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The effect of Scribble loss in the epithelium of Drosophila wing imaginal discs: developmental markers and intercellular interactions

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Abstract:

The apical-basal polarity is a fundamental property of epithelial tissue. The loss of polarity leads to tissue dysfunction and the loss of homeostasis. Previous findings highlighted that the conditional knockdown of Scribble, the key polarity determinant and the tumour suppressor, results in the progression of polarity loss from the mutants to neighbouring wild-type cells prior to neoplasia formation. However, the mechanisms behind such progression of polarity loss have not been addressed.

This thesis employs *Drosophila melanogaster* wing imaginal disc as a model to illuminate the molecular mechanism behind the progressed polarity loss and neoplasia formation in the case of Scribble knockdown. The results suggested that prolonged Scribble knockdown affects signalling networks and intercellular communications, promoting the primary loss of polarity and later neoplasia formation.

Keywords:
Drosophila melanogaster, wing imaginal disc, epithelial polarity, Scribble, Nubbin, Wingless, apoptosis, engulfment, intercellular bridges, ECM remodelling

CERCS:

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

Scribble funktsiooni kaotuse mõju äädikakärbse imaginaaldiskide epiteelile: arengulised markerid ja rakkudevahelised interaktsioonid

Lühikokkuvõte:

Rakkude apikaalne-basaalne polarisatsioon on iseloomulik epiteliaalsetele kudedele. Häired raku polarisatsioonis viivad kudede väärtalitlusteni ja homöostaasi kaoni. Varasemad uuringud on näidanud, et konditsionaalne Scribble valgu (polarisatsiooni võtmekomponent ja tuumorsupressor) funktsiooni vähendamine rakkudes põhjustab progresseeruvalt polarisatsiooni kadu ümbritsevates normaalsetes naaberrakkudes. Vaatamata sellele on mehhanism, mis sellist rakkude käitumist põhjustab, varasemalt käsitlemata.

Antud bakalaureusetöös kasutatakse äädikakärbse *Drosophila melanogaster* arenevat tiiba mudelina, mille abil püütakse selgitada molekulaarset mehhanismi, mis põhjustab progresseeruvat polarisatsiooni kadu ja neoplaasia teket kui Scribble valk on konditsionaalselt alla surutud. Tulemused näitavad, et pikaajaline Scrib valgu alla surumine mõjutab signaaliradade võrgustikku ja rakkudevahelist kommunikatsiooni, võimendades esmalt polaarsuse kadu ja hiljem neoplaasia formeerumist.

Võtmesõnad:

Drosophila melanogaster, tiiva imaginaaldisk, epiteliaalne polaarsus, Scribble, Nubbin, Wingless, apoptoos, rakkude eemaldamine, intertsellulaarsed sillad, ECM remodelleerimine

CERCS:

B350 Arengubioloogia, loomade kasv, ontogenees, embrüoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

GAL4 – the GAl4/UAS+GAL80ts system component responsible for tissue specificity.

UAS – an upstream activating sequence that starts the expression of the gene of interest in response to GAL4 binding.

GAL80ts – the repressor of GAL4, where binding prevents the activation of the gene of interest; the temperature-sensitive form is used to achieve the temporal control in the system.

RNAi – RNA interference system, where the introduction of a sequence of hairpin dsRNA complementary to the endogenous gene of interest results in its silencing.

ptc – *patched* gene's regulatory sequence controls expression of GAL4, resulting in the expression of proteins of interest within a stripe of cells at the anterior/posterior in wing imaginal discs.

Scrib – the protein that acts as the basolateral determinant and tumour-suppressor in the epithelial cells of *D. melanogaster*.

GFP – green fluorescent protein, the readout of Scrib knockdown cells.

Nubbin – the transcription factor that plays a role in growth control; the repressor of Wingless.

Wingless – a morphogen that affects the rate of proliferation in a concentration-dependent manner.

Myc – *D. melanogaster* Myc protein, where the family of Myc is proto-oncogenes is involved in cell proliferation, apoptosis, differentiation, and neoplasia in vertebrates and invertebrates.

Peanut – the readout of cytokinesis failure.

Draper – the engulfment receptor.

Histone two A gamma – the histone that is phosphorylated in response to DNA damage.

Kelch – the component of cytoplasmic bridges in the wing imaginal discs.

Mmp1 – Matrix metalloproteinase 1, *D. melanogaster'*s representative of MMPs that can cleave almost every protein component of the extracellular matrix; in wing imaginal discs, the readout of tumours' progression.

