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Analysis of cell-cell communication involved in the control of proliferation in fruit fly *Drosophila melanogaster* wing imaginal discs

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Supervisor

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INFOLEHT

Äädikakärbse Drosophila melanogster tiiva imaginaaldiski epiteelrakkude proliferatsiooni kontrolliga seotud rakkudevaheline kommunikatsioon

Apiko-basaalne polarisatsioon (ABP) on olulise tähtsusega epiteliaalsete kudede kujunemisel ja funktsionaalsuse säilimisel. Epiteliaalse morfogeneesi uurimiseks on mudelina laialdaselt kasutatav äädikakärbse (*Drosophila melanogaster)* tiiva eellase – tiiva imaginaaldiski arengumehhanismid. Tiivadiski epiteeli ABP eest vastutavad polariseeritult paiknevad Par3, Crumbs ja Scribble valkkompleksid, millest antud bakalaureusetöö fookuses on Scribble kompleks. Ehkki tuumorsupressor Scribble funktsiooni on hästi uuritud erinevates teadustöödes, on siiski teadmata millisel moel *scribble* mutantsed rakud tiivadiskis suhtlevad ümbritsevate metsiktüüpi rakkudega enne neoplaasia moodustumist? Mis määrab, kas rakk muutub neoplastiliseks või mitte? Käesoleva töö eesmärk on välja selgitada *scribble* mutantsete rakukloonide suuruse mõju neoplaasia moodustumisele tiivadiskis.

Srcribble RNAi klonaalse analüüsi tulemusena selgus, et enamik väikeseid *scribble* kloone ellimineeritakse arengu käigus, seega väike hulk *Scrib* mutantseid rakke ei ole piisav neoplaasia moodustamiseks, samas kui suuremate kloonide puhul toimub rakkude kontrollimatu jagunemine ja neoplaasia moodustumine. Kokkuvõtteks, töö tulemused näitasid, et *scrib* mutantsete rakkude teatud kriitiline hulk on määrava tähtsusega neoplaasia moodustumisel. Saadud leiud on olulised mõistmaks epiteliaalset morfogeneesi ja kudede patogeneetilisi muutusi.

Märksõnad: äädikakärbes, *Drosophila melanogaster*, tiiva imaginaaldisk, Scribble, apiko-basaalne polaarsus

CERCS (B350): Arengubioloogia, loomade kasv, ontogenees, embrüoloogia

Analysis of cell-cell communication involved in the control of proliferation in fruit fly *Drosophila melanogaster* wing imaginal discs

Apico-basal polarity (ABP) establishment and its maintenance is important for epithelial morphogenesis and epithelium function. Well-established model for studying epithelial morphogenesis is a wing primordium – wing imaginal disc of fruit fly *Drosophila melanogaster*, which composed with polarized epithelial cells. Epithelial architecture of polarized imaginal disc cells is regulated by Par, Crumbs and Scribble complexes. This thesis is focused on the Scribble. Although the function of the tumour suppressor Scribble has been well studied in various scientific works, it is still unknown how *scribble* mutant cells in the wing disc communicate with wild-type cells before neoplasia formation? What determines whether a cell becomes neoplastic or not? Main aim of thesis is to elucidate how the clone size of *scribble* mutant cells impacts neoplasia formation. As a result, when conventional RNAi clone analysis has been carried out, small *scribble* RNAi clones are largely eliminated, suggesting that a small number of *scribble* mutant cells are not sufficiently competent for neoplasia formation, but larger scribble mutant cell populations become competent for overproliferation, ultimately leading to neoplasia formation. Taken together, these findings suggest that achieving a critical amount of mutant cells appears to be critical for the development of neoplastic competence. Further understanding these mechanisms will shed light on epithelial tissue dynamics and have broader implications for cancer research.

Keywords: Fruit fly, *Drosophila melanogaster*, wing imaginal disc, Scribble, apicobasal polarity

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

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TERMS AND ABREVEATIONS

ABP - apical-basal polarity

AJs - adherens junctions

ALM - apical margin of the lateral membrane

Baz - Bazooka

Crb – Crumbs

ct - control

D - days (e.g. 3D - 3 days)

DAPI - 4’,6-diamidino-2-phenylindole

Df - deficiency line

Dlg - Discs-large

dsRNA - double-stranded RNA

ELMO - engulfment and cell motility, a Ced-12 homolog

FA - formaldehyde

FLpase - Flp recombinase

FRT - FLPpase recognition target

Gal4 - galactose-responsive transcriptional activator in UAS/Gal4 system

$Gal80^{ts}$ - galactose-responsive temperature sensitive repressor of Gal4

GFP - green fluorescent protein

GUK - guanylate kinase

hsp70 - the 70 kilodalton heatshock proteins

hr - hours (e.g. 72hr - 72 hours)

JNK - c-Jun-N-terminal kinase

KD - knock-down

LAP - leucyl aminopeptidase

Lgl - Lethal giant larvae

LRR - leucine-rich repeat 7

MAGUK - membrane-associated guanylate kinase

Mbc - myoblast city, a Ced-5/ DOCK180 homolog

nTGS - three neoplastic tumour suppressor genes

Par - partitioning-defective complex

Patj - PALS1-associated homologue

aPKC - atypical protein kinase C

PBS - phosphate-buffered saline

PBT - buffer used for imaging

PDZ - post synaptic density protein (PSD95),Drosophila disc large tumour suppressor (DlgA), and zonula occludens-1 protein (zo-1)

RISC - RNA-induced silencing complex

RNAi - RNA interference

RT - room temperature

Scrib – Scribble

SH3 - schnurri-3

siRNA - small interfering RNA

SJs - septate junctions

Tb - Tubby

TJs - tight junctions

UAS - upstream activating sequence

Wg - Wingless

WT - wild type

Wts - Warts

YAP - Yes-associated protein

Yki - Yorkie

Cell autonomous - A genetic trait in multicellular organisms in which only genotypically mutant cells exhibit the mutant phenotype. (GenScript Biology Terms Dictionary; https://www.genscript.com/biology-glossary/8965/cell-autonomous#)

Cell nonautonomous – A genetic trait in which genotypically mutant cells cause other cells (regardless of their genotype) to exhibit a mutant phenotype. (GenScript Biology Terms Dictionary; https://www.genscript.com/biology-glossary/8965/cell-autonomous#)

INTODUCTION

Drosophila melanogaster is a popular model organism in developmental biology research. It has earned this distinction due to its generally simple organism, fast developmental cycle, and compact genome, which is 60% homologous to that of humans. Commonly used fly structures are the imaginal discs, which act as precursors to adult structures like eyes and wings.

Wing imaginal discs, composed of epithelial cells, are one of the cornerstones in cancer research. Apico-basal polarity (ABP) is a key component in epithelial cell morphogenesis. Polarity complexes and pathways can be tied back to human epithelial tissue since *Drosophila* has many homologous structures. (Milán et al., 1996; Tripathi & Irvine, 2022). The focus is on the Scribble polarity complex, located in the basolateral cell domain, as it is involved in the localisation of other polarity complexes like Par and Crumbs, in the apical domain, (Assémat et al., 2008; Bilder & Perrimon, 2000) as well as the regulation of the cell-cycle through the Salvador-Hippo-Pathway (Pan et al., 2018).

It is known that the loss of *scribble* can lead to neoplasia formation and overgrowth, since it directly affects ABP regulation and cell proliferation (Bilder, 2004). The discord is also connected to intercellular contacts which also envelopes cellular communication (Y. Huang et al., 2023).

Recent studies aimed to determine if there is synergy between Scribble and gene candidates involved in ABP control and cell-cell communication (Fischbach, 2022; Y. Huang et al., 2023). Still certain questions remain about the nature and type of communication between *scribble* mutant cells and wild-type cells. The aim of this study is to investigate how the size of mutant cells impacts neoplasia formation in the wing imaginal discs.

The main goal is to establish a protocol for RNAi clonal analysis method utilising the FLP/FRT system (Germani et al., 2018), as well as to study larger clones by combining previously mentioned method with Gal4/UAS/Gal80^{ts} (Barwell et al., 2017) and an RNAi in vivo approach (Kennerdell & Carthew, 2000).

1 LITERATURE REVIEW

1.1 Drosophila melanogaster as a model organism

Drosophila melanogaster, commonly referred to as the fruit fly, has been a cornerstone model organism in unveiling deeper insights in the field of cell and developmental biology, genetics, and the study of human diseases. The average lifespan of a fruit fly is 40 to 50 days with a 10-day transformation from a fertilized egg to a sexually mature adult under optimal conditions. After 24-hour of embryogenesis, the organism enters a 3-stage larval period during which it feeds to conserve energy for further development. No adult organs are present at these stages; however, they will soon develop from 19 imaginal discs after the larvae covers themselves in a chitinous protective pupal case or a puparium and undergoes the pupal stage over the next 4-5 days. During metamorphosis, the larval tissue breaks down, allowing the adult structures to form. After a process termed eclosion, the young fruit fly emerges and completes an 8 to 12-hour maturation and become ready to repeat the cycle of life once more (Hales et al., 2015).

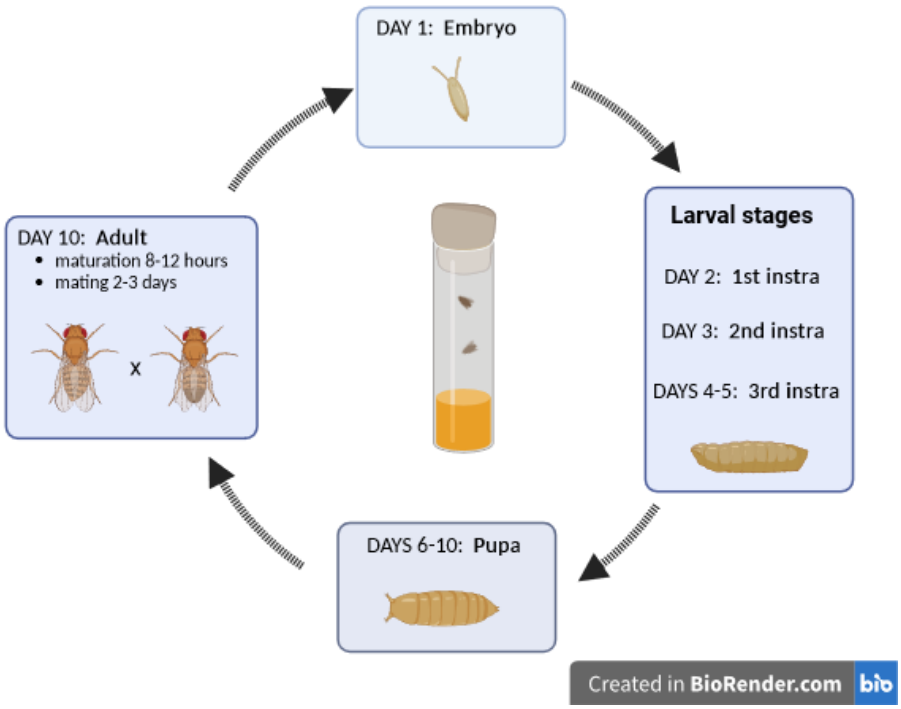

Figure 1. D. melanogaster life cycle. Cultured in vials with food, D. melanogaster transform from embryo to adult in 9-10 days if kept in 25°C. After 24-hour embryogenesis, the undeveloped fly enters 3 larval stages: first, second and third instra larval stages. It spends around 4-5 days as larva in food. On 5th day 3rd instra larva crawls on the walls of the vial, where it spends 1-2 days preparing for pupation. Days 6-10 metamorphosis occurs; and from

darkened pupa case develops an adult fly. It finishes its maturation in 8-12 hours and resets the cycle by mating. Adapted from Hales et al.(2015).

1.1.1 Genome

The *Drosophila melanogaster* genome is carried by 4 pairs of chromosomes. Of the four pairs two are the large metacentric autosomes. Their arms are referred to as 2L, 3L for the left arm and 2R, 3R for the right respectively. Two remaining pairs are the sex chromosomes and a very small autosome called dot chromosome (Hales et al., 2015). As an organism with a compact genome, *Drosophila melanogaster* has become a great model and was the second multicellular organism to have its genome sequenced. The construction of a large database soon followed with the *modENCODE* project in 2007 being the most notable (Boley et al., 2014).

1.1.2 Wing imaginal disc

The *Drosophila* imaginal wing disc is famously known in developmental biology community as valuable study model in research of tissue pattering, growth control, tumourgenetics, cell polarity and proliferation. The imaginal wing disc's simple design makes it easy to genetically manipulate without outright killing the fly (Tripathi & Irvine, 2022).

The early imaginal wing disc is a relatively flat tissue composed of clusters of undifferentiated cells. Its formation takes place in the embryo from ~30 cells located in the second thoracic segment. When the young fly enters the larval stage the cell clusters undergo rapid proliferation and differentiation, which results in a structure of ~35000 cells. Despite the imaginal disc being like any other larval tissue, its cells remain diploidal. The 3rd instra larval imaginal wing disc is a sac-like structure, mostly consisting of a thin continuous layer of compactly packed cuboidal epithelial cells (Milán et al., 1996; Tripathi & Irvine, 2022).

