par), and Crumbs (Crb) complexes (Su *et al.*, 2013). Par and Crumbs are located in the apical compartment and Scrib is located in the basolateral compartment (Figure 5) (Enomoto and Igaki, 2011). In addition to maintaining cell polarity and acting as tumour suppressors, they are also responsible for assembling and maintaining correct junctional alignments between cells (Su *et al.*, 2013). All polarity proteins of epithelial cells function as major suppressors of metastasis through different pathways to maintain epithelial characteristics. This means the disruption of one complex or protein will lead to the disruption of others and the loss of ABP is inevitable (Jung *et al.*, 2019)

The Scrib complex consists of tumour suppressor genes *scribble* (*scrib*), *disc large* (*dlg*), and *lethal giant larvae* (*lgl*), which are responsible for the proper functioning of ABP (Wodarz, 2000).

Mutations in those genes are shown to cause disruptions in ABP resulting in overproliferation of epithelial cells and neuroblasts and eventually leading to the development of neoplasia (Bilder, 2004).

Par is one of the most studied polarity complexes, and it is located in the apical domain of the epithelial cells. It consists of Par6, Par3 (Bazooka in *Drosophila*), and atypical protein kinase C (aPKC). Lgl is excluded from the apical region through the phosphorylation by the aPKC (Figure 5A). The Par complex is inactive in the basolateral region because the Lgl binds to Par and inhibits aPKC (Figure 5B). The Crb complex consists of tumour suppressor proteins Pals1 (Stardust and a member of the membrane-associated guanylate kinase (MAGUK) protein family in *Drosophila*) and Patj, which is a tight junction (TJ) protein (Rust and Wodarz, 2021; Su *et al.*, 2013).

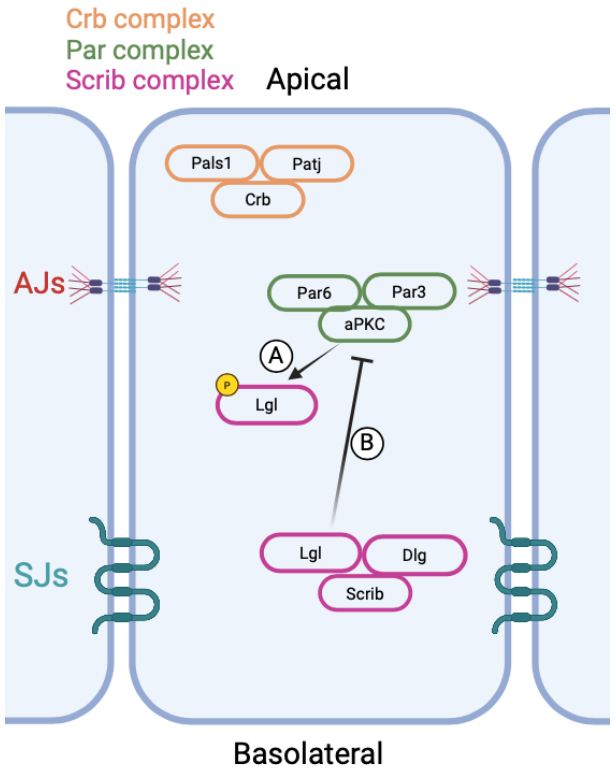


Figure 5. Drosophila apicobasal polarity-determination complexes and their components in epithelial cells. The three main complexes determining the ABP are Crb (orange), Par (green), and Scrib (pink). Crb and Par are located in the apical region of the epithelial cell and Scrib in the basolateral region. The Par complex kinase aPKC phosphorylates Lgl (A) restricting it in the basolateral side of the cell. At the basolateral side of the cell, Lgl inhibits aPKC (B). Adherence junctions (AJs, red) and septate junctions (SJs, blue) are the main junctions between epithelial cells. Figure created with https://biorender.com.

Epithelial cell polarity is established early during embryonic development and it is vital for ensuring correct tissue architecture and determining cell fate. Each epithelial cell is divided into two domains: the apical domain, which is responsible for exchanging molecules with other cells and the environment, and the basolateral domain, which is responsible for attachment to the extracellular matrix and exchanging molecules with neighbouring cells. The ABP follows the apico-basal axis, which crosses the two domains (Campanale *et al.*, 2017; Le Bivic, 2005; Wilson, 2011).

1.3.2 Cell junctions

Two main cell-cell junctions in *Drosophila* epithelial cells are adherence junctions (AJs) and septate junctions (SJs) (Röper, 2015). Epithelial cells are polarized along the apical-basal axis and establish distinct membrane domains - apical, basal, and lateral. AJs provide strength and cohesion at the interface of apical and lateral domains, while SJs (TJs in vertebrae) prevent the passage of molecules and maintain segregation between apical and basolateral membrane components (Rodriguez-Boulan and Macara, 2014). In addition, the tricellular junctions (TCJs) are also found in *Drosophila* epithelial cells where three cells meet (Schulte *et al.*, 2006).

AJs are conserved structures that mediate cell-cell adhesion in invertebrates and vertebrates. They form continuous adhesive belts at the interfaces of cell-cell contacts and are apical to the zonula adherens (ZA). The main components of epithelial AJs are clusters of dimeric E-cadherin, which is a calcium-dependent, homophilic cell-cell adhesion receptor, and is responsible for the structural and functional core of AJs (Brüser and Bogdan, 2017).

SJs are formed during mid-embryogenesis (Hildebrandt et al., 2015). The core complex of SJs forms of around 30 proteins that include transmembrane proteins like Neurexin IV, Neuroglian, ATP alpha, Nervana 2, Megatrachea, Sinous, cytoplasmic adaptor proteins Coracle, and Varicose (Oshima and Fehon, 2011). The Scribble complex is also essential for the correct formation of SJs, which are basal to the ZA (Bahri *et al.*, 2010). Research has shown that genetic interactions between Scrib and α-Catenin are the core components of SJs. Also, the Scrib-SJ pathway is essential for correct tissue growth and homeostasis in the wing imaginal disc (Huang *et al.*, 2022). SJs restrict the free diffusion of solutes through the paracellular route (Furuse and Tsukita, 2006). In addition, they polarize the plasma membrane, make bridges to communicate with neighbouring cells, and participate in the underlying cortical cytoskeleton along the apical-basal axis (Cereijido *et al.*,

2008; Miyoshi and Takai, 2008). SJs create a tight membrane barrier, limiting the diffusion of molecules in the extracellular space (Badouel and McNeill, 2009). SJs are also critical factors in maintaining tissue architecture and function, as loss of *dlg1* has been shown to result in abnormal growth and fusion of imaginal discs (Woods and Bryant, 1989).

TCJs are formed when three adjacent cells in polarized epithelia converge to their pleated SJs. Transmembrane protein Gliotactin associates with Dlg and is essential for the formation of the TCJs. They are components of a larger protein complex that links the converging SJs with the TCJs to create the transepithelial barrier (Schulte *et al.*, 2006).

1.3.3 Scribble

The Scribble (Scrib) complex was first identified for its role in maintaining ABP and epithelial integrity in *Drosophila melanogaster*. Now we know that Scrib plays a role in assembling and positioning diverse multiprotein complexes in different processes like planar polarity, adhesion, oriented cell division, and synaptogenesis (Bonello and Peifer, 2019). Scrib complex consists of proteins Scrib, Dlg and Lgl. It is located in the apicobasal apex of epithelial cells. If any of these proteins are disrupted, the maintenance of ABP is unattainable and the overgrowth of the wing imaginal disc will occur (Woods *et al.*, 1996).

Dlg was the first protein in the MAGUK family that was ever discovered (Ghosh et al., 2018). It consists of PDZ (post synaptic density protein (PSD95), *Drosophila* disc large tumour suppressor (DlgA), and Zonula occludens-1 protein (Zo-1)) domains, Src Homology 3 domain, catalytically inactive guanylate kinase domain, and Dlg protein domain (Dimitratos *et al.*, 1999). One of the most important functions of Dlg is to form SJ in *Drosophila* (Papagiannouli and Mechler, 2010). Lgl regulates cell division in the wing imaginal discs and optic centres of the brain. If the *lgl* is mutated, it can lead to abnormal cell division and the development of malignant neuroblastomas in the larval brain and imaginal discs which will result in the death of the fly in the third instar larvae stage or early pupal stage (Tepass *et al.*, 2001). Scrib is a cytoplasmic protein that contains multiple copies of leucine-rich repeats (LRR) and PDZ. They are the key to many different protein-protein interactions and often affect the localization of the Scribble complex in the epithelial cell. PDZ domains are also the mediators of Scrib and ligand interactions. Additionally, Scrib is responsible for the correct formation of SJ (Bilder and Perrimon, 2000; Javorsky *et al.*, 2023).

The sequence analysis of the *scrib* locus revealed seven ethyl methanesulfonate–induced alleles (Zeitler et al., 2004). Six of these have premature termination codons, which cause nonsense mutations (*scrib*$^{2-7}$). In addition, *scrib*^{1} presents a missense mutation as it alters leucine 223, in LRR 10, to glutamine (Figure 6.1). *Drosophila*'s wing imaginal disc screening shows that the severity of APB loss depends on the variant of partially lost Scrib protein (Figure 6.2). The mutant proteins that do not retain the LRR are not capable of forming an epithelial monolayer whereas the ones with a correct LRR and partial PDZ domains are. PDZ domains seem to be in control of cell proliferation. *scrib*^{2}, which does not have LRR nor PDZ domains, is considered a null allele, as it causes the most severe disruption in ABP resulting in neoplasia (Zeitler *et al.*, 2004).