DAPI – 4′,6-diamidino-2-phenylindole, is a fluorescent stain that binds strongly to adenine–thymine-rich regions in DNA.

INTRODUCTION

The epithelial tissue is characterised by its apical-basal polarity, which determines its tissue architecture. This characteristic of epithelial cells appeared to be evolutionarily conserved (reviewed in Knust & Bossinger, 2002; Nelson, 2003; Tepass et al., 2001). Therefore, the loss of polarity results in tissue dysfunction and no homeostasis in both invertebrates and vertebrates (Bilder, 2004; Klezovitch et al., 2004; Williams et al., 2017). Sub-compartments establish the polarity along the apical-basal axis, where Scribble complex was crucial for restricting apical proteins from the basolateral surface (Bonello & Peifer, 2019). One of the components of this complex is Scribble (Scrib), found to play a role of basolateral determinant and tumour suppressor. In wing imaginal discs, the mutation in *scrib* results in uncontrollable overgrowth, neoplasia formation. Moreover, the clones of *scrib* mutants were found to be eliminated through cell competition. They were eliminated by healthy cells (Brumby & Richardson, 2003; Igaki et al., 2006, 2009; Pagliarini & Xu, 2003). However, the tumour cells were found to affect healthy cells. It was observed that the conditional knockdown of Scrib within the *patched* (*ptc*) region resulted in the depletion of the Scrib in mutant cells and the flanking cells prior to the severe neoplasia formation (Gui et al., 2021). It indicates that active cell communications occur between cells that lost polarity and healthy cells, where the former affects the integrity of the latter. Therefore, there is a gap in how the loss of polarity progress from the mutant cells to the healthy cells and results in the neoplasia formation.

Here we assembled the protocols for two distinct stages of neoplasia that would provide a promising platform of how Scrib loss affects tissue homeostasis. Firstly, we investigate how Scrib loss affects the morphology of wing imaginal discs. Next, we illuminated the possible tools of cell elimination that exist at different stages of neoplasia. Finally, we considered the effect of intercellular communications, like cytoplasmic bridges and extracellular matrix remodelling. Therefore, the received data could provide a promising platform to elucidate the mechanism of cell elimination and intercellular communications in epithelial tissues with the loss of polarity.

1 LITERATURE REVIEW

1.1 *Drosophila melanogaster* as a model organism

The fruit fly *Drosophila melanogaster* has been widely employed as a model for elucidating aspects of human diseases. The short generation time remains one of the main advantages of this model organism (Fig. 1.1). In more detail, *D. melanogaster* is an insect with a larval and a pupal stage before an adult stage. At 25°C, embryos hatch in 22–24 hours after laying. After this, first, second and third instar larvae feed on the substrate to use the received energy to grow and save it for future changes. At this stage, adult organs like wings, legs, eyes, and genital ducts are not present yet. Nevertheless, the precursor structures, imaginal discs, are present. These epithelial tissues undergo rapid growth: ~30 to 35,000 cells in four days for wing imaginal discs (reviewed in Tripathi & Irvine, 2022). However, they do not differentiate during the larvae stage. Critically, the shared similarities of disc cells with the epithelial cells that protect most human organs highlights the relevance of discoveries made with imaginal discs for shedding light on the aspects of human diseases (reviewed in Beira & Paro, 2016; Herranz et al., 2016).

On the other hand, the development of genetic tools allowed scientists to study the consequences of manipulating genes and protein interactions within the different stages of fly development. Specifically, manipulation of imaginal discs could elucidate clock-like precision cell divisions, growth, and territorial specification within the epithelial tissue. One of such experimental systems could be a cell-specific conditional knockdown of the protein of interest that combines GAL4/UAS+GAL80ts and RNA interference (RNAi) (Fig. 1.2). The GAL4/UAS+GAL80ts system provides both temporal control and cell-type specificity, allowing the study of proteins' conditional expression within the epithelial monolayer of imaginal discs. On the other hand, RNAi is used to achieve the conditional silencing of the protein of interest (Fig. 1.3). Therefore, the system of GAl4/UAS+GAL80ts together with RNAi provides an excellent opportunity to study the conditional knockdown of protein of interest at different time points of imaginal discs' development.