Along with cell division, the fates of certain areas, like notum and the hinge, are determined by intricate signalling pathways. This transformation brings the imaginal disc's simple structure to an adult organ with complex epithelial morphology (Tripathi & Irvine, 2022).

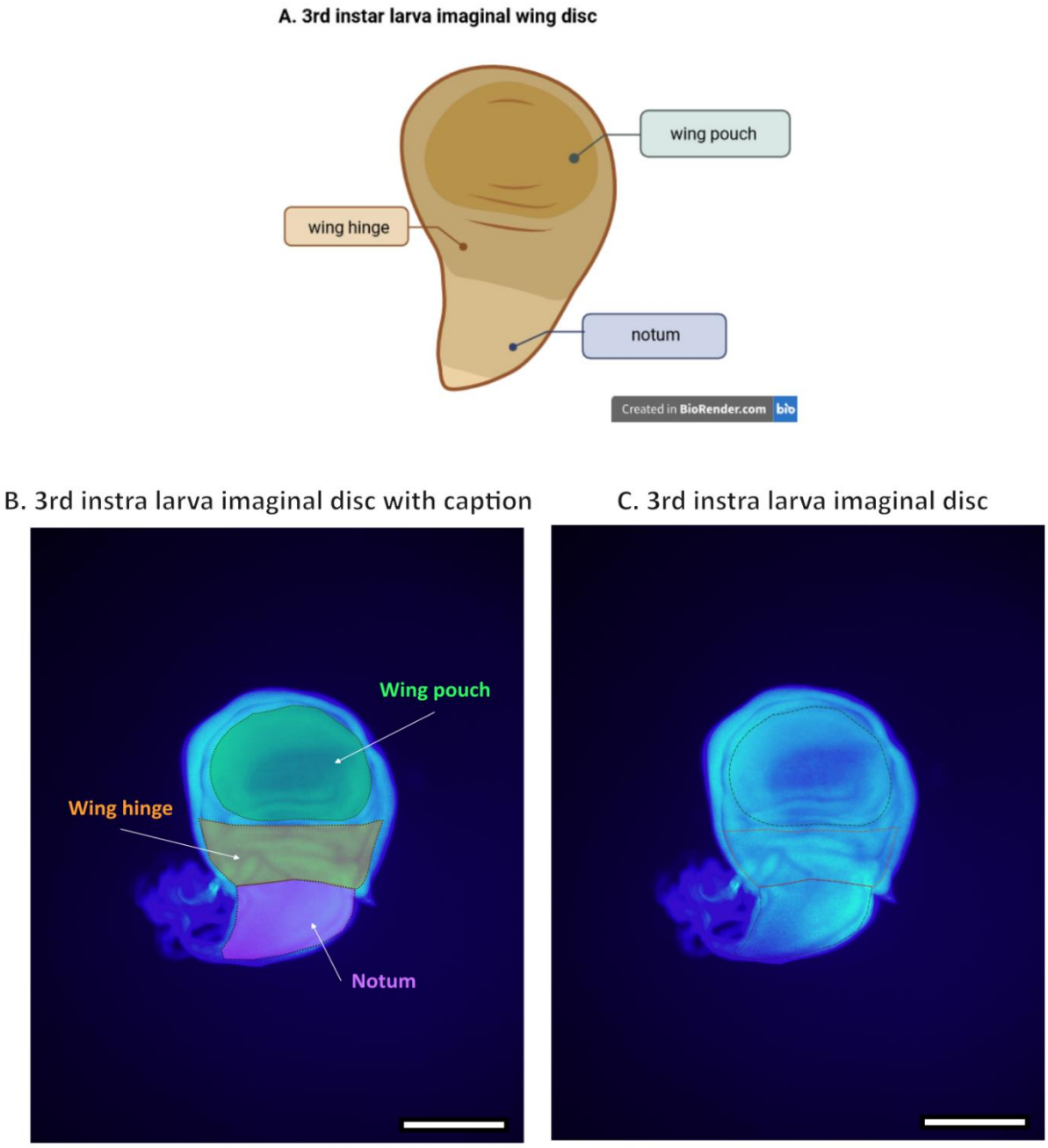

Figure 2. D. melanogaster late 3rd instra larval imaginal wing disc regions. A-C. The main regions of late 3rd instra larval wing disc are: wing pouch, from which the wing blade emerges; wing hinge, that gives form most of the structures at the base of the wing; and notum, that gives rise to the back of the fly (Tripathi & Irvine, 2022). Images B. and C. scale: 50 µm (magnification x20). Figure created with https://biorender.com and https://inkscape.org/.

1.1.3 Epithelial cells and cell-cell adherence aka junctions

In general, epithelial cells have three distinct domains: the apical, that projects towards the external surface, the lateral, where cells interconnect, and the basal, that connects the whole cell to the basement membrane (L Mescher, 2016). Like all epithelia, it is characterized by its tight cell-cell adhesion (also known as junctions) and apico-basal polarity (ABP), that is governed by asymmetric distribution of proteins in epithelial cells (Su et al., 2012). It has been proved, that faulty function of signalling pathways in two specific junctional domains, located on the apical and basolateral part of the wing disc, causes the loss of the ABP. In the most probable situation, this may result in the formation of tumour cells, which can either be eliminated by neighbouring healthy cells or spread further damaging the disc even more (Bilder et al., 2000)

The wing disc epithelium has two significant junctions: adherence junctions (AJs), located in the apical compartment of the cell, and septate junctions (SJs), located basally from the adherence junction. The AJs functionality spans from physically connecting cells to acting as a cell size regulator (Farhadifar et al., 2007). The SJs act as a barrier, blocking the direct flow between the apical and basolateral surfaces, and aiding the AJ in maintaining the ABP (Tepass et al., 2001).

1.2 The Scribble polarity complex

One of the essential multidomain protein complexes that regulates the overproliferation of epithelial cells is the Scribble complex. It consists of three neoplastic tumour suppressor genes (nTSG), that code three proteins – Lethal giant larvae (*Lgl*), Disc-large (*Dlg*) and Scribble(*scrib*). They are localized in the basolateral domain of the epithelia cell and their function depends on it. Together three of them cooperate in a common pathway encoding cytoplasmic proteins crucial to the establishment and maintenance of epithelial apico-basal polarity (Bilder, 2004; Bilder et al., 2000; Humbert et al., 2008)

Dlg product is a scaffolding protein homologue to the MAGUK (membrane-associated guanylate kinase) family. It consists of three PDZ domains, an SH3 domain, and a GUK domain (Bilder, 2004). It is usually located at the septate junction and mutation of Dlg leads to its loss and formation of ectopic adherence junction (Woods et al., 1996). Additionally, it plays

important role in providing stable association of Scribble to cell membrane and secures signalling components (Bilder et al., 2000; Hough et al., 1997).

Lgl stands out from both Scribble and Dlg as it is not particularly a scaffolding protein, although its function is directly tied to its localisation at the lateral membrane, just above AJ (Bilder et al., 2000; Jacob et al., 1987; Manfruelli et al., 1996). It consists of four WD40 motifs or β-propeller domains that are involved in protein-protein interactions. Since Lgl is not bound to a specific place in cell, its activity is dependent on whether it is phosphorylated or not (Humbert et al., 2008; Wirtz-Peitz & Knoblich, 2006; Yamanaka & Ohno, 2008). Lgl phosphorylation status is determined by aPKC and binding to aPKC and Par-6, which are proteins of the Par protein complex alongside Par-3 (Bazooka in Drosophila). When Lgl is phosphorylated, it is inactive and cannot localise to apical margin of the lateral membrane (ALM) where Scribble and Dlg are positioned (Betschinger et al., 2005; Bilder, 2004; Bilder et al., 2000; Humbert et al., 2008). Lgl also plays a role in vesicle targeting, promoting right localisation of growth factors and cellular adhesion molecules (Bilder et al., 2000; Greaves, 2000).

scrib encodes a multi-PDZ scaffolding protein and leucine-rich-repeat (LRR) protein and is also being referred to as LAP protein. PDZ and LRRs domains of Scribble are centred around mediating protein-protein interactions giving this protein a pivotal role in regulation of cellular protein distribution and stabilisation of plasma membrane. It can be found at septate junction alongside Dlg, that aids Scribble in recruiting Lgl to the membrane, proving the interwoven function of the three (Bilder, 2004; Bilder et al., 2000; Bilder & Perrimon, 2000; Humbert et al., 2008; Zeitler et al., 2004).

1.2.1 The Hippo complex

The Hippo complex governs the Salvador-Wart-Hippo (SHW) pathway that is core in managing organ size by cell proliferation and apoptosis. As of current date, it consist of eleven proteins and several side effectors (K. Harvey & Tapon, 2007). The most notable components are a module of serine/threonine kinases Warts (*wts)*, in charge of the amount and direction of cell proliferation (Justice et al., 1995), and Hippo (*hpo*) (K. F. Harvey et al., 2003), restricting cell growth. In addition there are two other proteins: a scaffold protein Salvador (*sav*) (Tapon et al., 2002) and Mob as tumor suppressor (*Mts*) (Lai et al., 2005), that control cell cycle and cell death. Alongside other genes, for example *scrib*, the SHW pathway oversees the normal cellular cycle and renewal in tissues. Its involvement in tumour suppression can be described

by its role in regulation of Warts binding partner and a transcriptional co-activator of growth-promoting genes Yki *(yorki*) (K. Harvey & Tapon, 2007; J. Huang et al., 2005).

1.2.2 Scribble as a tumour suppressor

One of core examples of *scrib* being an essential tumour suppressor gene is its relation to *yorkie*. Scribble contributes to activation of Hippo and a cascade of reactions within SHW pathway. Hippo phosphorylates Salvador directing it to bind with Warts and Hippo. In close proximity to Hippo, Warts is also phosphorylated, and connected to Mats, increasing its kinase power. Phosphorylated Warts creates a 14-3-3 binding site with Yki, which leads to Yki being localised in cytoplasm. Further steps of Yki regulation consist of ubiquitination and degradation (Oh & Irvine, 2010; Pan et al., 2018). In situations where Scribble is not present, the pathway is faulty, which leads to activation of Yki and pro-proliferation of the cell. This causes a massive tissue overgrowth without possibility of auto-apoptotic removal of mutant cells (Gui et al., 2021; J. Huang et al., 2005; Pan et al., 2018).

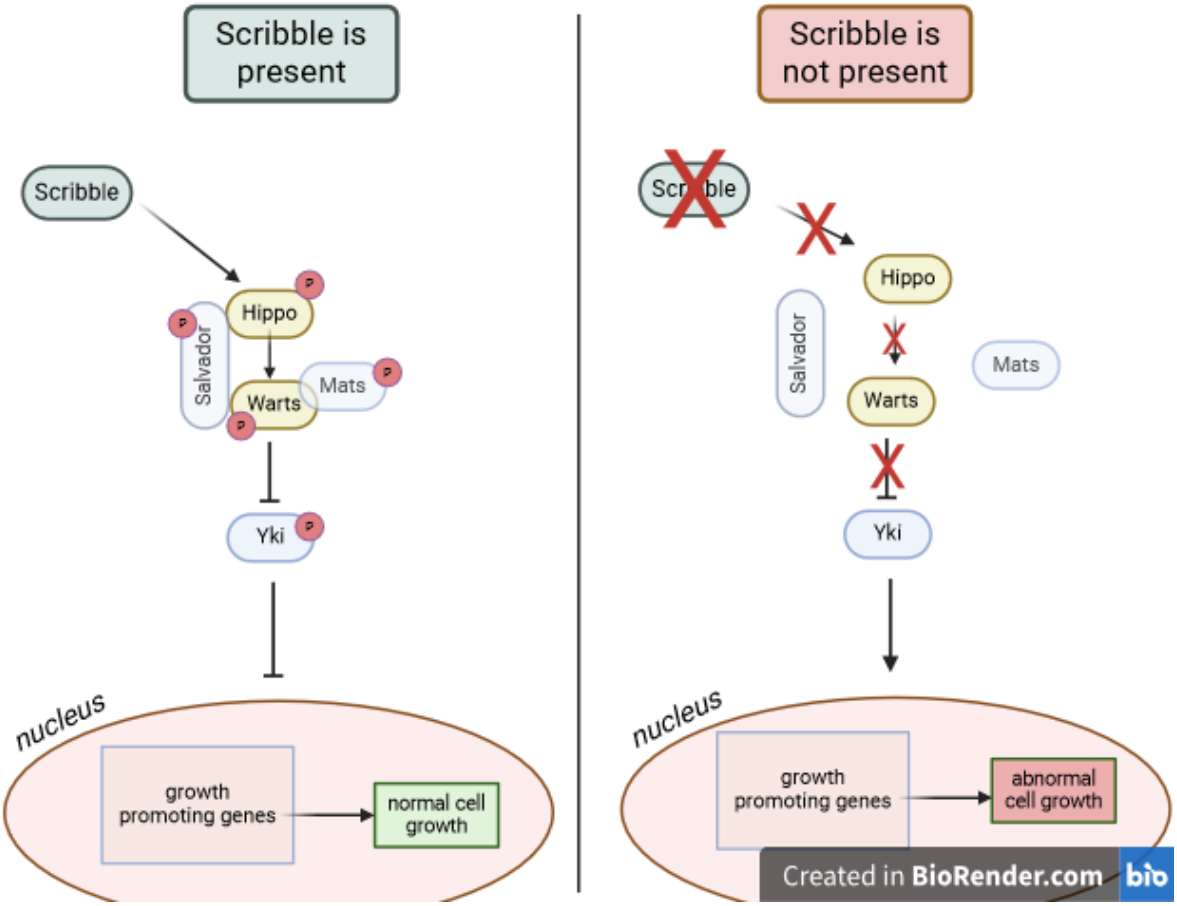

Figure 3. Yorkie activity regulation by Scribble complex and SHW pathway. When present, Scribble activates Hippo and a cascade of reactions lead to activation and phosphorylation of

Warts. Warts phosphorylates Yrki which leads to degradation of Yrk. When Scribble is missing from the pathway, the correct reactions are not regulated. This allows for uncontrollable activity of Yki without the maintenance cell growth and death (Oh & Irvine, 2010; Pan et al., 2018). Adapted from Oh & Irvine (2010).