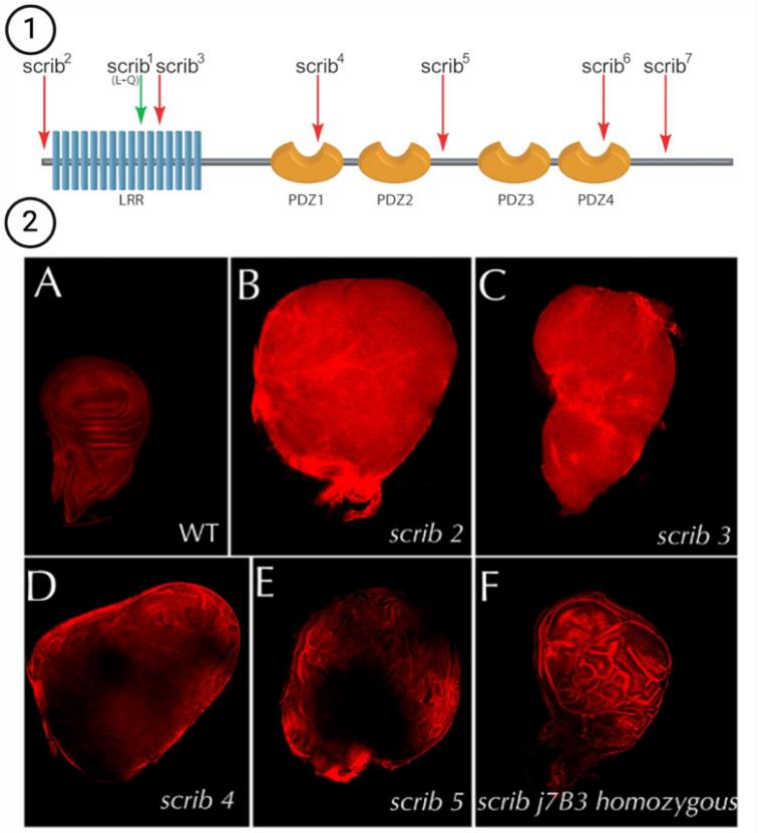


Figure 6. *scribble* variants and their impact on imaginal wing disc phenotype. Scrib is one of three proteins in the Scribble complex, which has an impact on ABP homeostasis. (1) Seven alleles on *scrib*. All these alleles have a mutation either a missense or nonsense mutation. *scrib*$^{2-7}$ have a premature termination codon (marked with red arrows) and *scrib*^{1} has a missense mutation (marked with green arrow). (2) Phenotype of Drosophila melanogaster wing imaginal discs with normal scrib (A) and various scrib mutant alleles (B-F). Every variant of *scrib*, besides the wild type (A), shows the wing disc overgrowth and overproliferation of epithelial tissue caused by the loss of ABP (B- *scrib*^{2}, C- *scrib*^{3}, D- *scrib*^{4}, E- *scrib*^{5}, F- *scrib*^{j7B3}). Figure adapted and modified from Zeitler *et al.*, 2004.

Furthermore, it has been discovered that Scrib has an impact on the Hippo signalling pathway, which controls tissue growth both in mammals and *Drosophila* (Chen *et al.*, 2010). In fruit flies the growth is controlled by the repression of a transcriptional co-activator Yorkie (Yki), the homolog of the mammalian Yes-associated protein (Oh and Irvine, 2010). Scrib has been shown to play a role in the Hippo pathway as it interacts with Hippo signalling downstream of Fat, an atypical cadherin, to regulate Warts (Wts) levels and activity. Scrib interacts and activates Wts by phosphorylating it on the apical domain. Phosphorylated Wts in turn phosphorylates and inactivates the co-transcriptional activator Yki, by restricting it in the cytoplasm (Figure 7A). When the Scrib complex is disrupted, it causes Yki to translocate into the nucleus, where it binds to the Scalloped (Sd) and activates the transcription of Yki-dependent genes (Figure 7B). Yki-dependent genes responsible for cell proliferation are then activated, leading to the overgrowth of the tissue. Therefore, the reliance of Scrib to phosphorylate Yki and prevent its translocation into the nucleus is a critical aspect of its tumour suppressive role, and dysregulation of Scrib leads to a compromised Hippo signalling pathway (Badouel and McNeill, 2009; Enomoto and Igaki, 2011; Richardson and Portela, 2017; Verghese *et al.*, 2012).

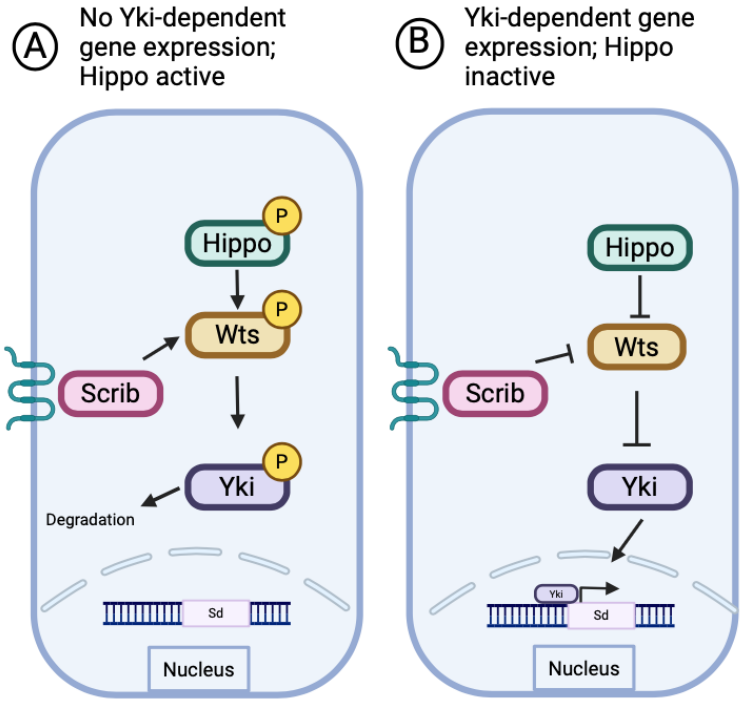


Figure 7. The mechanism of Hippo signalling pathway. (A) Active Hippo signalling pathway. Hippo and Scrib interaction leads to the phosphorylation of Warts (Wts). This in turn

phosphorylates Yorkie (Yki), leading to the subsequent degradation of Yki. As a result, Yki is unable to translocate into the nucleus and bind to the Scalloped (Sd) region and no Yki-dependent gene expression occurs. (B) Inactive Hippo signalling pathway. As a result of the disruption of the Scrib complex, Wts is not phosphorylated and no subsequent Yki phosphorylation occurs. This allows unphosphorylated Yki to translocate into the nucleus and bind to Sd leading to the expression of Yki-dependent genes. Figure created with https://biorender.com.

1.4 Wing imaginal disc

Drosophila imaginal discs are the larval primordia of adult structures such as eyes, legs, antennae, genitalia, and wings (Hariharan and Serras, 2017). They represent integral clusters of undifferentiated monolayer of epithelial cells during embryogenesis that invaginate into structural units during the metamorphosis (Gui *et al.*, 2019).

The wing imaginal disc of *Drosophila melanogaster* is widely recognized as an important experimental system for studying the control of tissue growth by cell proliferation and apoptosis (Handke *et al.*, 2014). Wing disc forms during embryogenesis, and it consists of around 30 cells. However, during the larval stages, these cells undergo extensive proliferation, resulting in the wing disc consisting of 35,000 cells at the end of the third instar larval stage (Tripathi and Irvine, 2022). Thereby wing imaginal disc is an excellent system to study tissue growth control as extensive proliferation occurs in a relatively short time. During the pupal stage, the wing undergoes morphogenetic processes and forms into the adult wing and notum. Importantly, the wings of *Drosophila* are excellent research objects to identify and characterise different mutations that have an impact on wing size, shape, and posture because the flies do not need wings to survive or reproduce in laboratory culture (Tripathi and Irvine, 2022).

In the wing imaginal disc, tissue morphogens play a crucial role in both the anterior-posterior (A-P) and dorsal-ventral (D-V) axis, particularly in the pouch region. Engrailed (En) directs the Hedgehog (Hh) signalling in the posterior compartment. In addition, the Hh secretion enables short-range signalling, thus regulating vital genes such as *wingless (Wg)* (encoding a Wnt family member) or *decapentaplegic (Dpp)* (a homolog of the TGFβ family of growth factors) in neighbouring cells (Figure 8.). This activation of Dpp at the A-P boundary results in the expression of Dpp (Beira and Paro, 2016). Patched (Ptc), a co-receptor to Hh, controls Hh signalling and gradient formation. The maximal intensity of the Ptc is located in the border of the A-P compartment and regulated by Dpp (Brigui *et al.*, 2015).

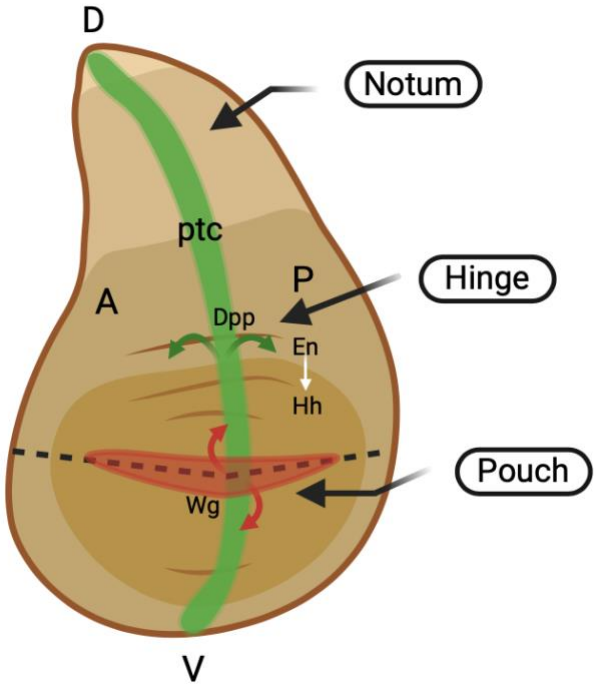


Figure 8. *Drosophila* imaginal wing disc. The epithelial cells of the imaginal wing disc can be categorized into three distinct subregions: the pouch region on the ventral (V) side, the hinge region in the middle and notum on the dorsal (D) side. The disc is split in half by the Patched (Ptc) compartment (green), which is controlled by the Dpp and creates anterior (A) and posterior (P) sides. Wingless (Wg) is produced in the D-V boundary and it is responsible for wing development (red). En is expressed in the posterior cells and it directs the expression of Hh, which can cross the A-P boundary and induce the expression of Dpp. Figure created with https://biorender.com.

1.5 Deficiency lines

Deficiency lines (Dfs) are chromosomal deletions in a specific region of the genome, which means it contains only one copy of the targeted genes. This leaves the fly stock in a haploid state for these genes in the genome region (Cook *et al.*, 2012).

Deficiencies provide definitive null alleles for the genes they cover. They are used to test the severity of a particular allele. The classical test for defining an amorphic or null allele is to evaluate whether the homozygous phenotype is as severe as when the allele is hemizygous with a deletion (St Johnston, 2002). The deficiency kit is a quick and efficient method for mapping mutations to small genomic regions by crossing the mutant gene stock of interest to a set of deficiency stocks and analysing the lack of complementation. Deletions are also useful in identifying genes that interact with a gene of interest through enhancer/suppressor screens, which weaken or strengthen

the phenotype of a mutation when the dosage of an interacting gene is decreased (Roote and Russell, 2012).