Finally, analysis of proteins of interest within the tissues like imaginal discs becomes more accessible not only due to their genetic plasticity, but also with the employment of advancements in imaging tools. A higher resolution microscope provides the platform to analyse cell-to-cell interactions at the tissue level. Discs themselves are transparent with

flat morphology tissues that can be relatively easy dissected and stained, simplifying visualisation of the proteins of interest under the microscope.

To conclude, *D. melanogaster* provides a promising platform to study the aspects of diseases due to its short regeneration time and advancements in genetic and imaging tools. The precursor structures in larval stages, wing imaginal discs, allow studying the epithelial development with the help of the GAL4/UAS+GAL80ts system, their genetic plasticity and relative ease of dissection and staining against the protein of interest.

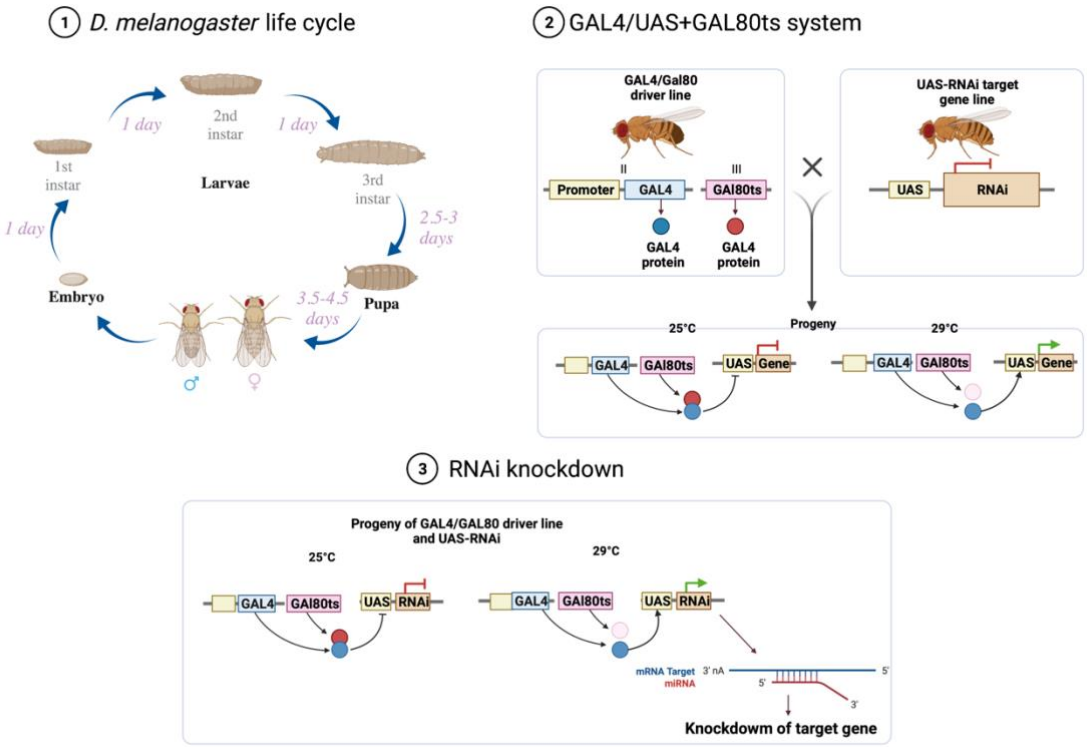


Figure 1: ***D. melanogaster* as a model organism.** 1. The model organism has four stages: embryos, larval, pupal and adult stages. The calculations of days are made based on room temperature of 25°C. 2. It is composed of gene GAL4, encoding a protein that regulates the transcription of genes under the control of Upstream Activating Sequence (UAS). Thus, genes encoded downstream UAS are expressed only when GAL4 is bound, as it recruits transcription machinery to the site to induce gene expression (Duffy, 2002). In fruit fly, to achieve tissue-specificity, the GAL4 gene is placed under a tissue-specific promoter. Moreover, to achieve modularity, GAL80ts gene could be added to the system. A gene encodes for a protein that physically binds to GAL4 and represses its activity. Its expression is temperature-sensitive. At 25°C, GAL80ts acts as a repressor of GAL4, stopping its recruitment of transcriptional machinery. However, at 29°C, it is inactive, degraded. Therefore, the employment of the GAL4/UAS+GAL80ts system results in the modularity of expression based on tissue type and temperature. 3. This mechanism was discovered in *Caenorhabditis elegans*, where the introduction of dsRNA resulted in silenced gene expression (Fire et al., 1998). In *D. melanogaster*, a sequence of hairpin dsRNA complementary to the endogenous gene of interest is introduced into the chromosome under the control of the GAL4/UAS system. It results in tissue-specific

silencing and with the addition of GAl80ts - in temperature sensitivity. The figure was created with BioRender.com.