1.2.3 Apico-basal polarity

The Scribble complex is not the only complex contributing to the stability of apico-basal polarity. In total there are three distinct polarity protein modules located at different parts of the epithelia cell, forming apicobasal axis and operating the cellular architecture. These protein complexes are: apical complexes, consisting of Crumbs and Par, that function as antagonist in relation to the basolateral module, Scribble complex (Assémat et al., 2008). In turn, the Scribble complex acts as a apical determinant for both Crumbs and Par (Humbert et al., 2008).

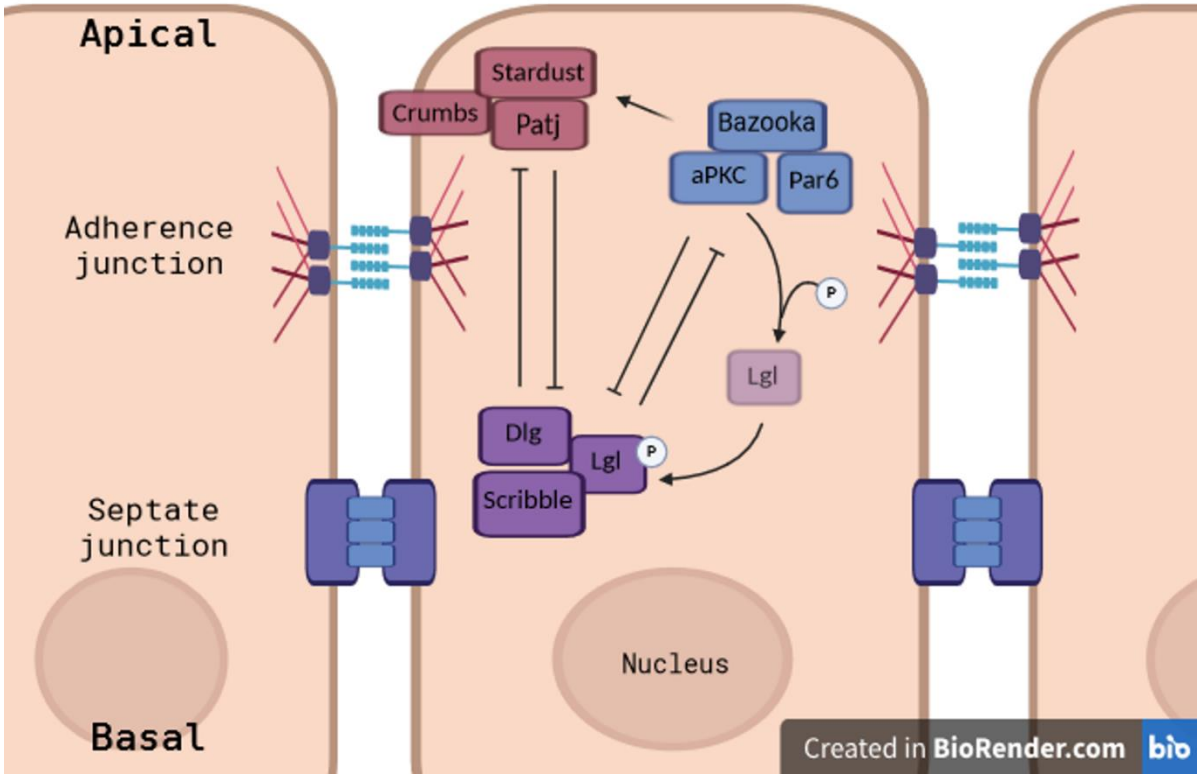

Figure 4. Apico-Basal polarity maintenance. Three polarity modules activity polarise the epithelial cells along apicobasal axis. The Cumbs complex (Stardust, Crumbs and Patj; red) and th Par complex (Bazooka, aPKC and Par6; blue) are located at the apical side of the cell. The Scribble complex (Scribble, Dlg, Lgl; purple) is located basolaterally at the SJ. Mutual antagonistic interactions between modules contribute to activity restriction. The Scribble complex antagonises both the Crumbs and the Par complex, whereas the Par complex promotes activity of the Crumbs complex. One important reaction between the Scribble and Par complexes is aPKC phosphorylation of Lgl that directs it localisation with the rest

components of the Scribble complex (Humbert et al., 2008). Figure adapted from Elsum et al. (2012).

Subsequently the role of the Scribble complex not only lies in maintenance of ABP, but in negative regulation of cell proliferation (Bilder et al., 2000). Consequences of loss of Scribble are an activation of Yrk mediated transcription of growth-promoting genes, expansion of apical domain, as well as a non-autonomous mispolarisation of anterior flanking cells, too. The overproliferation caused by ABP loss is exacerbated further due activation of Yrk that leads to expansion of Scribble loss. The significant observation to point out is this process is affected by dosage of Scribble (Bilder, 2004; Gui et al., 2021; Zeitler et al., 2004).

1.2.4 Cell competition

An interesting occurrence was described by Morata & Ripoll (1975), where cells with the cytoplasmic ribosomal protein mutation called “Minute”, although viable, could not survive in mosaic tissue with wild type cells. From this observation was deducted a term “cell competition”, a surveillance mechanism used in detection and elimination of viable, but suboptimal cells. After more experimentation and research, it was suggested that mutations in other genes have a possibility to also lead to cell competition, some possessing “super-competition”. Super-competition is described as the ability to eliminate surrounding WT cells in mosaic tissues. Thus, cells partaking in the cell-competition were named “loser cells”, due to them being “less fit” and eliminated by the “winner cells”, that are “more fit” for the replacement. Cell competition between wild type and mutant cells is usually resolved with apoptosis induced by the “winner cells”. It may occur in cells that possess any kind of malignant mutation, have rapid growth, or affected by neoplastic tumour suppressor defects. Cells with mutations in the Scribble complex are also subjugated to cell-competition (Baker, 2017; Fahey-Lozano et al., 2019; Merino et al., 2016).

1.2.5 Cell elimination

The observations of Scribble cells in mosaic tissue (also known as *scrib* or *scribble* clones) led to notice of changed characteristic in mutant cells. Rather than overgrow and effect surrounding wild-type cells, polarity-deficient cells were dealt through cell apoptosis, caused by exerted antitumor factor by the healthy neighbours. Through experiments using mutations in other polarity genes, it was suggested that elimination of *scrib* clones is regulated by an

active system rather than a passive aftereffect caused by dysregulation and overgrowth (Kanda & Igaki, 2020). In general, there are 3 non-exclusive modes of competition brought up by Merino et al. (2016): “extracellular survival factors, competition through fitness fingerprints, and competition through mechanical stress.” It is revealed, *scrib* clone elimination has two pathways. The first is c-Jun-N-terminal kinase (JNK) mediated apoptosis through activation of Drosophila tumour necrosis factor (TNF) family member Eiger and ELMO (engulfment and cell motility, a Ced-12 homolog)/Mbc (myoblast city, a Ced-5/ DOCK180 homolog)-mediated engulfment pathway. Eiger-JNK signalling is regulated by endocytosis in the *scrib* clones, which subsequently triggers the activation of endosomal activation of Eiger-JNK signalling leading to cell death (Igaki et al., 2002, 2009). This pathway is also present in surrounding WT cells, but won’t end in their apoptosis, but rather an upregulation of ELMO/Mbc mediated phagocytosis of nearby damaged cells (Ohsawa et al., 2011).

1.2.6 Cell communication

In previous study performed by Y. Huang et al. (2023), it was postulated that although ABP of sub-optimal cells was damaged by mutations in *scrib* gene, the tissue homeostasis can be restored via cellular communication with surrounding WT cells through genetical interactions of α-Catenin (α-Cat) with SJs components. Nevertheless, the rescue is very context dependent. The study shown three outcomes reserved for *scrib* mutant clones that dependent on the level of loss of function of the *scrib* gene. For *scrib* null clones ($scrib^{2}$/+), they were eliminated by cell competition, due to them missing minimal Scribble protein elements. Hypermorphic cells ($scrib^{2}$/$scrib^{5}$) had sufficient elements to have their polarity maintained by intercellular communication with surrounding optimal WT cells.

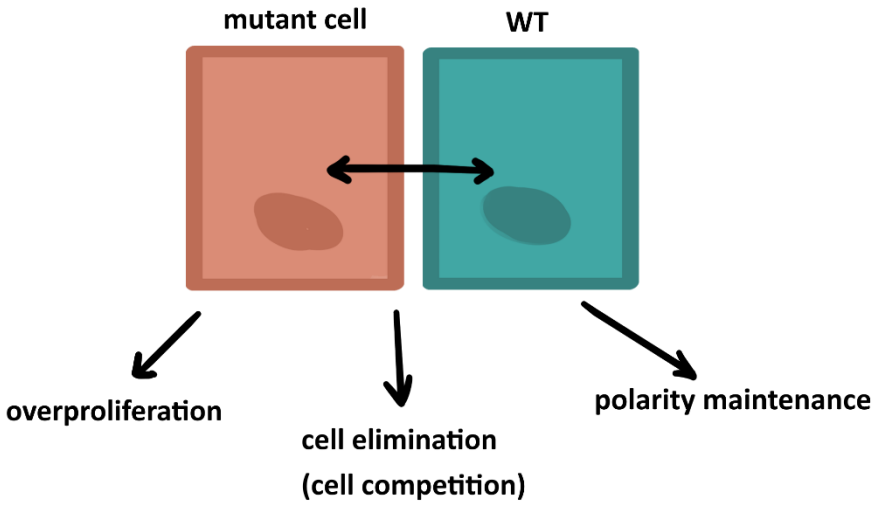

Figure 5. Cell communication between sub-optimal and optimal cells. There are three context-dependent manners of cell communication between sub-optimal and optimal cells. Either the mutant cell is eliminated via cell competition or restored to its optimal status with aid of neighbouring WT cells. In other cases, mutant cell starts overproliferating, potentially damaging the surrounding cells. Figure created with https://krita.org/en/

1.3 Genetic Toolkit

1.3.1 Balancer chromosomes

Balancer chromosomes or “balancers” are a common tool in *Drosophila* work and has been in use for almost 100 years. They are engineered chromosomes with multiple inverted and rearranged sequences (Miller et al., 2019). The original balancers contained only single inversion making them efficient, but not entirely safe from genetic material exchange. To overcome this setback an overlap of inversions and/or combination of inversions were used (Kaufman, 2017). The need in balancer chromosomes arose when it became clear that introduced recessive mutation could be eliminated from the stick population due to a genetic drift. The main feature of balancers is to prevent heterozygous or homozygous mutated alleles from being lost via genetical recombination (Bloomington Drosophila Stock Centre (https://bdsc.indiana.edu/)).

Balancers features include maintenance of sterile or lethal mutations from being excluded from inbred population and keeping the linear integrity of the genome. Some of the balancers also carry a dominant visible marker, making the carrier flies to be easily distinguished from the rest of the stock. In some specific screens a set of recessive visible mutations are used, as well as, fluorescent markers, like GFP and LacZ, added to existing balancers as another indicator (Kaufman, 2017).