To facilitate gene mapping there are deficiency "kits" available that cover the genome with a minimum number of lines. However, these kits cannot be systematically analysed for phenotypes because embryos homozygous for many deficiencies in these kits fail to develop due to the loss of key gene products encoded within the deficiency (Wright *et al.*, 2010).

Thousands of *Drosophila* strains have been created for various research purposes, and they can be obtained from Bloomington or Kyoto Drosophila Stock Centers. These stock centres provide comprehensive information about each strain, including its genetic background, phenotype, and associated publications, which are linked to the FlyBase database (https://flybase.org).

2. EXPERIMENTAL PART

2.1 The aims of the thesis

One of the ongoing projects in the host laboratory is to identify genes cooperating with Scrib in maintaining tissue homeostasis. Identification of these genes is crucial for comprehending the mechanisms underlying intercellular communication, cooperation, and epithelial morphogenesis.
The previous results in the laboratory have established protocols of conditional *scrib* knockdown and dosage dependency of *scrib* in RNAi (Fischbach, 2022; Huang *et al.*, 2022). Taking it into consideration, the hypothesis for this thesis is that Scribble may have synergy with other genes related to the early progression of neoplasia. To further investigate this, we plan to finish the screening of the entire third chromosome to narrow down the chromosomal regions and identify new candidate genes.
The key factor for accurate results is a proper protocol, which includes precise timing and temperature conditions.
Thus the aims of this thesis are as follows:

- Establish an updated screening protocol incorporating revised conditions.
- Perform a partial screening of the third chromosome's right arm using the deficiency lines to detect the chromosomal regions containing potential candidate genes which have a synergy with Scrib and are involved in ABP establishment.
- Conduct a more detailed screening of the previously confirmed deficiency line areas in the left arm of the third chromosome, which have ABP disruption and result in neoplasia phenotype.

2.2 Materials and methods

2.2.1 Fly lines

This study on *Drosophila* imaginal wing discs is possible only because of the availability of genetic tools and techniques, such as mutagenesis, transgenic technologies, and RNAi. The ongoing project focuses on the Df screening, which was made possible by the deficiency toolkit obtained from the Bloomington *Drosophila* Stock Center (BDSC) (https://bdsc.indiana.edu/stocks/df/dfkit-info.html). The precise genotypes must be determined, thus various fly flies were used (Table 2). In addition to Dfs, fly lines for the UAS/Gal4/Gal80ts system, controls and balancer chromosomes were utilised.

Table 2. Fly lines used in the experiments.

Line number in Bloomington	Genotype	Type, purpose
N/A	+/+; Ptc-Gal4, UAS-GFP, ex-LacZ/CyO; *scrib* RNAi, $Gal80^{TS}$/*scrib* RNAi, $Gal80^{TS}$	Host stock, used for Df crosses
8105 [Df(3R)ED6232]	w[1118]; Df(3R)ED6232, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED6232/TM6C, cu[1] Sb[1]	Df containing *scrib* locus, used as a sensitized control
7681 [Df(3R)Exel6202]	w[1118]; Df(3R)Exel6202, P{w[+mC]=XP-U}Exel6202/TM6B, Tb[1]	Df, primary screening
7737 [Df(3R)Exel6270]	w[1118]; Df(3R)Exel6270, P{w[+mC]=XP-U}Exel6270/TM6B, Tb[1]	Df, primary screening
8923 [Df(3R)ED6085]	w[1118]; Df(3R)ED6085, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED6085/TM2	Df, primary screening
9090 [Df(3R)ED5644]	w[1118]; Df(3R)ED5644, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED5644/TM6C, cu[1] Sb[1]	Df, primary screening

9226 [Df(3R)ED5100]	w[1118]; Df(3R)ED5100, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED5100/TM6C, cu[1] Sb[1]	Df, primary screening
24139 [Df(3R)ED5938]	w[1118]; Df(3R)ED5938, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED5938/TM6C, cu[1] Sb[1]	Df, primary screening
24516 [Df(3R)ED50003]	w[1118]; Df(3R)ED50003, P{w[+mW.Scer\FRT.hs3]=3’.RS5+3.3’}ED50003/TM6C, cu[1] Sb[1]	Df, primary screening
24965 [Df(3R)BSC461]	w[1118]; Df(3R)BSC461/TM6C, Sb[1] cu[1]	Df, primary screening
24968 [Df(3R)BSC464]	w[1118]; Df(3R)BSC464/TM6C, Sb[1] cu[1]	Df, primary screening
24973 [Df(3R)BSC469]	w[1118]; Df(3R)BSC469/TM6C, Sb[1] cu[1]	Df, primary screening
24980 [Df(3R)BSC476]	w[1118]; Df(3R)BSC476/TM6C, Sb[1] cu[1]	Df, primary screening
24990 [Df(3R)BSC486]	w[1118]; Df(3R)BSC486/TM6C, Sb[1] cu[1]	Df, primary screening
25696 [Df(3R)BSC621]	w[1118]; Df(3R)BSC621/TM6C, cu[1] Sb[1]	Df, primary screening
25740 [Df(3R)BSC650]	w[1118]; Df(3R)BSC650/TM6C, Sb[1] cu[1]	Df, primary screening
26580 [Df(3R)BSC728]	w[1118]; Df(3R)BSC728, P+PBac{w[+mC]=XP3.RB5}BSC728/TM6C, Sb[1] cu[1]	Df, primary screening
26838 [Df(3R)BSC740]	w[1118]; Df(3R)BSC740, P+PBac{w[+mC]=XP3.RB5}BSC740/TM6C, Sb[1] cu[1]	Df, primary screening
26839 [Df(3R)BSC741]	w[1118]; Df(3R)BSC741/TM6C, Sb[1] cu[1]	Df, primary screening

26847 [Df(3R)BSC749]	w[1118]; Df(3R)BSC749, P+PBac{w[+mC]=XP3.WH3}BSC749/TM6C, Sb[1] cu[1]	Df, primary screening
27404 [Df(3R)FDD-0317950]	w[1118]; Df(3R)FDD-0317950/TM6C, Sb[1] cu[1]	Df, primary screening
7566 [Df(3L)Exel6087]	w[1118]; Df(3L)Exel6087, P{w[+mC]=XP-U}Exel6087/TM6B, Tb[1]	Df, secondary screening
8050 [Df(3L)ED4196]	w[1118]; Df(3L)ED4196, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED4196/TM2	Df, secondary screening
8054 [Df(3L)ED4256]	w[1118]; Df(3L)ED4256, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED4256/TM6C, cu[1] Sb[1]	Df, secondary screening
9609 [Df(3L)BSC178]	w[1118]; Df(3L)BSC178/TM6B, Tb[+]	Df, secondary screening
23761 [Mi{ET1}Zasp52[MB01837]]	y[1] w[67c23]; Mi{GFP[E.3xP3]=ET1}Zasp52[MB01837]	Df, secondary screening
26522 [Df(3L)BSC670]	w[1118]; Df(3L)BSC670, P+PBac{w[+mC]=XP3.RB5}BSC670/TM6C, Sb[1] cu[1]	Df, secondary screening

2.2.2 Fly crosses

The main crosses used in this study are Dfs crossed with the *scrib* RNAi host stock. The host stock was generated especially for this experiment by our lab (Fischbach, 2022). It consists of the GAL4 gene with UAS, which is a transcriptor enhancer on which GAL4 will bind and activate the transcription of the gene of interest (*scrib* RNAi). In addition, the timing of the expression is controlled by $GAL80^{TS}$ which is a repressor of GAL4. With this system, it is possible to conduct conditional KD of *scrib* RNAi in the Patched (Ptc) compartment which originates from the middle of the wing imaginal disc. The GFP fluorescent protein is crucial for confirming the presence of *scrib* RNAi. The host stock containing *scrib* RNAi is crossed with the Df over the Tm6Tb balancer,

which is needed for genotypic identification of the desired progeny during third instar larvae. The desired genotype (Figure 8a) consists of *scrib* RNAi-Gal80^{TS} for the conditional KD of *scrib* under the deficiency line on the third chromosome. The identification of the desired genotype on the third chromosome is possible due to the absence of a Tm6Tb balancer (Figure 8a,b). The identification of the desired genotype on the second chromosome is possible by the GFP fluorescents under the fluorescent stereo microscope (Figure 8a,c).

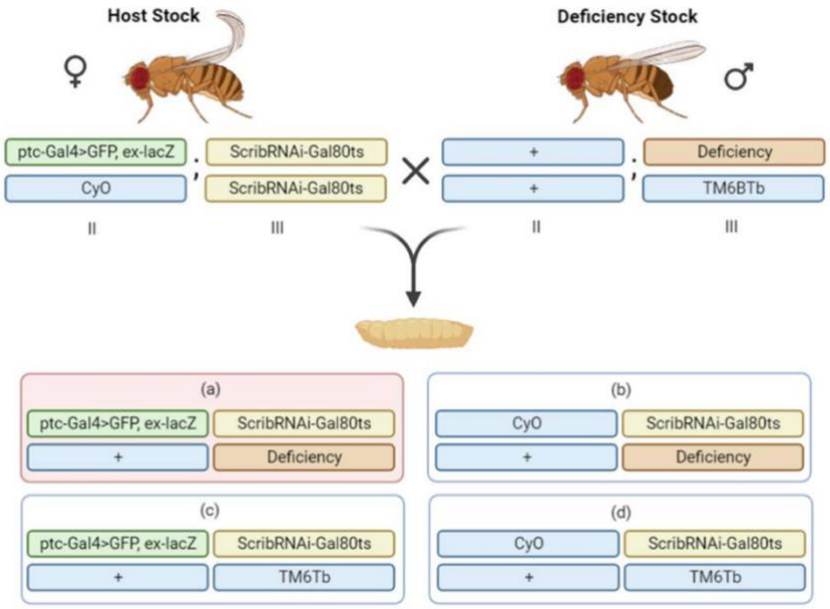


Figure 8. Expected progeny genotypes of screening crosses. The host stock (*ptc*-Gal4>UAS-GFP, ex-LacZ/CyO; scrib RNAi, Gal80^{TS}) is being crossed with the Df stock under the Tm6Tb balancer. There are four possible genotypic outcomes (a-d). The desired genotype (a) contains a deficiency line, active scrib RNAi due to the presence of both parts of the UAS-GAL4 system and GFP fluorescence. Genotypes containing the Tm6Tb balancer (c and d) can be avoided by not picking the short and chubby larvae. The genotypes that do not contain the GFP (b and d) can be excluded by observing the larvae under the fluorescence stereomicroscope. II refers to the second chromosome and III refers to the chromosome. The + sign means the chromosome does not include any added genes. Figure adapted from Fischbach, 2022.