1.2 Wing imaginal disc

Imaginal discs provide a versatile tool to study the functional contribution of genes to epithelial development. Wing discs are the largest among them (Marren & Mabey, 2010) and overproliferation and uncontrollable growth are one of the problems to tackle. To shed light on this, one should look at how the wing imaginal disc transforms into the adult wing. It starts from a cluster of embryonic epidermis that later, by a series of changing expression domains, divide and create the precursor structures of adult organs in the larvae stage and mature organs during the pupal stage. In general, the disc can be divided into notum, hinge and pouch region, where the pouch's cells divide and give rise to the adult wing (Fig. 2.2). The specification of wings starts from embryonic stage 11 (5 - 5.5 hours) with thoracic imaginal disc primordia (TP) (Fig. 2.1a) (B. Cohen et al., 1993; S. M. Cohen, 1990). This region gives rise to both leg and wing disc cells, where clusters of ~25–30 embryonic cells end up in the wing disc primordia. The early wing disc is a flat sac of cuboidal epithelial cells, with the apical sides toward the lumen. On one side, cells are flattened, forming a thin squamous epithelium or peripodial epithelium (PE). On the other side, they are elongated apical-basally as their density increases, forming a columnar epithelium. Interestingly, most of the growth, and ultimately most of the cells of the wing disc are in the columnar epithelium (reviewed in Tripathi & Irvine, 2022).

By the late third instar, columnar cells become densely packed and increasingly tall, particularly in the central region of the disc (pouch region) that will give rise to the wing. These columnar cells become pseudostratified (the nuclei are at different heights) (Fig. 2.2). It is a physical necessity to maintain an epithelium monolayer, as the widths of the cells become less than the width of the nucleus caused by the planar divisions. Three distinct folds form the hinge–notum, hinge–hinge, and hinge–pouch folds (Fig. 2.2).

Later, the wing undergoes morphogenesis. Firstly, it is converted to a pupal wing by eversion. In more detail, the imaginal discs turn inside out, such that appendages extend out of the body cavity. The apical surfaces are on the surface of the developing animal rather than facing a lumen. After eversion, the wing continues to elongate and expand, creating a mature organ from a 2D to a 3D shape (Gui et al., 2019) (Fig. 2.3).

Therefore, as a single columnar epithelial layer in which cells undergo rapid growth during larvae development, the disc itself can provide a promising model for studying growth defects, like uncontrollable overgrowth, neoplasia formation.