1.3.2 UAS/Gal4/Gal80^{ts} system

One of the mostly used transgenic systems in developmental research and cell fate mapping is a UAS/Gal4/Gal80^{ts}. In 1993 Brand and Perrimon constructed a P-element vector with cloned yeast transcription factor Gal4, that can be integrated at random site in the genome. Cloned Gal4 factor is enhancerless, meaning it can be controlled by many different genomic enhancers. They also added an upstream activation sequence (UAS). Gal4 protein can bind to UAS that is also connected to general promoter and a cloning site. This system allows to insert any desired gene in a cloning site and perform an array of genetic manipulations. In practice, one strain must have Gal4 which expression is partially restricted and be mated with the second strain that has any chosen UAS-gene construct (Brand & Perrimon, 1993).

However, the original UAS/Gal4 system was very limited to temporal control. Different approaches were proposed, such as “tet-off” using tetracycline-dependent transactivator system Tet-off GAL80 transgene, but they were restricting to certain developmental stages (Bello et al., 1998). Thus GAL80^{ts}, a thermosensitive GAL80, variant was introduced. The logic behind its function is connected to temperature shift, as at 18°C the Gal80^{ts} is active and repressed Gal4 mediated expression. When the temperature was shifted to more permissive 29°C Gal80^{ts} became inactive, allowing for transcriptional activation of Gal4 (Barwell et al., 2017).

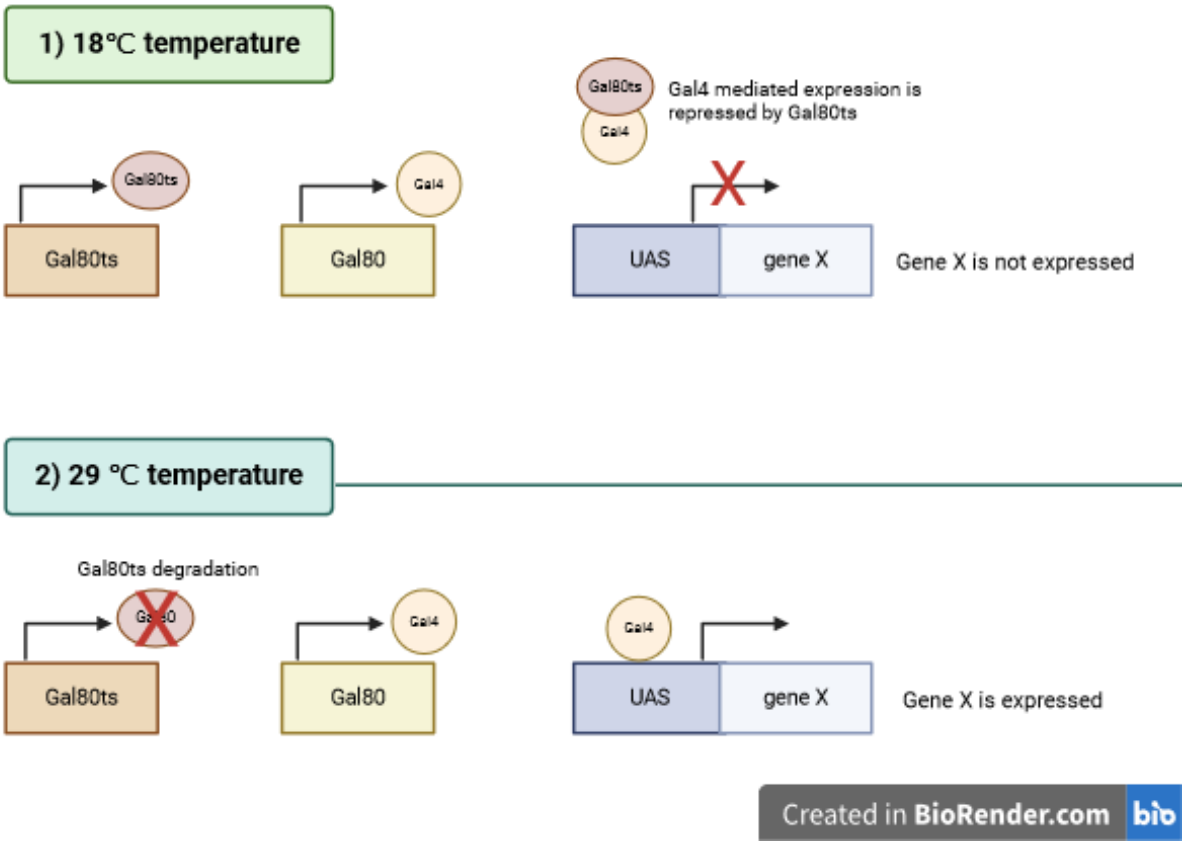

Figure 6. Gal4/UAS/Gal80^{ts} system. Gal4 produces eponymous protein that binds to UAS sequence and promotes expression of gene of interest. Gal80^{ts} is a temperature-sensitive protein that permits Gal4 mediated promotion by binding to it. 1) At 18°C, Gal4 is repressed, and gene of interest is not expressed. 2) When temperature is shifted to more permissive 29°C, Gal80^{ts} is degraded and allows for Gal4 to bind to upstream sequence UAS, activating the expression of downstream gene.

1.3.3 RNA interference

As was mentioned previously, the partial loss of expression of one of the genes in the Scribble complex causes neoplastic distribution of epithelial cells. Using this knowledge, application of

gene silencing with RNA-mediated interference proved useful in determining gene function. First discovered by Fire et al. (1998) in the nematode worm Caenorhabditis elegans, sequence specific gene silencing was developed further.

The core of the mechanism lies in production of double-stranded RNA by viral replication or the transcription of transposable elements and it's recognition by cellular enzymes (Choudhary et al., 2007). RNAi mechanism is triggered by dsRNA. It is recognised by Dicer enzymes and processed into ∼22-nucleotid small interfering RNAs (siRNA). The next step is performed by RNA-induced silencing complex (RISC), which incorporates siRNAs and unwinds them to later use as a guide in degradation of target mRNA leading to transcription inhibition or, in some cases, to chromatin remodelling (Hannon, 2002).

To effectively utilise this method with spatial and temporal control, transgenic approach was created by Kennerdell & Carthew (2000). UAS/Gal4 system was combined with RNAi that allows to flexibly inhibit gene expression at any stage of *Drosophila*'s life cycle.

1.3.4 Genetic mosaicism and clonal analysis

As termed by Germani et al. (2018) "Genetic mosaics individuals composed of cells with at least two different genotypes." This phenomenon proved effective in characterising genes viable in early stages of development, research in cell competition and mutations in genes that otherwise would be lethal if applied to the whole organism (Blair, 2003; Hales et al., 2015).

To generate tissue composed of patches of homozygous cells that originates from a single ancestral cell, in other words clones, a FLP-out technique was innovated by combining two different systems. The first method was developed by Struhl and Basler in 1993 to test functions of Wingless (Wgl) (Struhl & Basler, 1993) and later this knowledge was used by Golic and Lindquist in 1989, to formalise the FRT/FLP cassette (Golic & Lindquist, 1989). The second part of the system is derived from Gal4/UAS transcriptional expression (Blair, 2003; Brand & Perrimon, 1993).

The FLP-out technique is based around the fact that when two FRT sites are located on the same chromosome and their direction is similar, Flp recombinase (FLpase) will perform an extrusion of the DNA fragment located between two FRT sites, connecting two fragments in the end of the process. With the removal of the fragment, the termination signal and marker or other regulation gene are removed also. The fusion of FLpase and hsp70 heatshock

promoter allows for manual expression of FLPase without a requirement for a specific cell cycle phase and can be used to experiment on postmitotic tissue (Blair, 2003; Germani et al., 2018; Golic & Lindquist, 1989).

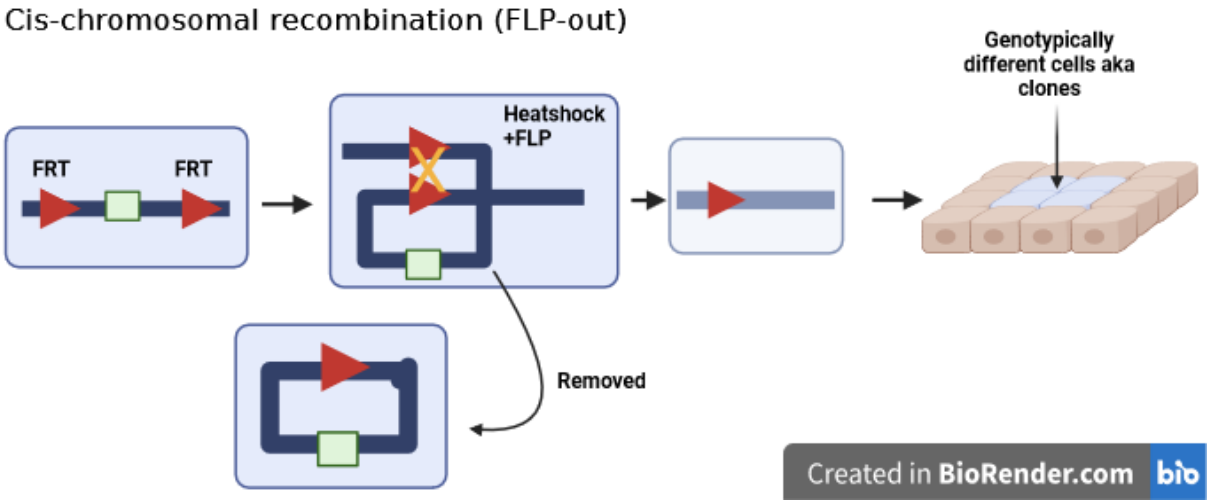

Figure 7. Cis-chromosomal recombination (FLP-out system). Gene or marker (green box) is located between two FRT sites (red arrows). With application of heatshock, FLP-recombinase is expressed and promotes recombination between two FRT sites. In this process, gene or marker is removed. Cells, where cis-chromosomal recombination was active (grey cells), have different genotype from the whole tissue and are referred to as clones. Adapted from Germani et al. (2018).

The Gal4/UAS system be added to have the resultant mosaic cell express or not to express the gene in the UAS construct (Blair, 2003; Brand & Perrimon, 1993). In normal conditions, STOP sequence between FRT sites permits Gal4 expressions and subsequently transcription of UAS construct. When heatshock is performed, FLP removes the FRT cassette alongside STOP sequence and activation of Gal4/UAs system. This leads to cells with active Gal4/UAS system to express GFP and other genes in the UAS construct. Those cells are called clones. Cells where recombination didn't occur, remain as a control tissue and are referred to as wild-type cells (Lee et al., 2012).

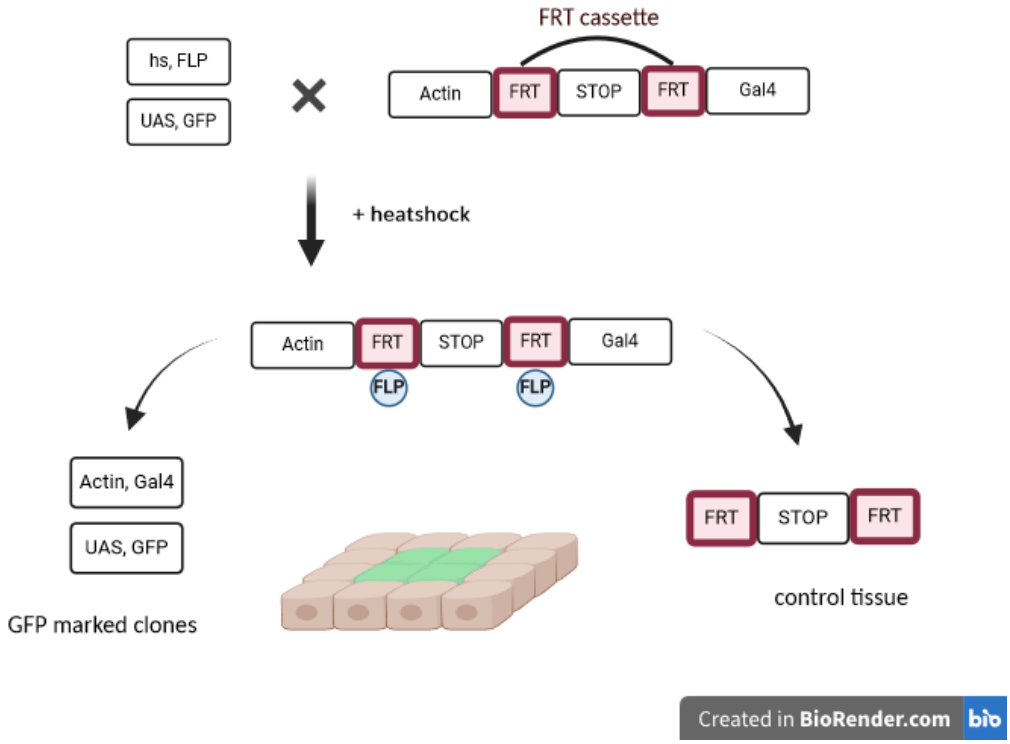

Figure 8. Flip-out method combined with Gal4/UAS system. STOP sequence between FRT sites (marked red) permits for transcription of Gal4. FLP (marked blue) removes the FRT cassete alongside STOP sequence. Cells where FRT cassete is removed become clones (marked purple). Cells where recombination didn't occur, remain as a control tissue and are referred to as wild-type cells (marked beige) Adapted from Lee et al. (2012). Figure created with https://biorender.com

2 AIM OF THE STUDY

The ongoing projects in the host lab's previous works utilized RNAi-mediated knockdown (KD) of *Scribble* via the patched (ptc)-driver, which manifested as a streak of cells mapping the anterior-posterior part of the wing disc. The method was used to identify the genes that synergize with *scrib,* contributing to neoplasia formation (Fischbach, 2022). The results yielded a great deal of gene candidates and also raised a question concerning why the boundary WT cells around the ptc-region were subjugated to the loss of Scribble. Two working hypotheses are considered: either the boundary WT cells experienced early-stage RNAi but regained their optimal condition through cellular refinement, leading to the subsequent loss of Scribble; or the mutant cells exhibit a nonautonomous genetic trait that influence WT neighboring cells to lose Scribble via cell communication.