To ensure the accuracy of the resulting offspring, female virgins from the host stock must be used for crossing instead of mature flies as they may already be inseminated. Hence, it is important to identify and collect the virgins for crossing, the most reliable characteristics of the virgins are the ventral abdomen spot called meconium, larger body size and lighter body colour. It is also possible to identify so-called super-virgins as their wings are yet to unfold.

Previous studies have revealed that the RNAi-mediated KD of *scrib*, driven by the Ptc-GAL4, sufficiently induced Yki-dependent cell overproliferation in the *Drosophila* wing imaginal disc. In

addition, studies have shown that the local loss of *scrib* depends on the dosage of *scrib*. Using the previously discovered information two controls were introduced to the screening protocol. The sensitised control, which contains one copy of *scrib*, is obtained by crossing the host stock with Df 8105 line that is deficient in *scrib* locus and shows severe neoplasia (*scrib*RNAi/*scrib*-)(one-copy RNAi control). The other control contains two copies of *scrib* and is obtained by crossing the host stock with Df line stock (*scrib*RNAi/+)(two-copy RNAi control). The *scrib*RNAi/+ larvae from that cross are the ones under the Tm6Tb balancer and with the GFP expression (Figure 8C). The contrast between the two-copy RNAi (*ptc*-Gal4, UAS-GFP, ex-LacZ/CyO; *scrib* RNAi, $Gal80^{TS}$/Tm6Tb) and one-copy RNAi (*ptc*-Gal4, UAS-GFP, ex-LacZ/+; *scrib* RNAi, $Gal80^{TS}$/Df 8105) controls presents a contrast, which allows observing the severity of neoplasia on the screened Dfs used in the primary and secondary screenings. This comparison is possible as the sensitised control (one-copy RNAi) and the main Df × *scrib* RNAi cross both contain the deficiency regions. Although the ex-LacZ was not used in this thesis the ex-LacZ is present in the genome of the host stock to identify Yki activity within the tissue. The staining of LacZ provides a specific indication of the location where Yki is activated.

2.2.3 Conditional KD

Prior to the experiment, a cross is made by using the host stock of virgin females (25-30 flies) with the Df line stock and Df 8105 males (10-15 flies). It is important to add yeast to the vials because it facilitates egg laying. The vials are then placed into the 25ºC incubator for 2 days (D) for the flies to mate, subsequently resulting in more progeny at the start of the experiment.

At the start of the experiment, flies are transferred to the new vial for a 12-hour egg-laying period at 25ºC. This period is crucial to ensure the larvae are at a relatively same age during the sample collection process. After egg-laying the flies are removed and the vials with eggs are transferred to the 18ºC incubator for 3,5D (Figure 9A). Subsequently, a temperature shift occurs when the developing larvae are transferred into a 29ºC incubator for 3D (Figure 9B). This temperature shift allows the creation of a conditional KD of *scrib* RNAi because of the UAS/GAL4/$GAL80^{TS}$ system. In a total of 6,5D after the egg-laying, the third instar stage larvae are collected.

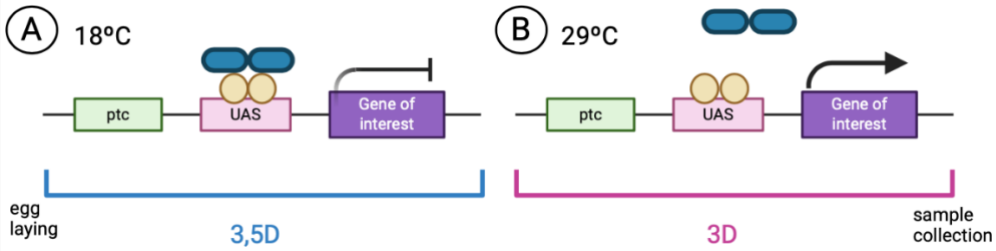


Figure 9. Experimental plan for conditional knockdown using GAL80^{TS}. (A) After egg-laying, the larvae are kept at 18ºC for 3,5 days (D). At this temperature the GAL80^{TS} (blue) acts as a repressor to GAL4 (yellow) and the gene of interest downstream is not expressed. (B) Vials are transferred to 29ºC for 3D to implement conditional KD of *scrib*. During this time the GAL80^{TS} becomes inactive and dissociates from GAL4, the transcriptional activator role of GAL4 restores and the expression of the gene of interest occurs. Figure created with biorender.com.

In a previous research conducted by Fischbach (Fischbach, 2022), she proposed various protocols on the number of days between the egg-laying and sample collection and the most suitable protocol of 4D at 18°C and 2D at 29°C was established. Many experiments were conducted in this thesis under these conditions. However, it was observed that these conditions were unsuitable after the controls were introduced. Different conditions were tried to determine the most appropriate one for both the controls and the Dfs.

2.2.4 Sample preparation

After conducting the conditional KD protocol (Figure 9), the first step of the experiment is to collect the non-Tb third instar stage larvae from the walls of the vial, transfer them to a 35mm Petri dish containing 1xPBS (phosphate-buffered saline) solution, and place it on ice to slow down the mobility of the larvae. The Petri dish with the larvae is placed under a fluorescent stereomicroscope Leica M205 FA to assess the salivary glands for the GFP signal. The larvae without the GFP expression are discarded. For the two-copy RNAi control, the Tb third instar stage larvae are also collected from the walls of the vial and checked for the GFP signal under the microscope. Preferably 10 or more GFP-positive larvae are collected and dissected.

The second step is the primary dissection. This procedure takes place under a stereomicroscope on a silicone pad filled with 1xPBS. The larva is placed in the PBS and 1/3 of the posterior part of the body is removed with forceps so that the wing discs located laterally near the anterior side are not damaged and would move towards the anterior side. The intestines and most organs are removed

until the wing discs are uncovered. The discs bound to the head are carefully transferred into a 1.5 ml Eppendorf tube with 1xPBS and placed on ice until all larvae are dissected.

All the dissected larvae are then fixed. This step is required for cell structure preservation. The 1xPBS is removed from the Eppendorf tube and 1xPBT (buffer based on PBS and non-ionic detergent Tween 20 (Roche®)) is added with formaldehyde (Sigma-Aldrich®). The concentration of the fixation solution is 3.7%. The imaginal wing discs bound to the head are kept in the fixation solution for 20 minutes at room temperature (RT) and then rinsed with 1 ml of 1xPBT three times.

The fourth step is the secondary dissection, which also takes place under a stereomicroscope on a silicone pad filled with 1xPBS. The imaginal wing discs, which are bound to the head are placed in the 1xPBS and the imaginal wing discs are extracted using the forceps. The extracted wing discs are transferred into a 1.5 ml Eppendorf tube containing 1xPBT until all the wing discs are extracted.

After the wing discs are dissected, they are stained with DAPI (Thermofisher®, D1306). The concentration of DAPI and staining time can vary between the following conditions 1:300 ratio for 2-hour staining at RT, 1:300 ratio for staining overnight at 4°C, or 1:100 ratio for 1 hour at RT. After the staining the PBT-DAPI solution is removed and the discs are rinsed with 1 ml 1xPBT three times.

After staining, the wing imaginal discs are mounted, which prepares the stained discs for microscopy. The microscopy slide and cover glass are cleaned with 70% ethanol and dried with Kimtech® wipes. The wing discs are pipetted on the glass slide and the excess 1xPBT is removed with the wipe after the discs are placed correctly onto the microscopy slide. When the excess liquid is removed 16 µl of mounting solution (70% glycerol) is added to the discs before the cover glass is placed on top. The edges of the cover glass are sealed with transparent nail polish.

2.2.5 Imaging and image analysis

The images of *Drosophila* wing imaginal discs are taken with Olympus BX51 Fluorescence Microscope with a 20x magnification objective. The microscope uses an Hg lamp to excite the fluorescent part of the disc like GFP and DAPI. Hence, the GFP is pH sensitive and the images have to be taken within three days after the mounting. After three days the mounting solution could get too acidic and cause the loss of the GFP fluorescent.

Thus, two images are taken for each disc, one for DAPI and the other for GFP signal. They have to be merged for accurate image analysis in the following image analysis step. ImageJ/Fiji is the software that is used for merging the channels and more (https://fiji.sc/). To sharpen the images in Fiji, it is possible to download plugins like DeconvolutionLab and PSF generator, which make it feasible to enhance the focus of images. After the merged images are created it is possible to analyse the imaginal wing discs further.

The analysis of the *Drosophila* wing imaginal discs is based on a scale generated by Lea Fischbach (Figure 10) (Fischbach, 2022). The stage of tissue overgrowth is determined by observing the expansion of the GFP stripe, which indicates the *scrib* RNAi cells, and the tissue architectural disruption, identifiable through DAPI staining.

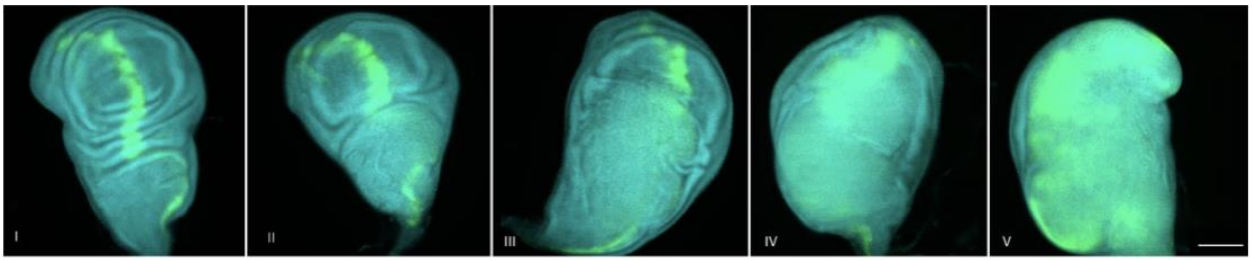


Figure 10. Examples of the neoplasia progression stages. From left (I) to right (V) are examples of the severity of tissue overproliferation. (I) no overgrowth; (II) mild overgrowth; (III) moderate overgrowth; (IV) severe overgrowth; (V) tumour. Scale=100µm. Adapted from Fischbach, 2022.