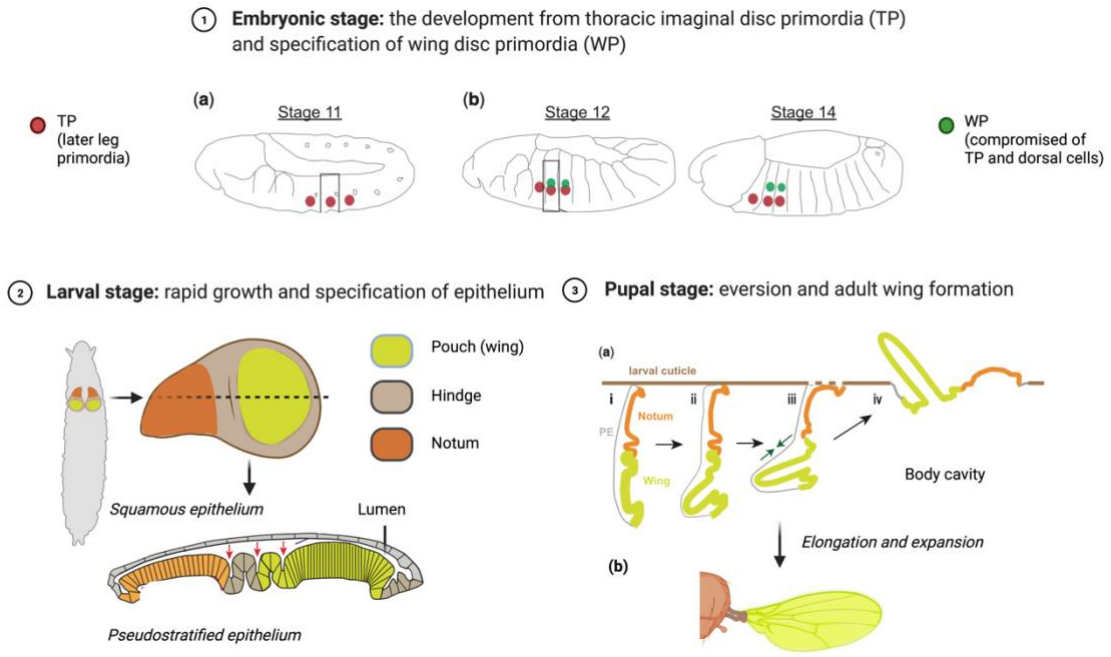


Figure 2: Development of wing imaginal discs (based on Tripathi & Irvine, 2022). 1. During embryonic stages, TP gives rise to leg primordia and wing primordia, where the separation of both is achieved on stage 14 (~10 hours). 2. The imaginal disc experiences rapid growth during larval stage, where initial monolayer of cuboidal cells give rise to the squamous and columnar epithelium. At the late third instar larvae, cells within the pouch region specialise to pseudostratified epithelial sheet. Moreover, the folds are formed. They are labelled by red arrow starting from hinge–notum, hinge–hinge, and hinge–pouch (from left to right) 3. (a) During pupal stage, wing disc undergoes eversion out of body cavity, where initial larval wing disc is attached to the cuticle by squamous epithelium or peripodial epithelium (PE) (i). (ii) Part of the PE attaches to the larval cuticle, and the wing pouch begins to elongate and flatten. (iii) The PE over the notum invades the larval cuticle and ruptures, generating an opening for the disc to evert through. The PE over the wing contracts (green arrows), in part through cells becoming columnar rather than squamous. (iv) The disc has everted through the hole created by invasion and rupture of the PE and larval cuticle. Eversion is driven by contraction of the remaining PE. (b) Finally, the adult structure is achieved by a series of elongation and expansion.

1.3 Apical-basal polarity of epithelial sheets in wing imaginal discs

One critical feature of epithelial cells is apical-basal polarity that serves as a barrier formation and control of tissue architecture. This characteristic of epithelial cells appeared to be conserved, where the underlying molecular mechanisms of such polarisation are surprisingly similar (reviewed in Knust & Bossinger, 2002; Nelson, 2003; Tepass et al., 2001). Therefore, the loss of polarity results in tissue dysfunction and no homeostasis in both invertebrates and vertebrates (Bilder, 2004; Klezovitch et al., 2004; Williams et al.,

2017). The polarity is maintained by establishing distinct sub-compartments along the apical-basal axis vectorising specific cellular functions.

In the wing imaginal disc, epithelial cells are physically connected through cell-cell junctions near their apical side. Adherens junctions, connected to the actin cytoskeleton, provide mechanical coupling between cells and regulate apical cell shape (Farhadifar et al., 2007). A paracellular diffusion barrier between apical and basal surfaces is established by septate junctions, just basal to the adherens junctions (Tepass et al., 2001). A series of conserved complexes were identified along the apical-basal axis with an apical compartment containing Crumbs (Bulgakova & Knust, 2009) and PAR (Nance & Zallen, 2011) and a basolateral - Scribble complex (Fig.3).

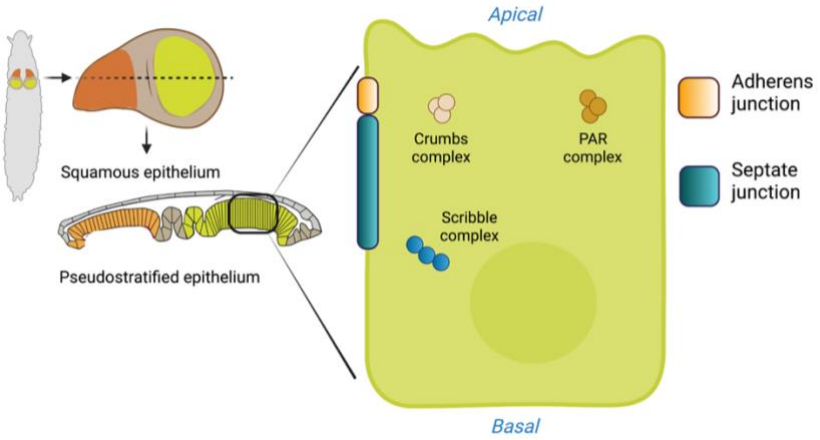


Figure 3: Apicobasal polarity: junctions and their complexes (based on Nakajima, 2021). The wing imaginal disc of late third instar larvae is composed of squamous and columnar epithelium, where the pouch region resembles the pseudostratified sheet. The cells within these epithelial sheets have a distinguished characteristic: apical-basal polarity, which is established by series of complexes. In apical site, Crumbs, and PAR complexes. On the other side, near septate junctions, Scribble complex.