Since the loss of Scribble causes a decline in the regulation of normal cell growth, as described in sections 1.2.1 and 1.2.2, we hypothesise that the size of mutant cells may contribute to their malignancy and competence in cell competition. It is presumed that clone size could influence whether mutant cells can outcompete WT cells or be eliminated by them.

For this study a more stringent method called RNAi clonal analysis was chosen. This method combines RNAi-mediated KD of the Scribble module with the temporal control of the FLP-out system, allowing for the generation of smaller *scrib* mutant clones ideal for this study.

Thus, the objectives of this thesis are to:

- Establish a protocol for utilising the RNAi clone method.
- Optimise the RNAi clone protocol to incorporate the Gal4/UAS/Gal80^{ts} system.
- To find out whether the size of the clone affects the mutant cells' competitiveness against WT cells.

3 EXPERIMENTATIONAL PART

3.1 Material and Methods

3.1.1 Fly lines

Most of the *Drosophila* lines utilized in this study are the fly stocks in the host lab or obtained from the stock centres, e.g. Bloomington *Drosophila* Stock Centre (BDSC; https://bdsc.indiana.edu/index.html).

Several fly lines were utilised. In short, RNAi and FLP/FRT lines, fly lines for UAS/Gal4/Gal80^{ts} system and balancers were used.

Table 1. Fly lines used in study.

Line number in Bloomington	Genotype	Type, purpose
35748	y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01490}attP2	Scribble RNAi line for positive control
36303	y[1] v[1]; P{y[+t7.7]=CaryP}attP2	Control RNAi line for negative control
NA	+/+; ptc-Gal4, UAS-GFP, ex LacZ/CyO; Scrib RNAi, Gal80^{ts} /Scrib RNAi, Gal80^{ts}	Gal80^{ts} carrier; used to implement Gal80^{ts} to Gal4/UAS system, lab stock
N/A	w; Sp/Cyo; MKRS/Tm6B	balancer, used for Gal80^{ts} carrier, lab stock
7	P{ry[+t7.2]=hsFLP}1, y[1] w[1118]; Dr[Mio]/TM3, ry[*] Sb[1]	FLP cassete and Gal4/UAs system carrier, #136, lab host stock

3.1.2 Fly crosses

The primary fly crosses used for the experiments were:

- #136: *hs-FLP/Sma6; actp>y>Gal4, GFP/Tb, Sb* × RNAi control – will be referred to as #136×RNAict or Negative control
- *hs-FLP/Sma6; actp>y>Gal4, GFP/Tb, Sb* × *scribble* RNAi – will be referred to as #136×*scribble* RNAi or Positive control
- *ptc>Gal4, UAS-GFP, exlacz; scribRNAi, Gal80^{ts}* × *w; Sp/Cyo; MKRS/Tm6B* = w; Sp/Cyo; ScribbleRNAi, gal80^{ts}/Tm6B
- #136: *hs-FLP/Sma6; Actp>y>Gal4, GFP/Tb, Sb* × *w; Sp/Cyo; scribble RNAi, Gal80^{ts}/Tm6B* – will be referred to as #136× *scribble* RNAI, Gal80^{ts} or Positive Gal80^{ts} control

Before setting up the experiment, male and female virgin flies were left to mate for 2-3 days in 18℃.

3.2 Imaging protocol

3.2.1 Heatshock

To successfully utilize FLP/FRT-induced mitotic recombination system heatshock should be performed during larval stages. Conditions for heatshock are incubation in 37℃ for 15 minutes.

3.2.2 Establishing protocol A

For establishing and testing RNAi clonal analysis method two crosses were used:

- #136: *hs-FLP/Sma6; actp>y>Gal4, GFP/Tb, Sb* × RNAi control as a Negative control
- #136: *hs-FLP/Sma6; actp>y>Gal4, GFP/Tb, Sb* × *scribble* RNAi as a Positive control

The overall structure of the protocol goes as follows: fly mating for 2-3 in 18℃, egg laying for 24 hours in 18℃, 1st incubation, that allows for the larvae to undergo first stages of development, heatshock for 15 minutes in 37℃. Then follows 2nd incubation, where larvae go thru remaining developmental stages before reaching 3rd instra larval stage.

The following three conditions were tested to decide on the most optimal time for 1st and 2nd incubations:

- **A'**: 1st incubation for 48 hours in RT, 2nd incubation for 72 hours in 25°C.
- **A''**: 1st incubation for 72 hours in RT, 2nd incubation for 48 hours in 25°C.

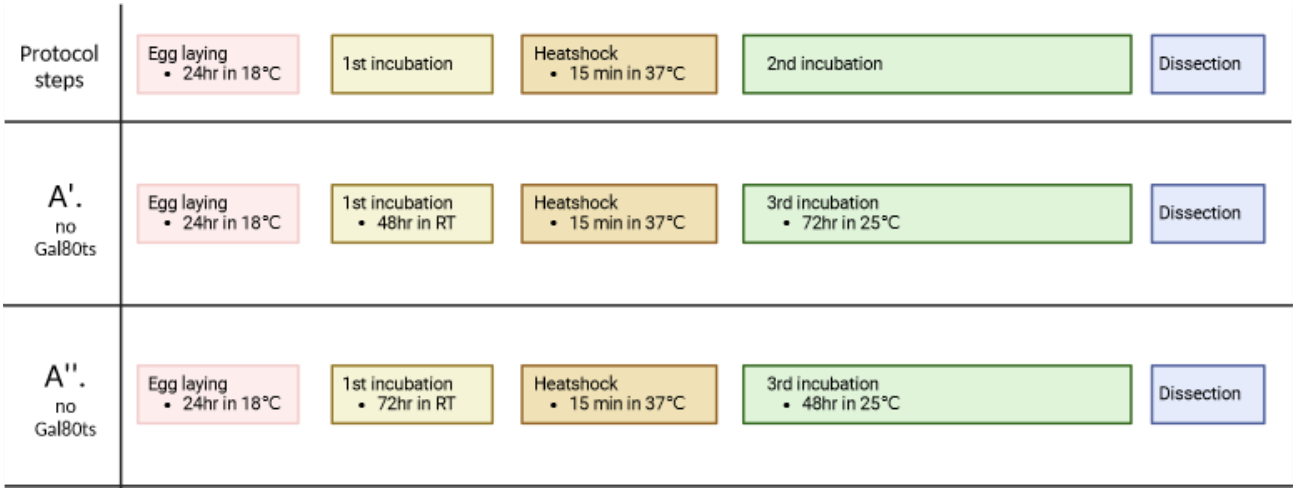

Figure 9. Establishing protocol A. Overall structure of the protocol remains the same throughout other protocols with the exception of different time periods for 1st and 2nd incubation.

3.2.3 Protocols for investigating different clone sizes

Protocol B and C were based on established conditions of protocol A, that were tested and described. Since protocol B and C use Gal4/UAS/Gal80^{ts} system, new conditions need to accommodate that. The overall structure of the protocol doesn't deviate from what was previously established other than changed 1st and 2nd incubation time and temperature.

For protocol B and C the 2nd incubation is divided into two sections. 1st part allows for the larvae and clones to grow after initiated heatshock. For the 2nd part, vials are placed in 29°C to activate degradation of Gal80^{ts} and expression of *scribble* RNAi and GFP carrying genes.

Following temperature and time conditions were used for protocol B and C:

- **Protocol B**: 1st incubation for 48 hours in RT, 2nd incubation for 48 hours in RT then for 48 hours in 29°C.
- **Protocol C:** 1st incubation for 48 hours in RT, 2nd incubation for 48 hours in RT then for 72 hours in 29°C.

In future references to specific protocols, the following labels will be used:

- **Protocol A:** (no Gal80^{ts}) Control A, when referring to negative control; Mutant A, when referring to #136×ScribbleRNAi prepared using protocol A.
- **Protocol B:** (with Gal80^{ts}); Control B, when referring to negative control prepared using protocol B; Mutant B, when referring to #136×ScribbleRNAI,gal80^{ts} prepared using protocol B
- **Protocol C:** (with Gal80^{ts}); Mutant C, when referring to #136×ScribbleRNAI,gal80^{ts} prepared using protocol C

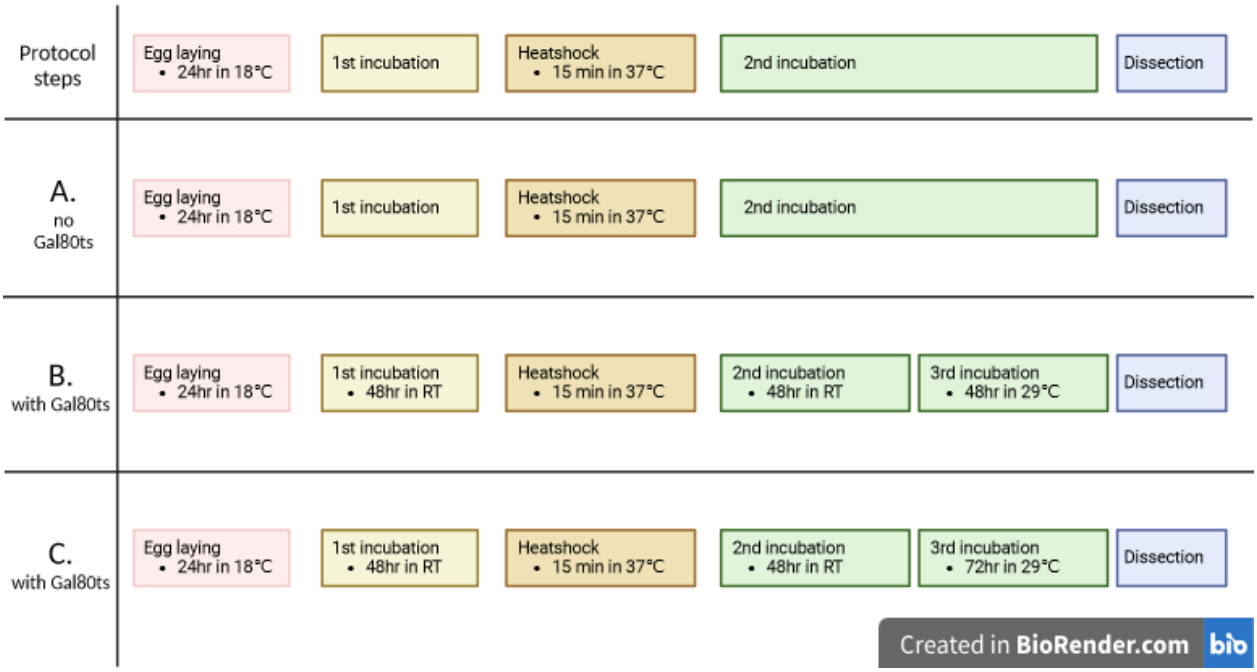

Figure 10. RNAi clone protocol variation. The first three stages of all three protocols remain the same to let fly embryos to enter early larval stages. The variation occurs in 2nd incubation stage (green) where, depending on fly crosses used, different growth conditions should be applied. Protocol A. uses Gal4/UAS system and doesn't need to temperature increased above 25℃ to allow for expression of ScribbleRNAi. B. and C. protocols use Gal4/UAS/Gal80^{ts} system and need to be placed in 29℃ to allow expression of ScribbleRNAi. Since protocol C is used to test cell malignancy, the time for 29℃ incubation is longer.

3.3 Preparations and dissection

Instruction on sample preparations were obtained in protocol by Antson et al. (2023).

To obtain desired samples, Non-tubby 3rd instar larvae was picked from the vial and transferred to the glass bottom dish containing 1x Phosphate-buffered saline (PBS) and immobilised by putting the dish on ice. To distinguish GFP positive non-tubby larvae from GFP negative, a

fluorescent stereomicroscope Leica M205 FA is used. The salivary glands are checked on for the presences of GFP signal. Signal positive larvae are selected.