2.3 Results

2.3.1 Establishing the protocol for screening

The conditions of the first protocol used in this thesis were 4D at 18ºC and 3D at 29 ºC. With this protocol, the two-copy RNAi control showed no overgrowth as expected and neither did the one-copy RNAi control (Figure 11A,A'). Thus it was not reliable to identify the candidate gene regions correctly.

The next protocol tested was 3D at 18ºC and 3D at 29ºC. With this protocol the one-copy RNAi control showed a severe neoplasia phenotype as expected, but so did the two-copy RNAi control (Figure 11B,B'). Therefore this protocol was not reliable for the correct identifications of candidate gene regions either.

As one of the protocols showed severe overgrowth in both one-copy RNAi and two-copy RNAi control (Figure 11A,A') and the other showed no overgrowth (Figure 11B,B'), an additional protocol was tested of 3,5D at 18ºC and 3D at 29ºC. With this protocol, the majority of the two-copy RNAi control's wing discs showed no overgrowth, while some showed light overgrowth and the one-copy RNAi control showed severe overgrowth and neoplasia phenotype (Figure 11C,C'). For this reason, the protocol of 3,5D at 18ºC and 3D at 29ºC was used for the primary and secondary screening.

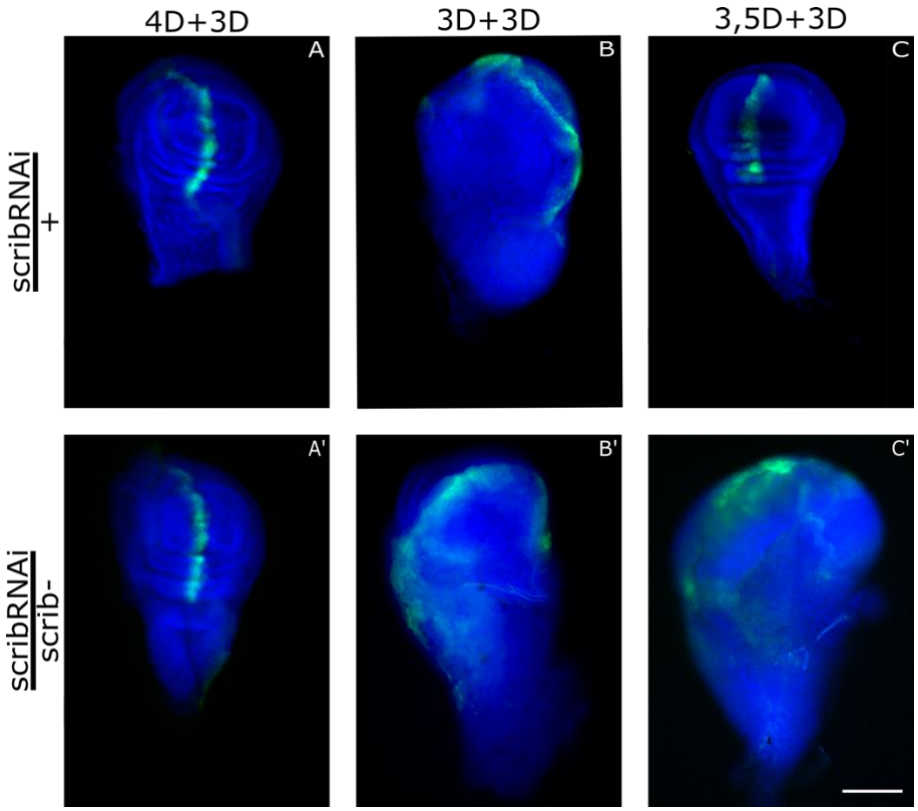


Figure 11. The levels of neoplasia in one-copy RNAi and two-copy RNAi control wing imaginal discs under different conditions. The top row represents the two-copy RNAi control (*scrib* RNAi host under a Tm6Tb balancer) collected under varying time conditions. The bottom row shows the one-copy RNAi control (Df 8105 under *scrib* RNAi host) collected under the same time conditions. At 4D+3D (D-days) conditions, neither two-copy RNAi nor one-copy RNAi control have neoplasia (A, A´). At 3D+3D both of them display a neoplastic phenotype (B, B´). At 3,5D+3D two-copy RNAi control has no neoplasia and one-copy RNAi control has tissue overgrowth (C, C). The scale bar is 100 μm.

2.3.2 Primary screening

101 initial lines with deficiency regions on the right arm of the third chromosome were obtained from the BDSC. Multiple randomly picked lines were screened for this thesis but 8 out of 19 were screened under the wrong conditions of 3D at 18ºC and 3D at 29ºC and their results are inconclusive. 11 lines were screened according to the updated protocol of 3,5D at 18ºC and 3D at 29ºC as discussed in section 3.2.1.

The results showed that eight of the Dfs do not contain genes essential to the maintenance of ABP with Scrib. Dfs with the negative result are Df 27404, 7737, 24965, 9090, 25740, 25696, 24516, 26839, and 24980 (Figure 12). Two lines were identified with a tumorous phenotype: Df 8923 and Df 24973. 50% of the wing imaginal discs of Df 8923 had an IV category overgrowth (Figure 10) and 19% had a V category overgrowth (Figure 12). For the Df 24973, 75% of the wing imaginal discs had IV category overgrowth and 25% had V category overgrowth (Figure 12).

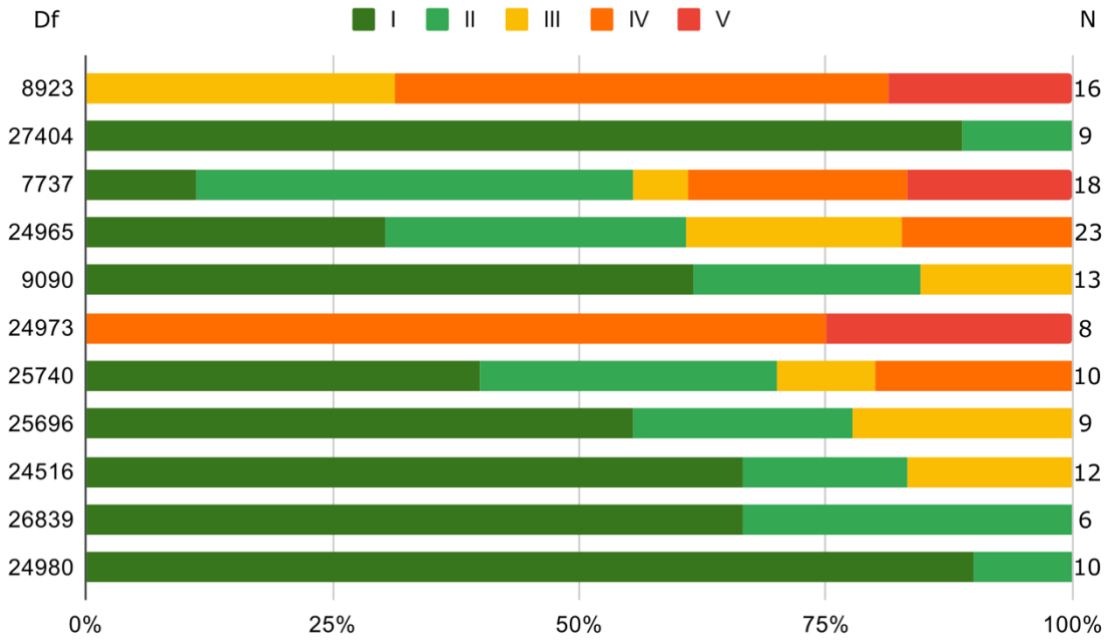


Figure 12. Statistical analysis of the results from primary screening. The figure shows the percentages of five neoplasia categories in every deficiency line (Df, top-left). The phenotypic identification method of the severity of neoplasia was taken from Fischbach (Fischbach, 2022). Dark green (I) shows no overgrowth, light green (II) mild overgrowth, yellow (III) moderate overgrowth, orange (IV) severe overgrowth, and red (V) tumourous overgrowth. N (top-right) denotes the total number of wing discs obtained for every Df.

Df 8923 was identified as a potential Df candidate as it showed III, IV, and V category severity of the neoplasia phenotype (Figure 12). The percentage of discs with severe tissue proliferation indicates the presence of potential gene(s) that synergize with Scribble and/or are crucial for maintaining epithelial homeostasis. In addition to the spread of the GFP signal from the Ptc compartment, DAPI staining revealed a disruption in the structural integrity in all of the collected wing discs (Figure 13A’’,B’’,C’’).