1.4 Scribble as apicobasal polarity and cell proliferation determinant

Scribble protein module comprises Scribble (Scrib), Discs Large (Dlg) and Lethal giant larvae (Lgl) acting as critical regulators of apicobasal polarity by restricting apical proteins from the basolateral surface (Bonello & Peifer, 2019). It was found that Scrib has two domains, leucine-rich repeats (LRR) and PDZ domains (Bilder & Perrimon, 2000). The PDZ domains are named in honour of proteins that share a common structural domain: the postsynaptic density protein (PSD95), Drosophila disc large tumour suppressor (DlgA), and zonula occludens-1 protein (ZO-1). Usually, proteins with such domains are localised to the basolateral surface of epithelial and appear to have a conserved function in polarity regulation. It was found that LRR repeats tether Scrib to the plasma membrane and are necessary and sufficient to organise a polarised epithelial monolayer (Zeitler et al., 2004).

Therefore, the activity of LRR regulates proliferation by cell polarisation. PDZ domain adds up the stated function but is not required.
Epithelia mutant for *scrib* mislocalises apical proteins and adherens junctions to ectopic basolateral sites. Furthermore, the mispolarised imaginal discs of mutant animals are dramatically overgrown and assume a number of other characteristics reminiscent of human malignant tumours, with rapid autonomous growth (Bilder et al., 2000; Brumby & Richardson, 2003; Gateff, 1978). In conclusion, Scrib plays a crucial role in maintaining cell polarity within epithelial sheets and as a tumour suppressor, as the mutation in *scrib* results in rapid growth and tumour formation.

1.5 Cell-cell interactions and their role in neoplasia formation

Tumorigenesis is a complex disease in which many facets of cell function, cell-cell, and tissue interaction play essential roles. In fruit flies' wing imaginal discs, the fate of tumour cells within the epithelium relies on the direct interactions with healthy cells. For example, the introduction of *scrib* clones to imaginal discs revealed that these patches of *scrib* mutant cells do not hyperproliferate but instead are eliminated by surrounding wild-type cells
(Brumby & Richardson, 2003; Igaki et al., 2006, 2009; Pagliarini & Xu, 2003).
Intriguingly, the mutant cells can overcome the elimination fate and start to progress, altering the healthy cells prior to neoplasia formation, uncontrollable overgrowth. It was found that the conditional knockdown of Scrib within the *patched* (*ptc*) region resulted in the depletion of Scrib in mutant cells and flanking cells, while the longer knockdown resulted in the severe neoplasia formation (Gui et al., 2021). It indicates that active progression of loss polarity occurs through tissue before the neoplasia formation. However, the mechanism of the progression is not defined. To illuminate it, cell-cell interactions, like cell elimination by apoptosis and engulfment, and cell behaviour could be studied. Apoptosis and engulfment were found to be required to produce tumours and drive metastasis. Apoptosis is a programmed cell death that occurs in physiological and pathological conditions, while engulfment is the clearance of these apoptotic cells. In *D. melanogaster*, it is achieved by both immune cells, macrophages, and epithelial cells (Pazdera et al., 1998; Sonnenfeld & Jacobs, 1995; Tepass et al., 1994). Moreover, the cellular behaviour is largely affected by the progression of the tumour, where the control is determined by cytoplasmic bridges and extracellular matrix (ECM) integrity. Therefore, to

study cell-cell interactions and changes in cellular behaviour, the correct candidates should be found.

The developmental determinants, Nubbin and Wingless, could be used to shed light on how Scrib loss affects tissue development. On the other hand, tissue homeostasis and tumour proliferation could be described by the following markers: DNA-packing protein, histone, phosphorylated in response to DNA damage, cytokinesis failure readout, Peanut, and pro-oncogene, Myc. Moreover, the interaction determinant could be Draper, engulfment receptor. Finally, the readout of altered cell behaviour could be Kelch, cytoplasmic bridges' component, and extracellular matrix (ECM) remodelling readout, Mmp1.