Obtain wing discs the “Inverted method” was performed. Roughly one third of the posterior end of the larva is removed. Carefully, pinching the anterior part of body with one of the forceps, most of the intestines were removed. Using other forceps, the larva was inverted by pulling it onto the forceps that were still pinching the anterior part. The wing disc were left attached to the body and placed in 1.5 mL Eppendorf tube containing 1xPBS.

3.3.1 Fixation

Next steps require the fixation of exposed wing discs using a 3.7% formaldehyde (FA) solution. Larvae bodies are transferred to a new 1.5 mL Eppendorf tube containing 900µL 1xPhosphate-buffered saline with 0.1% Tween 20 (1xPBT) and 100µL 37% FA. For the tissues to fixate properly the tubes are left in RT for 20 min.

After the fixation, the FA solution is carefully removed from the tube and the samples are washed thrice with 1xPBT.

3.3.2 Staining

For the primary screening, DAPI (Thermofisher®, D1306) was used to stain the wing disc. A solution of DAPI/PBT with 1:300 ratio is prepared, and samples were left overnight at 4°C or in RT for 2 hours.

3.3.3 Wing disc separation

The wing disc are still attached to anterior body of the larva and need additional isolation.

3.3.4 Mounting

Stained samples are mounted on microscopy slides, that were cleaned with 70% ethanol and dried with Kimtech® wipes beforehand. The excess liquid is removed with wipes and discs are covered with mounting solution (70% glycerol). Cover glass is carefully placed on top and sealed with nail polish.

3.3.5 Microscopy

Slides containing samples were examined using Olympus BX51 Fluorescence Microscope. Preferred magnification objective is 20X. Samples images were also taken using Olympus BX51 Fluorescence Microscope.

3.3.6 Image analysis

Obtained images are and analysed. Obtained images are colour adjusted edited using image processing software ImageJ (Schindelin et al., 2012).

3.3.7 Quantification process

Using ImageJ, the area of the wing disc was calculated. The same was done for the GFP+ clone of the same disc. The ratio of whole disc area and clone area was calculated. The results were converted into percentages for easier interpretation. Confidence interval was calculated through following formula:

$$CI = \overline{x} \mp \overline{z_c}\left(\frac{s}{\sqrt{n}}\right)$$

Equation 1: Confidence interval formula where:

CI - confidence interval

$\overline{x}$ – sample mean

$\overline{z_c}$ – Z value of confidence interval

s – sample standard deviation

n – number of elements in sample

4 RESULTS

4.1 Establishing protocol A

To test RNAi clonal analysis approach, protocol A was established.

As presented in Figure 11, different time conditions have influence on the appearance of clones. In version A', RNAi control clones are adjacent to each other. No smaller clones as seen for *scribble* RNAi clones are present. RNAict clones are localised more on the sides of the wing disc. Cells had less time to develop and migrate, since 1st incubation lasted only 48 hours. The last hours of the development during 2nd incubation (72 hours) were mostly taken up by cell maintenance in form of cell competition. This is why *scribble* mutant clones are less clustered together and smaller clones branch out from the bigger ones.

A'' version shows completely different phenotype from A'. Both Positive and Negative controls examine an abundance of smaller and larger clones that take most of the wing disc's area. The 1st incubation lasted 72 hours and cells were able to extend all over the disc. Positive control has some significance of larger clones localised on the sides of the disc. The normal cells were less connected even before the application of heatshock. There is a hight chance that any connecting clones were eliminated by cell maintenance during 48-hour 2nd incubation. That's why we see a different outcome for both A'' controls.

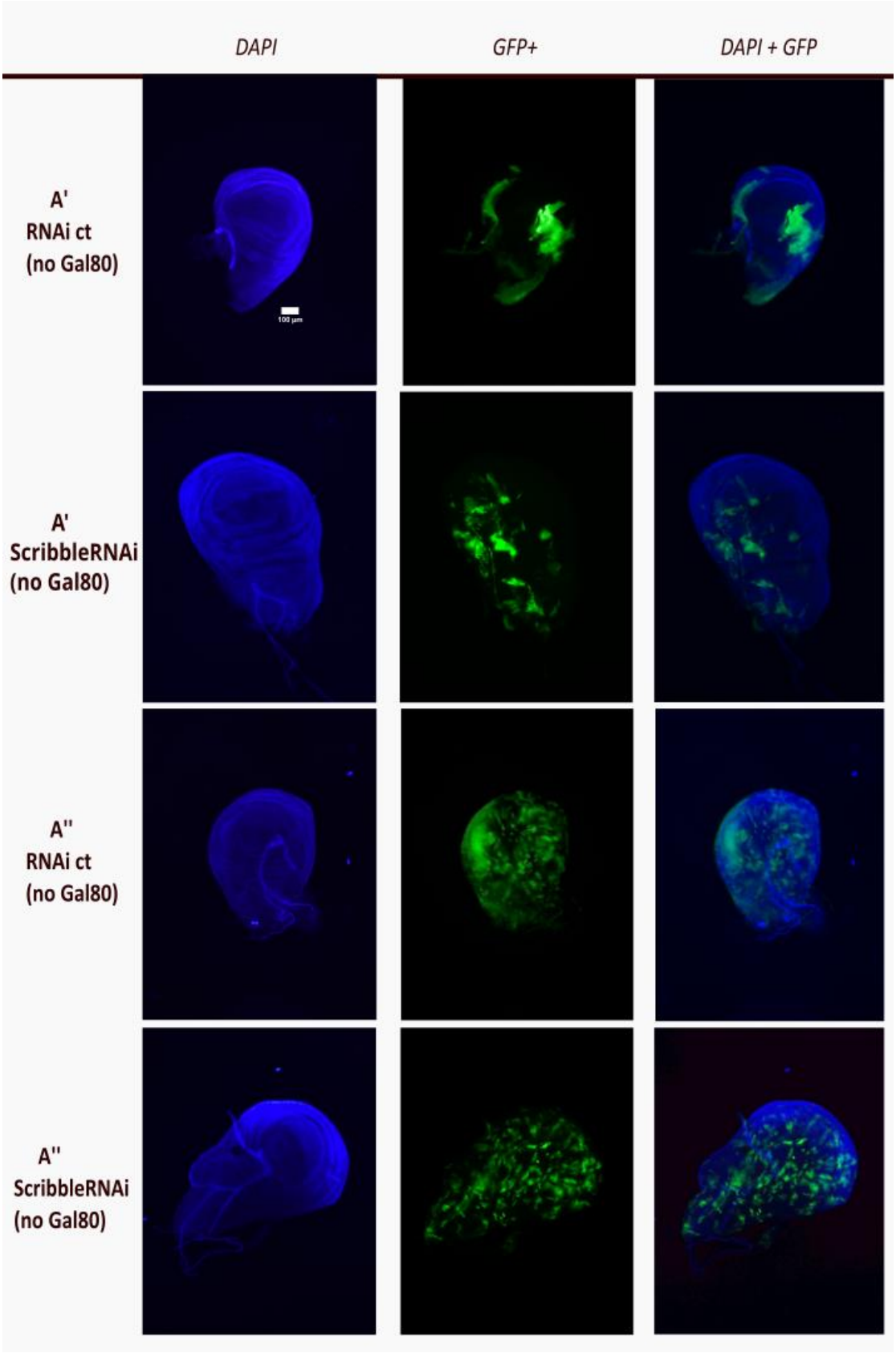

Figure 11. Establishing protocol A. RNAi ct as #136: hs-FLP/Sma6; Actp>y>gal4, GFP/Tb, Sb × RNAi control and ScribbleRNAI as #136: hs-FLP/Sma6; Actp>y>gal4, GFP/Tb, Sb × ScribbleRNAi dissected after 48hr in RT followed by 72hr in 25℃ (A'); or after 72hr in RT followed by 48hr in 25℃ (A''). Scale bar: 100µm (magnification x10).

4.2 Establishing protocol B

The main motivation behind altering established conditions in protocol A was to further investigate the proliferation of the mutant clones with application of Gal4/UAs/Gal80^{ts} system. This system allows to temporally control the synthesis of ScribbleRNAi by changing temperature conditions from RT to 29°C, where the Gal4/UAs system is active.

Results in Figure 12 clearly show the difference between both negative and positive controls.

Since after heatshock, cells were able to continue development without a threat of cell competition due to the repression of ScribbleRNAi synthesis by Gal80^{ts}, both controls of protocol B are bigger in comparison and cover the wider area of the disc. *Scribble* mutant clones branch more frequently. Undefined areas of the clone are also visible, which can be considered as clone cells being extruded or migrating to the lover levels of the epithelial tissues. The areas are more present in protocol B's positive control. Notable phenotypical difference in some Positive B controls is apparent deformation in wing disc's structure at the wing pouch lateral region (comparison in Appendices 1). It is safe to presume that a big clone was localised there similar to what can be seen in positive B control. Due to a sudden shift in cell homeostasis, surrounding WT cells cannot maintain or extrude the clone, so they resolved to eliminating it by apoptosis. This led to a missing piece of the tissue since it was almost at the last stage of development before metamorphosis.

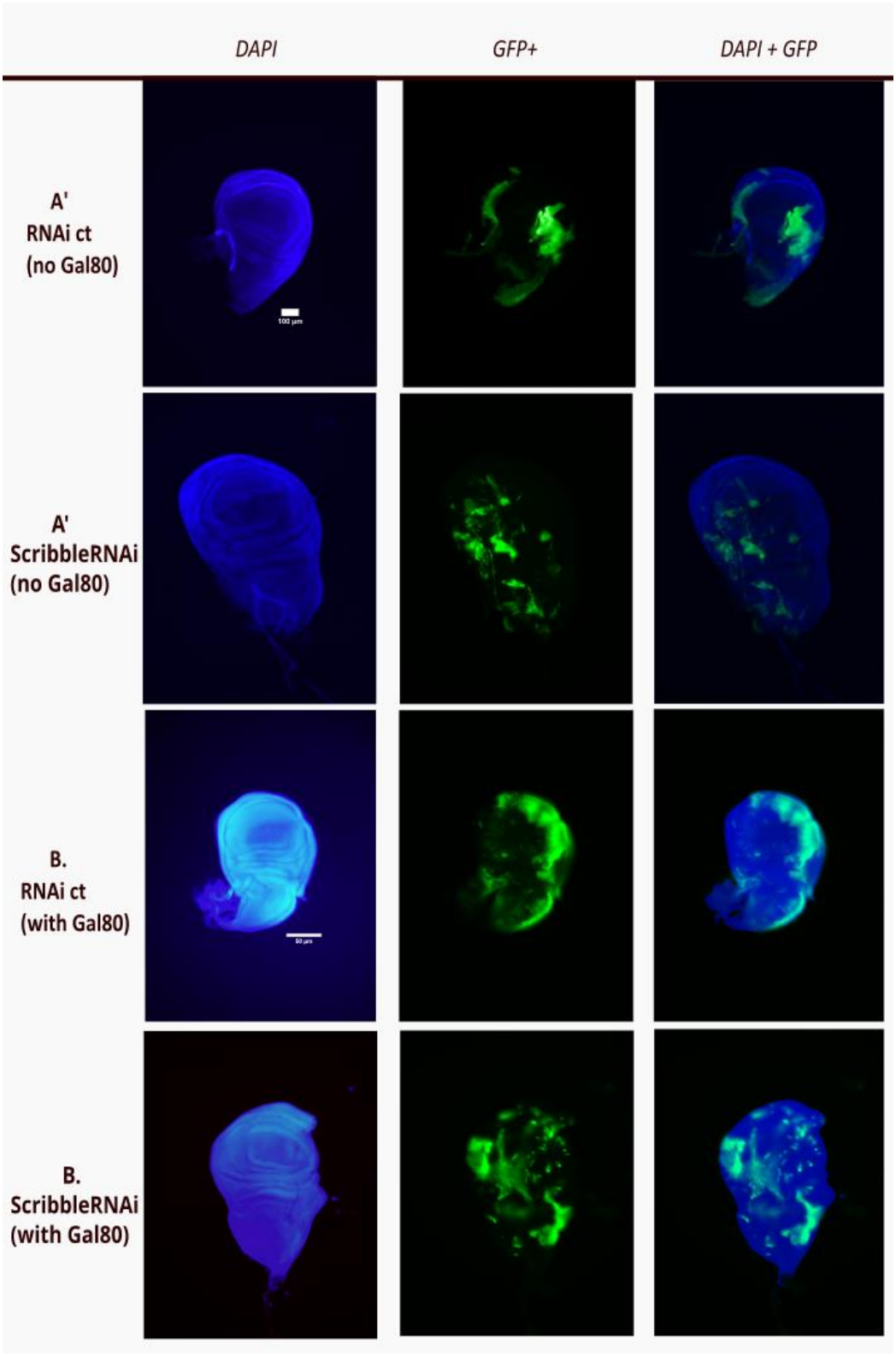

Figure 12. Results for protocol B establishment. RNAi ct as #136: hs-FLP/Sma6; Actp>y>gal4, GFP/Tb, Sb × RNAi control and ScribbleRNAI as #136: hs-FLP/Sma6; Actp>y>gal4, GFP/Tb, Sb × ScribbleRNAi crosses of protocol A. and protocol B. A') crosses of #136×RNAict and #136×ScribbleRNAi dissected after 72hr in 25°C. Scale bar: 100µm (magnification x10). B) crosses of #136×RNAict and #136×ScribbleRNAi,gal80^{ts} dissected after 48hr in 29°C.Scale bar: 50µm (magnification x20).