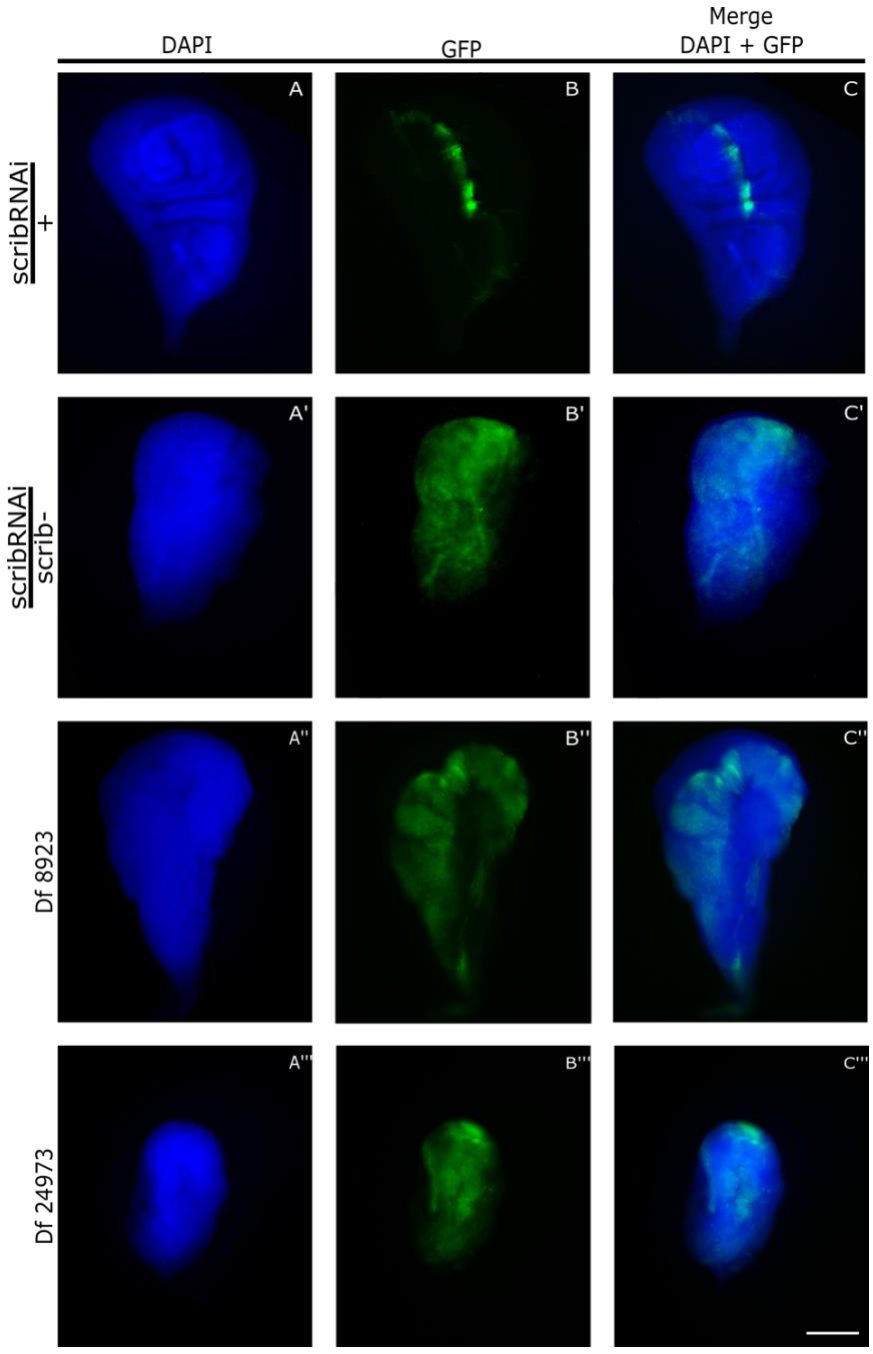


Figure 13. Screening results of two potential candidate deficiency lines. Df 8923 (A’’,B’’,C’’) and Df 24973 (A’’’,B’’’,C’’’) showed severe levels of neoplasia compared to the two-copy RNAi

control (A,B,C). Df 8105 under the *scrib* RNAi host was used as a one-copy RNAi control (A',B',C'). The DAPI staining (left), the GFP expression in the Ptc compartment (middle), and the merged channels (right) are presented. The scale bar (bottom-right) is 100 μm.

The Df 8923 consists of 108 inferred genes. The functions of 40 genes remain unknown, but the functions of five genes are interesting for this study as they have already been linked to either wing imaginal disc, cell competition, or regulation of apoptosis (Table 3). According to the tumour progression stages conducted by Fischbach (Fischbach, 2022), the Df 8923 belongs to the IV category of neoplastic severity (Figure 10).

Table 3. Interesting genes in the Df 8923.

Gene abbreviation	Known information
how	Molecular functions: enables mRNA 3'-UTR binding. Biological functions: it plays a role in cell adhesion, regulation of alternative mRNA splicing, regulation of embryonic development and apposition of dorsal and ventral imaginal disc-derived wing surfaces.
JMJD6	Molecular functions: histone H3R2 and H4R3 demethylase activity, P-TEFb complex binding. Biological functions: involved in cell competition in multicellular organism, negative regulation of apoptotic process and phagocytosis.
mats	Molecular functions: enables protein and protein kinase bindidng. Biological functions: it plays a role in apoptotic process, hippo signalling, and negative regulation of cell population proliferation.
sar1	Molecular functions: enables scaffold protein binding, GTP binding, GTPase activity. Biological functions: acts upstream of positive effect chitin-based cuticle development, it plays a role in positive regulation of dendrite morphogenesis and protein localization to plasma membrane.
tld	Molecular functions: enables collagen binding, metalloendopeptidase activity, calcium ion binding and zinc ion binding. Biological functions: it plays a role in amnioserosa formation, imaginal disc-derived wing vein morphogenesis, maternal specification of

	dorsal/ventral axis and positively regulates activin receptor signalling pathway and BMP signalling pathway.

The Df 24973 contains 101 inferred genes. The functions of 24 genes are still unknown, but three genes are particularly interesting as they have been linked to the maintenance of ABP, wing imaginal disc, and cell proliferation in previous research (Table 4). Most of the dissected wing imaginal discs belong to the IV neoplastic severity category according to the tumour progression stages previously categorised (Fischbach, 2022).

Table 4. Interesting genes in the Df 24973.

Gene abbreviation	Known information
csk	Molecular functions: enables ATP binding, non-membrane spanning protein tyrosine kinase activity and other kinase activity. Biological functions: it is involved in adherence junction maintenance, cell migration, epithelial structure maintenance, maintenance of ABP and negative regulation of growth.
Mrp4	Molecular functions: enables ATP binding, hydrolysis activity, and ATPase-coupled transmembrane transporter activity. Biological functions: it is involved in determination of adult lifespan, wing disc dorsal/ventral pattern formation, and response to anoxia, hypoxia, and oxcidative stress.
pros	Molecular functions: enables DNA binding transcription factor activity, which is RNA polymerase specific and sequence-specific DNA binding. Biological functions: it is involved in asymmetric neuroblast division, axon development and guidance, cell dedifferentiation, negative regulation of gene expression and cell population proliferation.

2.3.3 Secondary screening

The Df 23674 was identified as a candidate deficiency line by Lea Fischbach (Fischbach, 2022). It was chosen for a secondary screening to narrow down the region for a candidate gene. Collection

of the Bloomington Stock Center deficiency lines provides 17 stocks that overlap with the 23674 line. Five of them were ordered and used for the screening (Table 5).

Table 5. Dfs used for Df 23674 secondary screening. The table shows the Dfs used, the starting and ending point of the deletion on the left arm of the third chromosome, their deletion length, and the number of deleted genes.

Df	Starting point (nucleotide)	Ending point (nucleotide)	Deletion length (nucleotides)	Number of genes
BSC178 (9609)	1 368 841	1 534 423	165 582	31
ED4196 (8050)	639 583	1 478 937	839 354	133
ED4256 (8054)	1 546 104	1 586 663	40 559	9
Exel6087 (7566)	1 478 674	1 586 881	108 207	23
BSC670 (26522)	1 534 423	1 637 053	102 630	23
BSC289 (23674)	1 332 329	1 628 101	295 772	58

To narrow down the genomic region, four lines were screened for candidate line 23674 which included Df 8050, Df 9609, Df 7566, and Df 26522 (Figure 14). The lines were selected because they cover the whole Df 23674. Since Dfs 9609 and 8050 displayed no proliferative phenotype, it was reasonable to exclude eight of the Dfs without screening them, as the lines overlapped the previously mentioned two Dfs. Therefore, it was possible to confirm that those Dfs do not contain gene(s) involved in ABP homeostasis together with Scrib.

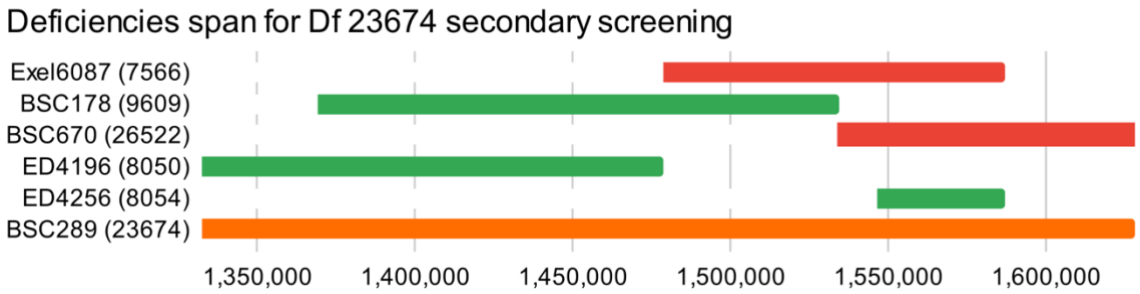
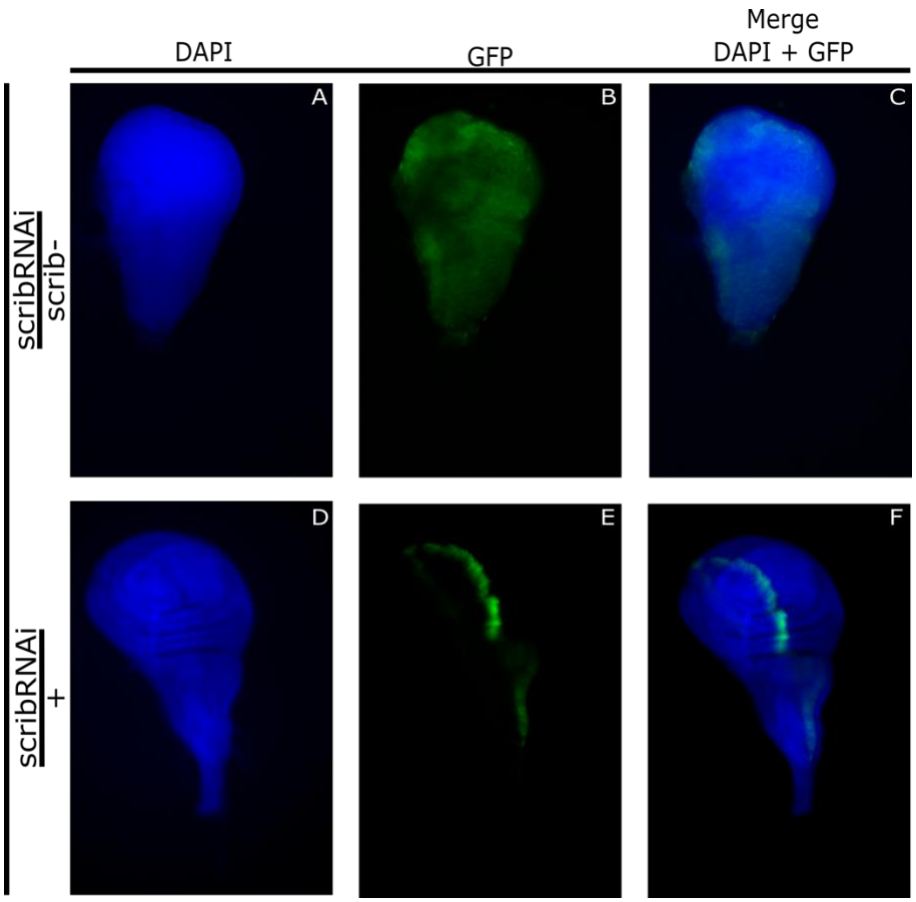
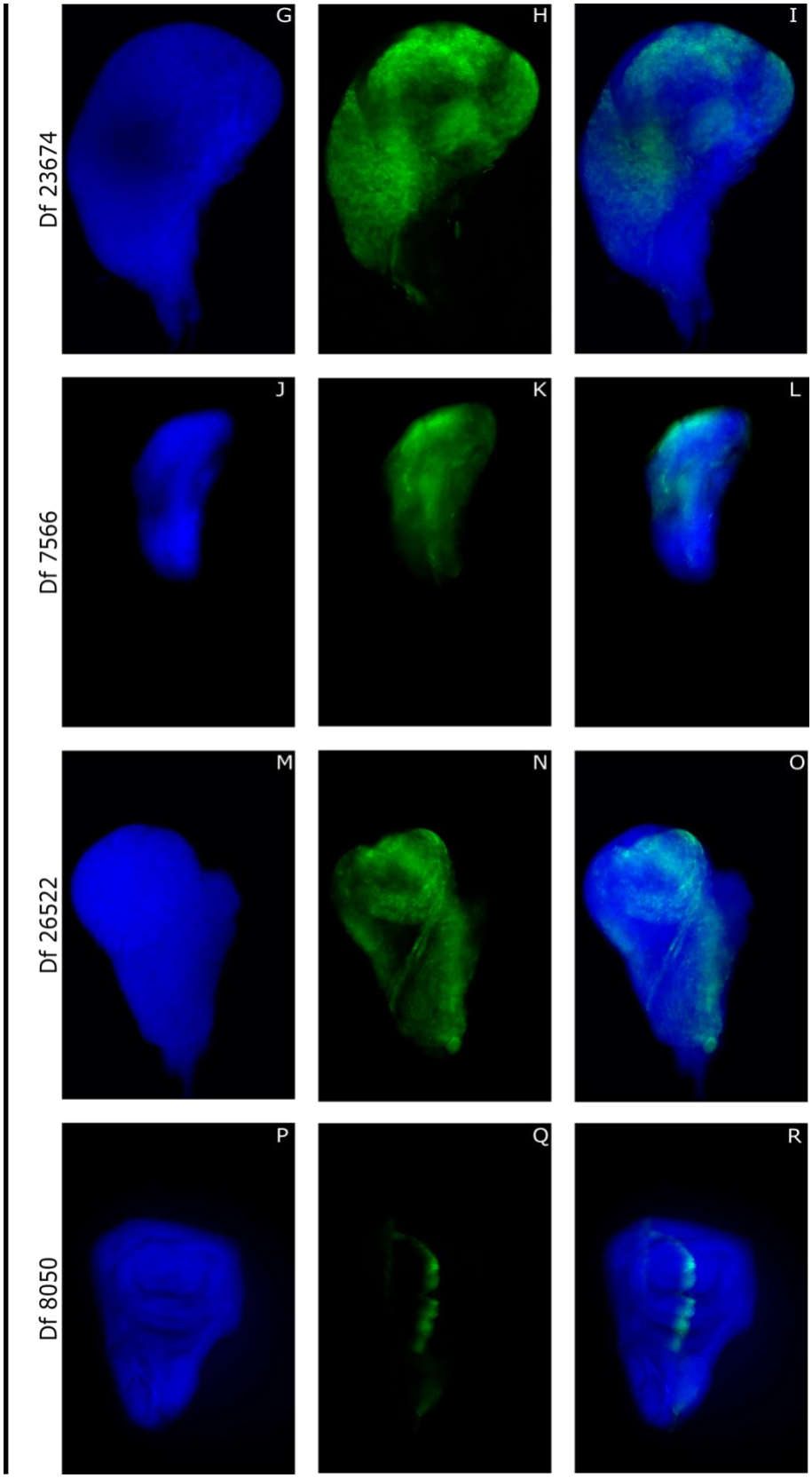