1.5.1 Development determinates

Nubbin is a transcription factor that plays a crucial role in the development of the wing. It is required for the normal growth and patterning of the wing. At the same time, in the imaginal disc, it was found to express a member of Pit-Oct-Unc (POU) family transcription factors (Ng et al., 1995). This family of transcription factors is characterised by conserved homeodomains, derived from the names of three mammalian transcription factors, the pituitary-specific Pit-1, the octamer-binding proteins Oct-1 and Oct-2, and the neural Unc-86 from *Caenorhabditis elegans*. Nubbin is found in the primordium of the adult wing, with the focus of activity in the hinge region. Importantly, it was found to play a role in growth control. Mutations in the *nubbin* result in a dramatic reduction of the size (Ng et al., 1995). The concentration of dying cells is higher in the hinge region but not substantial in the wing blade. The localised cell death is correlated with Wingless, a morphogen expressed in the dorsal/ventral (D/V) boundary of the wing disc to induce nested expression of target genes (Swarup & Verheyen, 2012). The phenotypically similar to *nubbin* mutant's hinge deletion can be achieved by the deletion of *wingless* in third instar larvae (Couso et al., 1994). Therefore, there is a requirement for *nubbin* function mediated through localised activation of Wingless expression.

It was found that Nubbin plays a role of Wingless repressor as the overexpression of Nubbin prevents Wingless activation in the D/V boundary (Neumann & Cohen, 1998). Wingless was found to constrain the growth of cells within the pouch, being the most critical during the early, rapid growth, phase of wing imaginal discs' development (second – third instar larvae, excluding late third instar) (Johnston & Sanders, 2003). On the other hand, the bimodal effect on proliferation by Wingless was revealed, where it inhibits

proliferation at the high abundance and stimulates proliferation at the intermediate abundance (Baena-Lopez et al., 2009). Therefore, Wingless affects the proliferation rate in a concentration-dependent manner, where Nubbin restricts Wingless expression to D/V boundary in third instar larvae, resulting in faster growth of neighbouring cells.
In conclusion, Nubbin has a growth control function and the repressor function of Wingless. At the same time, Wingless defines the proliferation rate in a concentration-dependent manner, where high abundance was found to result in lower rates.

1.5.2 Apoptosis – a tool of cell elimination

The role of apoptosis was found to be crucial to eliminate both the mutant cells and healthy cells in tumorous tissue. One of the proposed causes of apoptosis within mutant cells is DNA damage (Dalton & Yang, 2009; Hayashi & Karlseder, 2013). In *D. melanogaster*, one of the canonical eukaryotic histones, histone two A gamma variant located in the nucleosomes near the DNA break, is phosphorylated (Madigan et al., 2002). Therefore, the antibody against the phosphorylated histone could provide a promising assay for DNA damage detection (Lake et al., 2013).
Interestingly, the induction of DNA damage can be during and after mitotic failure, one example of which is cytokinesis failure. Septin family was found to mediate the cytokinesis, the process that physically separates two daughter cells at the end of cell division (Neufeld & Rubin, 1994). In *D. melanogaster*, the depletion of *peanut*, the representative of Septin family, by RNAi in the wing disc leads to cytokinesis failure (Eichenlaub et al., 2016). Genomic instability due to unequal chromosome distribution leads to aneuploidy, oncogenic in animal models (reviewed in Schvartzman et al., 2010). Intriguingly, to progress, mutant cells downregulate Peanut (Eichenlaub et al., 2016). Therefore, the Peanut expression is vital in preventing tumour progression, where the tumorous cells can downregulate its expression to progress. In more detail, the tumour cells with forced cytokinesis failure are initially eliminated by apoptosis. For example, the expression of *peanut RNAi* in the dorsal compartment of the wing disc caused a reduction in tissue size, associated with high levels of apoptosis (Gerlach et al., 2018). Therefore, the lack of Peanut leads to massive apoptosis. However, the tumour cells can overcome apoptosis and progress further (Gerlach et al., 2018). To summarise, the tumour cells initially experience the down-regulation of Peanut to eliminate by apoptosis. However, cells can overcome and progress.