4.3 Testing clone malignancy

To further investigate larger sized clones' effect on tissue protocol C was tested. As shown in Figure 13, protocol C *scrib* mutant clones are more compact and have slightly increased area in comparison to protocol B's negative and positive controls. Positive control C has the similar branching as in protocol B. This can be alluded to clone overproliferation, and mutant cells being extruded by WT cells in process of cell competition. The same wing disc pouch area disfigurement can be seen for both positive B and positive C2 controls, although some positive control wing disc retained normal structure phenotype.

A unique occurrence relates to protocol C conditions, where negative control larvae move on with metamorphosis. After 72 hours in 29℃, negative control larvae have already entered first stages of metamorphosis whilst positive control larvae are still at the 3rd instra stage or about to start pupation. The mutant clones affect not only cellular communication, but wing disc structure and developmental process.

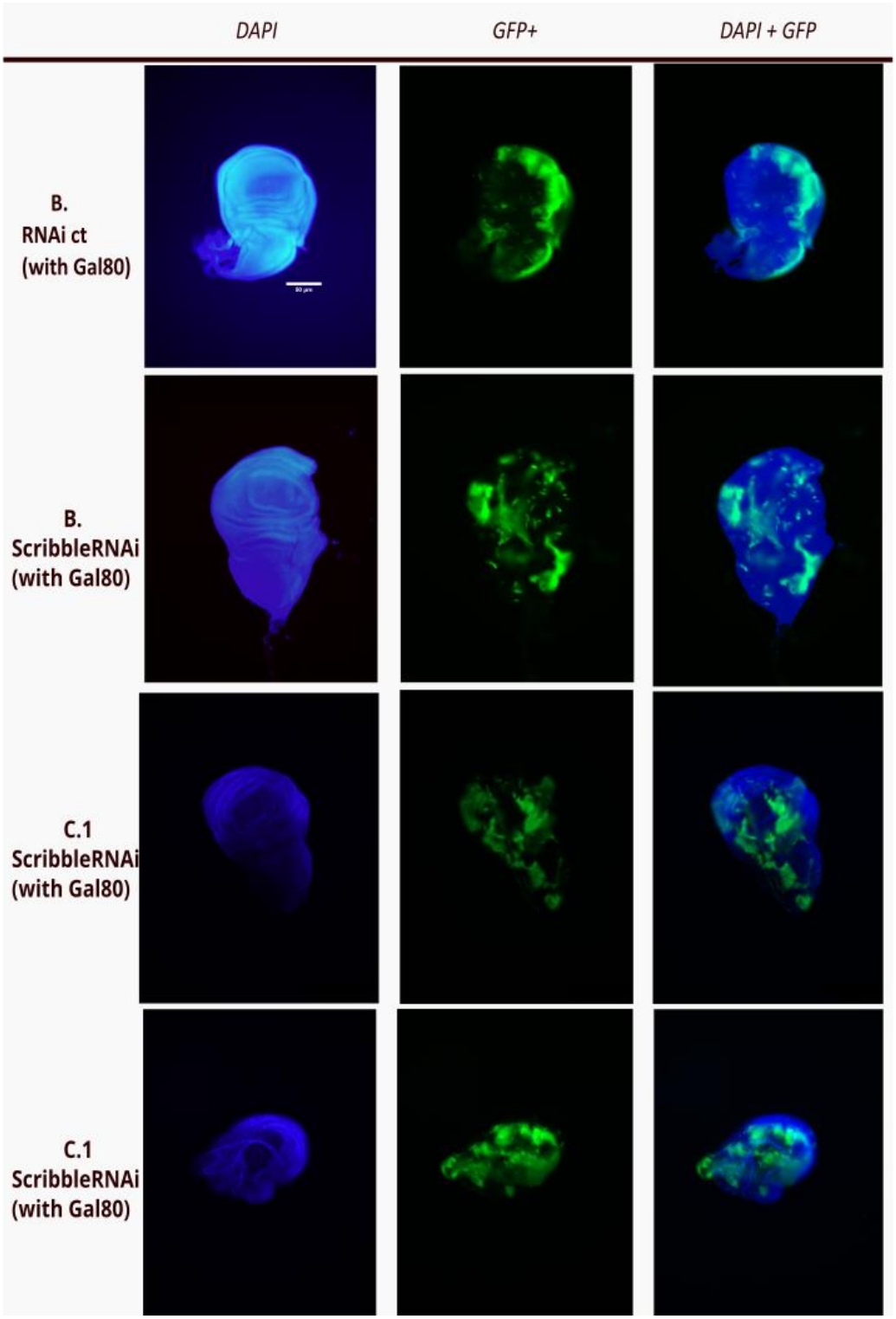

Figure 13. Results for clone malignancy testing. RNAi ct as #136: hs-FLP/Sma6; Actp>y>gal4, GFP/Tb, Sb × RNAi control and ScribbleRNAI as #136: hs-FLP/Sma6; Actp>y>gal4, GFP/Tb, Sb × ScribbleRNAi crosses of protocol B. and protocol C. B) crosses of #136×RNAict and

#136×ScribbleRNAi,gal80^{ts} dissected after 48hr in 29℃. C) #136×ScribbleRNAi,gal80^{ts} cross dissected after 72hr in 29℃. Protocol C RNAict is absent. Scale bar: 50µm (magnification x20).

4.4 Quantification

Quantification results show a deviation of size between protocol A, B and C results. Protocol A Control total GFP positive tissue size averages between 25.99% and 12.07% out of the whole tissue. Protocol A mutant GFP positive tissue size averages between 9.97% and 1.37% out of the whole tissue. For protocol B, Control B average mutant GFP positive tissue size lies between 35.32% and 16.88% out of the whole tissue; Mutant B's mutant GFP positive tissue size lies in interval of 54.72% and 16.59% out of the whole tissue. Mutant C mutant GFP positive tissue size lies in interval from 68.42% to 24.84% out of the whole tissue.

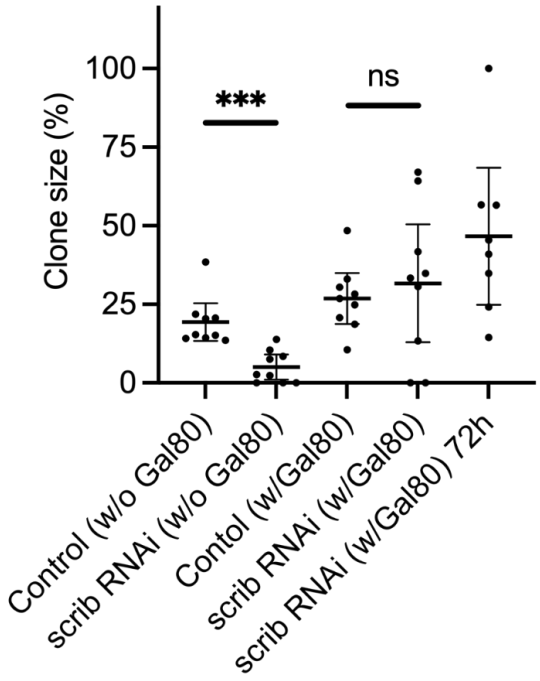

Figure 14. Quantification of the percentage of the clone size. Overall sample size is 8. Control and scrib mutant clones are marked on X axis. Clone size in percentages is marked on Y axis. Data are means G95% confidence intervals (CIs). Statistical significance was calculated by the unpaired t-test. *** $P \leq 0.05$. ns (not significant) $P > 0.05$.

Unpaired t-test showed that the difference of clone size between control and scrib mutant samples without gal80^{ts} is statistically significant ($P = 0.0003$). The difference of clone size between control and scrib mutant samples with gal80^{ts} is statistically not significant. ($P = 0.5949$)

5 DISCUSSION

RNAi clonal analysis is an efficient way for researching genetic interactions between mutant and wild type cells as well as for monitoring the processes of cellular communication and competition. Considering previous studies on fly deficiency lines (Fischbach, 2022) and cell communication between optimal and sub-optimal cells (Y. Huang et al., 2023), this method offers greater insight by creating clones of variant sizes.

5.1 Establishing RNAi clonal analysis protocol

Results between A' and A'' versions in Figure 11, show significant difference between tested conditions. Since A'' versions Positive and Negative controls' clone abundance and obscures the variation between two of them. The sheer number of them doesn't allow to distinguish an increase or decrease in number of clones. The version A' is considered the better choice. Thus, for protocol A following conditions for the 1st and 2nd incubation are chosen: 1st incubation for 48 hours in RT, 2nd incubation for 72 hours in 25°C.

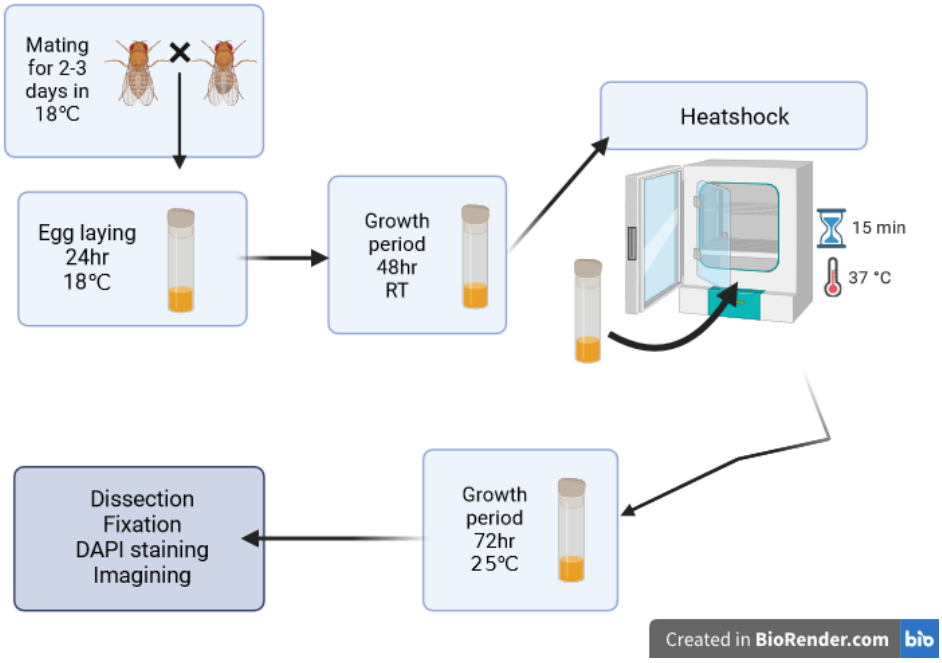

Figure 15. Detailed diagram for protocol A. 1st and 2nd incubation conditions implemented in protocol A to test RNAi clonal analysis method.

5.2 Smaller clones are eliminated in cell competition

Figure 12 proved the hypothesis on clone size influence on the further cellular process. Since protocol A *scrib* mutant clones were targeted by WT cells right after heatshock for next 72 hours (Figure 10), only small amount was able to resist being eliminated in cell competition and grow. This provides clear explanation for the outcomes regarding smaller clones that can be eliminated earlier in the developmental cycle by different loser cell elimination methods. For example, cell-autonomous activation of Eiger that leads to JNK-mediated engulfment signalling in normal imaginal cells (Ohsawa et al., 2011) or mechanically induced death (Merino et al., 2016).

It can be hypothesised that have the period after heatshock been longer than 72 hours the surrounding WT cells would have fully eliminated suboptimal cell colonies.

5.3 Mutant cell size can influence cell dynamics

Protocol A approved conditions for generating smaller clones, protocol B was needed to be established. Protocol B utilised temporal RNAi in vivo control by Gal4/UAS/Gal80^{ts} approach, *scrib* mutant clones had altered developmental environment. Since after heatshock clones were not targeted by WT cells, mutant cells were able to proliferate to larger sizes.