Figure 14. The genomic region of Df 23674 with overlapping deficiency lines. From the 17 Dfs overlapping the Df 23674 five were used for the finer screening of the candidate region. Three of them showed no signs of neoplasia (green) and two of them showed severe neoplastic phenotypes (red). The candidate Df 23674 is marked with orange.

The candidate region presented a neoplastic phenotype (Figure 15G-I) meaning it contains candidate genes. To infer the disc phenotype, other Dfs were screened and compared to one-copy RNAi and two-copy RNAi controls (Figure 16A-F). It is reasonable to say the Df 9609 and Df 8050 do not contain any synergic genes as they did not present any severe signs of neoplasia and can be excluded from the candidate region (Figure 15P-U). However, from these results, it can be concluded that the candidate gene is located in the overlapping region of Dfs 7655 and 26522, as they exhibited significant levels of neoplasia (Figure 15J-O). To further narrow down the region of interest, Df 8054 was utilized. Since this line did not demonstrate significant neoplastic formations, it is possible to eliminate it from consideration (Figure 15V-X).




Df 23674
Df 7566
Df 26522
Df 8050
G
H
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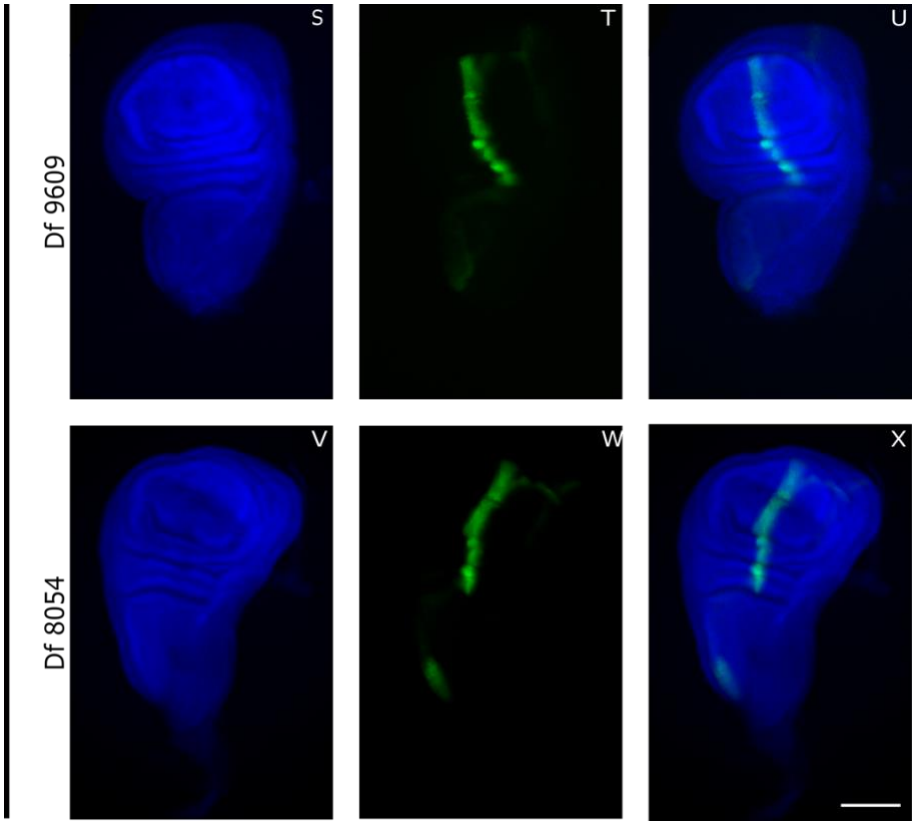

Figure 15. Results of secondary screening. The finer screening of the Df 23674 (G-I) showed that the Df 7566 and 26522 show severe levels of neoplasia (J-O). Df 8050, 8054, and 9609 showed no neoplastic phenotype (P-X). Df 8105 under *scrib* RNAi host was used as a one-copy RNAi control (A-C) and *scrib* RNAi host under a Tm6Tb balancer was used as a two-copy RNAi control (D-F). The staining with DAPI (left), the GFP expression (middle), and the merge of two channels (right) are presented. The scale bar is 100 μm.

Given that not all the wing imaginal discs from one Df have exactly the same phenotype, it is crucial to do a statistical analysis of the discs. Using Fischbach categorisation (Figure 10) the wing imaginal discs are divided into groups based on the severity of the neoplastic phenotype. The results are presented on a graph, which indicates the percentage of discs with a precise tumour progression stage (Figure 16).

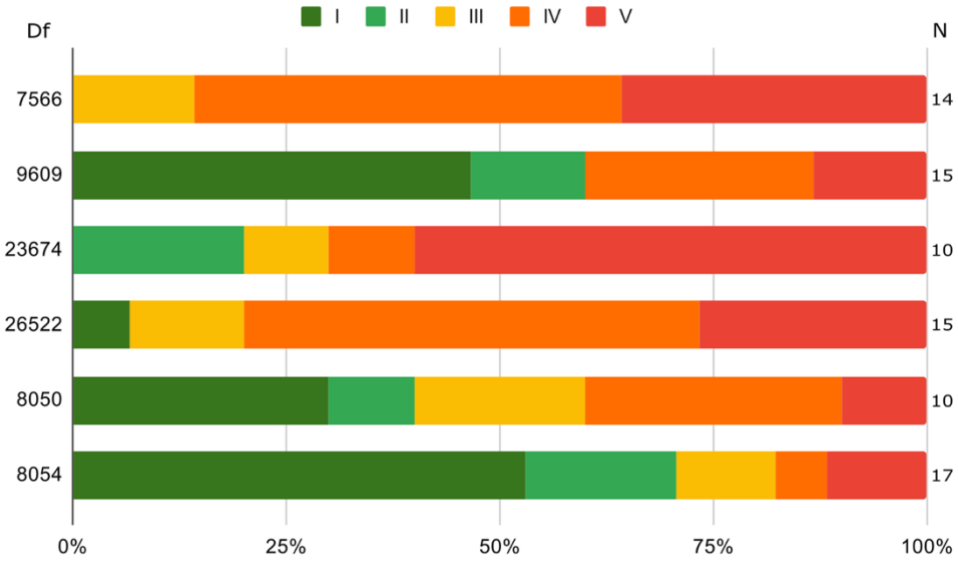

Figure 16. Statistical analysis of the results from secondary screening. The figure shows the percentages of five neoplasia categories in every deficiency line (Df, top-left). The phenotypic identification method of the severity of neoplasia was taken from Fischbach (Fischbach, 2022). Dark green (I) shows no overgrowth, light green (II) mild overgrowth, yellow (III) moderate overgrowth, orange (IV) severe overgrowth, and red (V) tumourous overgrowth. N (top-right) denotes the total number of wing discs obtained for every Df.