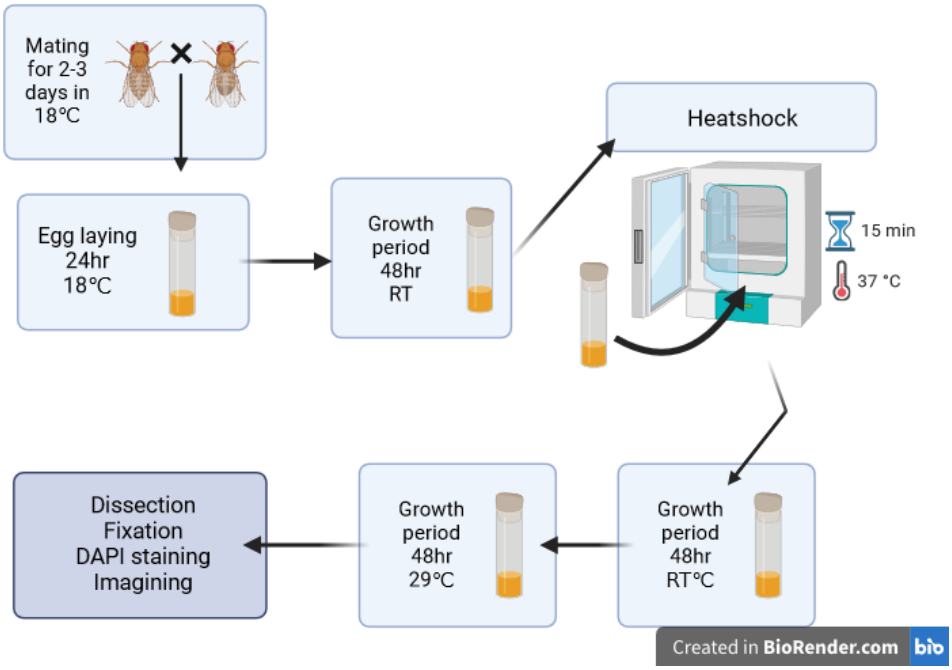

Figure 16. Detailed diagram for protocol B. Altered conditions of protocol A to establish protocol B: two-step 2nd incubation where vials stayed for 48 hours in RT and then for 48 hours in 29℃.

Both protocol B and C images (Figure 13) and quantification results (Figure 14) show this. This further proves that if mutant clones can achieve critical mass, they become more competent and can resist being eliminated. In the end, this can lead to neoplasia formation. This occurrence can be connected to competition for space. Faster-proliferation cells in the middle cause the stretching of more-lateral WT cells, making the elimination process more complex (Merino et al., 2016).

As shown by Y. Huang et al. (2023), the loss or gain of polarity can occur non-autonomously in context dependent manner. This can be applied to current findings. The critical mass of clones can subjugate disturbance of ABP of surrounding WT cells, while they in turn try to minimise the damaged area, succeeding in eliminating smaller clones or even aiding them in restoring ABP, if they have minimal Scribble protein elements.

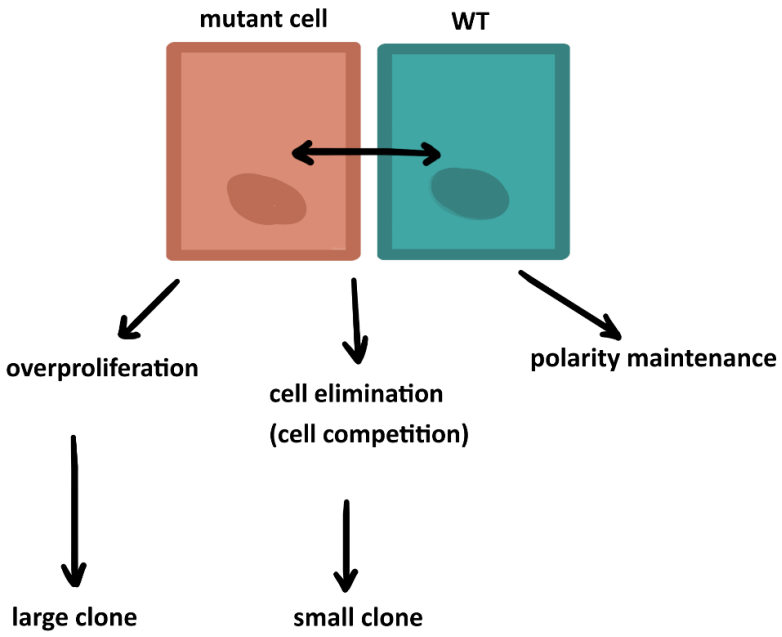

Figure 17. Cell communication and clone size. Three context-dependent manners of cell communication between sub-optimal and optimal cells can be connected to clone's size. Smaller clones just like *scrib null* cells are eliminated by act of cell competition. Large clones pose a more serios competition to WT cells through overproliferation, leading to neoplasia formation. Figure created with https://krita.org/en/

5.4 Mutant cell size can influence larvae development

During experimentations with protocol C, it was impossible to receive samples with #136xRNAict cross. By the end of 72hr in 29C, larvae have already transformed into pupas. This reveals another point of influence of large mutant clones. When subjugated to temperature shift from RT to 29℃ RNAict larvae's tissue homeostasis wasn't disturbed, meaning they moved on with fast, but otherwise normal, developmental cycle in heightened conditions. It can be said that *scrib* mutant clones with critical mass, like shown in Figure 13, disturbs not only wing disc structure, but also overall larvae development, slowing it down.

5.5 Future prospect

To finalise the findings of this study further experimentation should be considered. It is apparent that smaller sizes of mutant cell colonies are more influenced by neighbouring WT cells. However, we cannot conclude outright the outcome for larger clones. Experimentation with Scribble antibody staining should reveal if the hypothesis of the *scrib* mutant clones

affecting surrounding optimal cells in nonautonomous way is correct. Imagining using confocal microscope ought to show the behaviour of *scrib* mutant clones and if they migrate to lower layer hinting at overproliferation and malignancy.

SUMMARY

Research on cellular communication and homeostasis in suboptimal epithelial cells was performed using *Drosophila melanogaster* as a model organism. Two goals of this study were to establish a RNAi clonal analysis protocol, and then to investigate whether the clone size of the *scrib* mutant cells can affect tissue malignancy and competence in cellular maintenance processes.

The first part of experimentation was successfully achieved with the establishment of a protocol for using RNAi clonal analysis method. By using the first protocol as a reference point, two additional protocols were made by utilising the Gal4/UAS/Gal80^{ts} system and RNAi in vivo approaches. The tests showed a variety of data that shed light on how clone size *scribble* mutant cells affect tissue dynamics.

Data revealed that smaller *scrib* mutant clones were not able to resist elimination by surrounding wild-type cells. The mutant clones that achieved a substantial critical mass are mostly undisturbed by cell competition. Possessing a larger area across the whole wing disc tissue clearly shows that large sized cells overproliferate, become malignant and potentially affect the developmental timing.

By using Scribble antibody staining more in-depth data can be earned, which can prove whether the hypothesis that larger mutant cell colonies harm surrounding wild-type cells in non-autonomous way.

Extensions of RNAi clonal analysis approach have a great potential to unveil more about cell-cell communication between optimal and sub-optimal cells.

RESÜÜME

Äädikakärbse Drosophila melanogster tiiva imaginaaldiski epiteelrakkude proliferatsiooni kontrolliga seotud rakkudevaheline kommunikatsioon

Käesolevas uurimustöös keskenduti äädikakärbse tiivadiski suboptimaalsete epiteliaalsete rakkude omavahelisele kommunikatsioonile ja homeostaasile. Töös seati kaks eesmärki: 1) välja töötada RNAi klonaalne analüüs kasutades mudelina äädikakärbse tiivadiski 2) välja selgitada kas *scribble* mutantsete rakkude kloonide suurus on määrava tähtsusega tiiva epiteliaalse koe säilimisel ja selle muutumisel neoplastiliseks.

Bakalaurusetöös suudeti edukalt välja töötada protokoll RNAi klonaalse analüüsi jaoks, optimeerides Gal4/UAS/Gal80 süsteemi ja RNAi *in vivo* metoodikat, millega saab täpsemalt hinnata uuritavate rakukloonide suuruse mõju koe epiteliaalsele morfogeneesile. Antud töös leiti, et väiksemad *scribble* mutantsete rakkude kloonid elimineeritakse ümbritsevate metsik-tüüpi rakkude poolt. Kui mutantsete rakkude kloonid saavutavad teatava kriitilise suuruse, siis on nad märkimisväärselt võimelisemad suunama raku konkurentsi nii, et koe homöostaas on häiritud ja hakkab kujunema koe vohamine ning toimuvad muutused epiteliaalses morfogeneesis. See kas suuremad mutantsed kloonid mõjutavad ümbritsevaid rake mitte-autonoomse strateegia järgi on edasiste uuringute käigus vajalik välja selgitada.

Kokkuvõtvalt võib öelda, et RNAi klonaalne analüüs omab suurt potentsiaali uurimaks rakk-rakk kommunikatsiooni optimaalsete ja sub-optimaalsete rakkude vahel, andes olulist infot rakkude vahelise suhtluse ja konkurentsi mehhanismide kohta, mis heidavad valgust ka patoloogiliste protsesside mõistmisel.

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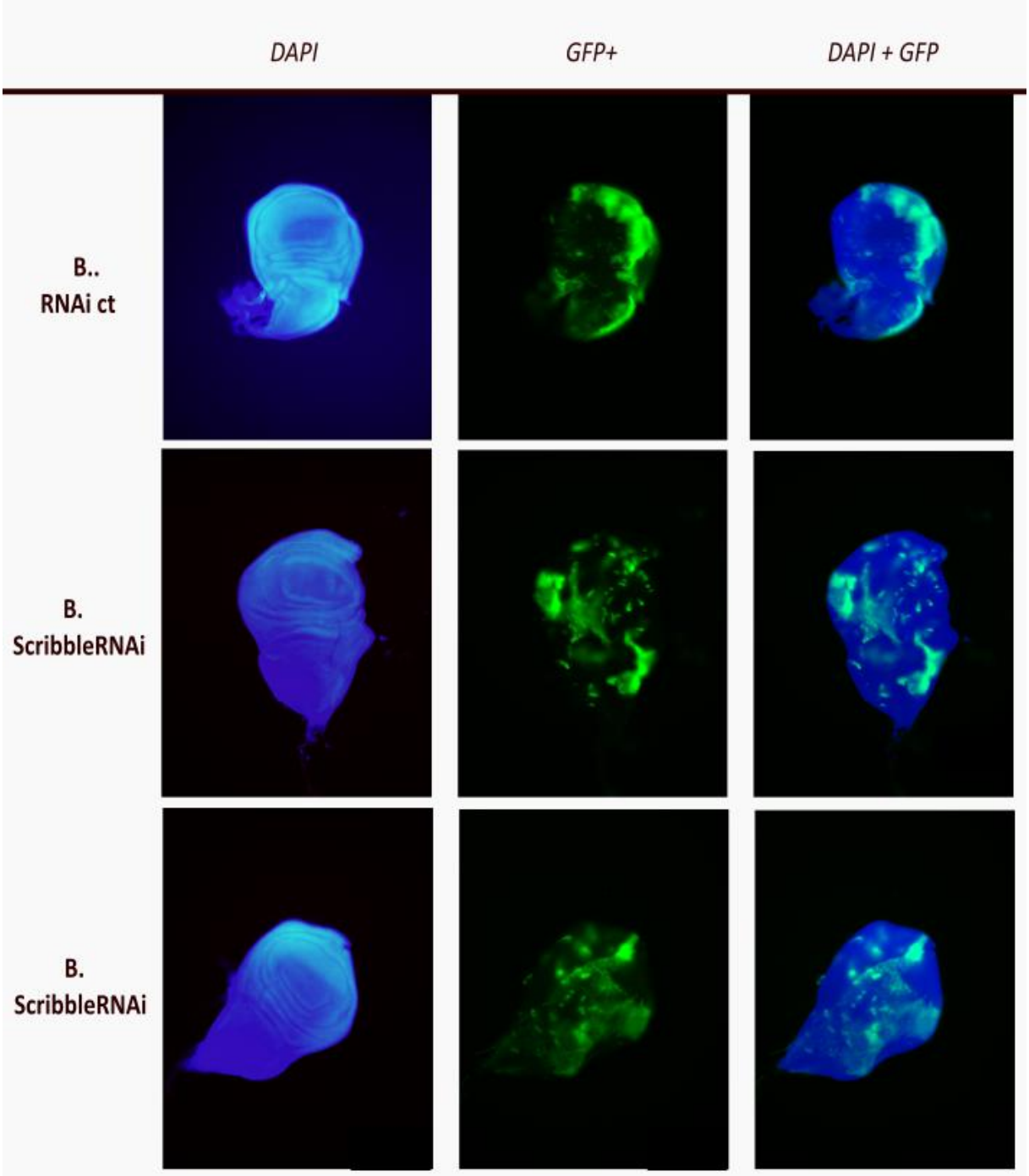
USED WEBSITES

1. GenScript Biology Terms Dictionary: https://www.genscript.com/biology-glossary/8965/cell-autonomous# (visited 28.05.2024)
2. Bloomington *Drosophila* Stock Centre: https://bdsc.indiana.edu/index.html (visited 28.05.2024)

APPENDICES



Appendices 1. Additional results for protocol B.

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