It is possible to identify 11 681 nucleotides of DNA that might contain the gene of interest. It is located between the end of the Df 9609 and the beginning of the Df 8054 and contains three genes (Table 6). None of the genes have been linked to the maintenance of ABP in previous research, but they are all protein-coding genes. The gene *CD12091* has a phosphatase activity, *pns* (*pinstripe*) is associated with Rab protein signal transduction but there is no information about the gene *CG32313*.

Table 6. Deficient genes in the obtained region

Gene abbreviation	Known information
CG12091	Molecular function: enables phosphoprotein and protein serine/theonine phosphatase activity. Biological function: involved in protein dephosphorylation. The phenotypic classes of alleles: viable, females are semi-fertile, some die during third instar larval stage, short lived, partially lethal, and some die during P-stage.

pns	Molecular function: enables guanyl-nucleotide exchange factor activity and small GTPase binding. Biological function: regulates Rab protein signal transduction. The phenotypic classes of alleles: viable, lethal.
CG32313	Molecular and cellular functions are not known. The phenotypic classes of alleles: viable; abnormal pain response.

2.4 Discussion

The screening of the *Drosophila* stocks deficient in a specific genomic region gives an insight into the genes crucial for the maintenance and/or establishment of the ABP and epithelial cell homeostasis together with Scrib (Fischbach, 2022). Disruptions in ABP will lead to overproliferation of epithelial cells which results in the formation of neoplasia (Bilder, 2004). Identification of the genes related to the maintenance of ABP is essential for understanding the mechanisms required for the proper intercellular alignment, cell communication, and epithelial morphogenesis (Huang *et al*., 2022).

The primary (right arm of the third chromosome) and secondary (left arm of the third chromosome) screening carried out in the thesis proved to be robust and well-adjusted. It can be used to pinpoint potential chromosome regions and further identify the genes vital for the maintenance of ABP. The genomic regions without the neoplastic phenotype can be excluded from further research and do not require additional screening, unlike the fly lines which provide the desired tumorous phenotype. The screening protocol still has some issues, as around 10-20% of the two-copy RNAi control wing imaginal discs continue to show neoplasia with the 3,5D+3D protocol (Figure 12). In addition, there was a variation in phenotypes due to different developmental timing and genetic backgrounds. However, some possible solutions can be explored. It is possible to change the initial incubation temperature conditions from 18°C to 20°C as it could significantly affect the phenotypic outcome and the developmental timing of the progeny. In addition, the time when the conditional KD occurs (at 29°C) could be shortened to 2D or 2,5D. As the 3D+3D protocol led to false positive results and 4D+3D produced no desired phenotype, it would be best to test the conditional KD timing (Figure 11). We can determine the most appropriate protocol for future screenings by comparing and contrasting different day and temperature conditions. This will help to change the rates of organism development and the progression of neoplasia formation after the KD of *scrib*.

The primary screening of 11 Dfs on the right arm of the third chromosome revealed that Df 8923 and Df 24973 contain genes important to the ABP homeostasis with Scrib as the Dfs showed severe levels of neoplasia (Figure 13). Other screened Dfs did not present the desired neoplasia phenotype and can be excluded from the secondary screening as in a high likelihood they do not possess candidate genes. (Figure 12). Further secondary screening of Df 8923 and Df 24973 will help to determine the possible candidate genes in the deleted regions. The Df 8923 consists of 108 genes and 5 of them – *how, JMJD6, mats, sar1*, and *tld* have been linked to the maintenance of ABP in

the previous research. Taking into consideration the findings of the previous research it is reasonable to start the secondary screening with a Df 27376 as it has a short deleted region containing the gene *how* (http://flybase.org). It is important to mention that although the function of other genes does not correlate with the described system, it is impossible to predict their interaction with Scrib. Therefore they could still be associated with the maintenance of ABP or tissue homeostasis. The Df 24973 consists of 101 genes including three interesting genes including *Csk, Mrp4* and *pros*. As the gene *Csk* is involved in the maintenance of the epithelial cell structure it is reasonable to start the secondary screening using the Df, which is short and includes the named gene, for example, the Df 7734.

The finer screening of the previously determined candidate region Df 23674 by Fischbach, 2022 helped to narrow down the region for the candidate genes in this thesis. The region starts from the end of the Df 9609 and ends at the beginning of the Df 8054 (Figure 14). It consists of 3 genes *CG12091*, *pns*, and *CG32313*. Previous research has shown that Rab5 endosomes are a localisation point for Thickveins, which is a bone morphogenetic protein (BMP)-type I receptor. This localisation is regulated by Scrib to optimize the signal transduction after receptor-ligand binding. The results showed that KD of *scrib* leads to loss of BMP signalling in the wing posterior crossvein region during the pupal wing development (Gui *et al*., 2016). As *pns* regulates Rab protein signal transduction this could indicate that Scrib interaction with the *pns* gene might lead to disruption of the tissue homeostasis. KD of *scrib* along with the deletion of *pns* might cause a loss of ABP during wing imaginal disc development. Moreover, there is no prior information available regarding the gene *CG32313*. Given this absence of data, it suggests the possibility of potential synergy with Scrib and should be investigated thoroughly. Lastly, there is no data, which would indicate that the gene *CG12091* has synergy with Scrib, but it cannot be predicted by the function of the gene. Therefore it is important to conduct a screening of each of the candidate genes. For the screening of the *pns* gene, it is possible to order fly stocks from BDSC, which have been generated for gene disruption like Df 15455, 85374, and 93318. There is also additional fly stock, which can be used for the screening of the gene *CG12091*, which is Df 86003, but there are no stocks, which could be used for the screening of the gene *CG32313* (https://bdsc.indiana.edu/).

It could be seen that Df 26522 has another overlapping area with candidate Df 23674, the region consisting of 10 genes. Nonetheless, excluding this small area doesn't alter the overall picture since the region containing the ABP homeostasis genes was identified in this study using the results of other Dfs. A screening of Df 24930 could cover a portion of the missing region and provide

conformational data about four genes in that region. However, a secondary screening allowed us to pinpoint a shorter region of interest based on the neoplasia phenotype (Figure 14).

The continuation of this project will involve a primary screening of all the Dfs, which cover the remaining parts of the right arm of the third chromosome. Furthermore, a finer secondary screening of the candidate Dfs lines discovered in this thesis and in the previous research conducted by other laboratory members. Newly identified genes from the continuing primary screening will be further investigated in the search for candidate genes that contribute to the establishment of ABP through homeostasis with Scrib. When all the candidate genes are identified, the second chromosome of *Drosophila* will be screened using the deficiency toolkit from BDSC.

SUMMARY

The fundamental goal of this thesis was to locate genomic regions containing the genes essential for the maintenance and/or establishment of the ABP and tissue homeostasis with *scrib*. To achieve this, the UAS/GAL4/GAL80^{TS} system was utilized to conduct conditional KD of *scrib* in the Ptc compartment of the *Drosophila* wing imaginal disc. Two deficiency lines from the primary screening, Df 8923 and Df 24973, showed tumorous overgrowth when crossed with the Scrib host stock. This indicates that these Dfs contain crucial gene(s) for the maintenance of ABP with Scrib. Additionally, the primary screening ruled out eight Dfs with a total of 323 genes that did not display a tumorous phenotype.

The secondary screening of the Df 23674 revealed three candidate genes in that region. Among them, *pns* is the most promising candidate as it is associated with Rab protein signal transduction, which is linked to Scrib. However, the other two genes, *CG12091* and *CG32313*, require further investigation as their synergy with Scrib cannot be predicted. The list of candidate genes involved in maintaining tissue homeostasis could offer insight into unknown mechanisms necessary for proper epithelial tissue development and growth control.

Through the research conducted in the thesis, it is possible to confirm that the 3,5 days at 18°C and 3 days at 29°C protocol has the most suitable conditions but it is not ideal. Additional protocol troubleshooting is required with different time and temperature conditions for a flawless method.

Apiko-basaalse polaarsuse säilitamiseks koostöös Scribble'iga vajalike geenide sõeluuring kasutades *Drosophila melanogaster'i* tiiva imaginaaldiski

Lonaly Jüriado

Summary

Raku polaarsusel on oluline roll koe homeostaasi säilitamises ja normaalse organite arengu tagamises kõikides hulkrakulistes organismides. Apikaalne-basaalne polaarusus (ABP) on epiteelrakkudele iseloomulik raku polaarsuse tüüp. ABP aitavad rakus tekitada ja säilitada kolm valgukompleksi: Partitioning defective, Crumbs ja Scribble kompleks. Scribble'il on palju erinevaid funktsioone, näiteks reguleerib Hippo signaalrida, mis kontrollib koe kasvu läbi raku jagunemise ja apoptoosi.

Viimased uuringud on näidanud, et Scribble mängib tähtsat rolli ABP loomises ja säilitamises läbi kogu koe säilitades koe homeöstaasi. Scribble funktsiooni häirimine võib põhjustada ABP kadumist ning läbi selle võib kujuneda kudede neoplaasia. Varasemalt on näidatud, et Scribble'i funktsiooni lokaalsest kadumisest põhjustatud neoplaasia teke on mõjutatud ka Scribble'i doosist antud koes. Antud bakalaureusetöös püstitati hüpotees, et Scribble valgu funktsiooni lokaalne kadumine on mõjutatud ka teistest geenidest, mis on sünergias *scribble* geeniga. Nende geenide avastamine, aitaks selgitada sünergilisi molekulaarseid mehhanisme, mis on normaalsete rakuvaheliste inteaktsioonide, koostöö ja epiteeli morfogeneesi alustaladeks.

Selle bakalaureusetöö põhiline eesmärk oli identifitseerida genoomi regioone, mis sisaldavad ABP säilitamiseks koostöös *scribble*'iga vajalikke geene. Selle saavutamiseks loodi geneetiline sõeluuring, mis kasutab UAS/GAL4/GAL80^{TS} süsteemi ning RNAi mehhanismiga *scribble* funktsiooni allasurumist *Drosophlia melanogaster'i* tiiva imaginaaldiski keskosas, kus asub *patced* regioon. Primaarse sõeluuringu tulemusena leiti kaks genoomi regiooni – Df 8923 ja Df 24973. Selleks kasutati deletsioone nendes spetsiifilistes genoomi regioonides, mis tähendab, et genoom on haploidne selles regioonis. Sekundaarse sõeluuringu tulemusena leiti kolm kandidaat geeni – *pns*, *CG12091* ja *CG32313*, mis võiks olulist rolli mängida ABP säilitamisel ning mille täpsemat funktsiooni arenevas tiivadiskis tuleks täpsemalt edasi uurida heitmaks valgust nii tiiva morfogeneesile kui ka patogeneetilistele protsessidele.

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