The progression of tumour was found to be caused by *Myc* proto-oncogene family, involved in cell proliferation, apoptosis, differentiation, and neoplasia in vertebrates and invertebrates. Its deregulation is prominent in cancer, making it a critical regulator of growth in flies and mammals (Iritani & Eisenman, 1999; Johnston et al., 1999; Trumpp et al., 2001). *D. melanogaster's* Myc protein (Myc) is encoded by the *dm* gene (*diminutive*, from the mutant phenotype) (Gallant et al., 1996). *myc* mRNA mainly present in the wing pouch, and at variable levels throughout the rest of the disc. However, it is not expressed in cells flanking the D/V boundary of the disc (Johnston et al., 1999). While the product of *myc* serves a plethora of functions in the wing imaginal discs. On the one hand, cellular growth is highly responsive to Myc levels. The mutants grow poorly, where the overexpression of this protein results in rapid cell growth. One of the regulators of Myc was proposed to be Wingless, which promotes Myc, thus, cell growth (Herranz et al., 2008; Johnston et al., 1999).

On the other hand, there are limits to Myc growth-stimulating activity, as one of the conserved functions of this protein family is the ability to trigger apoptosis. For example, signs of cell death are seen upon Myc overexpression in wing imaginal discs (Montero et al., 2008). Interestingly, the *myc* mutant clone introduced to the wing imaginal disc is outcompeted by surrounding non-mutant cells (Johnston et al., 1999). On the contrary, the overexpression of Myc leads to the death of surrounding wild-type cells (de la Cova et al., 2004; Moreno & Basler, 2004). Therefore, there is a clear role of Myc in the progression of tumour, where the mutant cells with overexpressed Myc cause apoptosis of the neighbouring cells.

In conclusion, one can summarise that the mutant cells primarily down-regulate Peanut, that could cause cytokinesis failure, promoting DNA damage and apoptosis. However, tumorous cells can overcome the apoptosis barrier and cause the cell death of neighbouring healthy cells. This could be accompanied by Myc's overexpression, which was found to promote cell death in the flanking cells.

1.5.2 Engulfment – a tool to limit the progression of mutant cells

Not only apoptosis, but also engulfment is required for mutant cells to produce tumours and metastasis, where Draper is a cell-surface receptor to recognise and engulf apoptotic cells. In wing imaginal discs, oncogenic neighbours were eliminated by the engulfment. For example, the *scrib* mutants are eliminated from epithelia when surrounded by wild-type tissue (Ohsawa et al., 2011). The opposite can be observed with the oncogenic cells,

which engulf the healthy cells, where Draper is crucial in such cell-cell interaction (Eichenlaub et al., 2016; Li & Baker, 2007). However, Draper was not affected by *scrib* deficiency in adult wings. Draper was modified by apical determinant, Crumbs, Lgl, but not by Scrib and Dlg (Fullard & Baker, 2015). Therefore, the role of Draper is vital for the engulfment in the wing imaginal disc; however, the interactions of Scrib and Draper is not confirmed in the adult wing, while no data is available for wing imaginal discs. Therefore, the extent to which the expression of Draper is crucial for the cell competition between *scrib* mutant and healthy cells is not covered in the imaginal wind discs.

1.5.3 Cytoplasmic bridges and ECM remodelling

The possible interactions between tumours and healthy cells are not limited to apoptosis and engulfment. There is a possible intercellular communication by cytoplasmic bridges. The incomplete cytokinesis causes them. These cytoplasmic bridges allow organelles and macromolecules to pass. In wing imaginal discs, it was found to form the intercellular communication to regulate cellular behaviour (Haglund et al., 2011). One of the components identified was Kelch. It produces a component of ring canals that regulates the flow of cytoplasm between cells (Xue & Cooley, 1993). Therefore, changes in the ring canals between cells can influence their behaviours.

Moreover, the high proliferation rate of tumour cells causes drastic changes in ECM as well. The readout of ECM remodelling can be a family of matrix metalloproteinases (MMPs) that can cleave almost every protein component of ECM. They are believed to be responsible for most of the matrix alterations required for normal and disease processes. MMP expression is upregulated in most cancers, significantly influencing tumour growth and progression (reviewed in Egeblad & Werb, 2002). In *D. melanogaster*, fly tumours express the secreted Matrix metalloproteinase 1 *(*Mmp1*).* Mmp1 degrades the basement membrane of the imaginal disc, allowing tumour cells to migrate and invade (Beaucher et al., 2007; Uhlirova & Bohmann, 2006). In normal wing discs, Mmp1 is not expressed in the proliferating epithelium and is only detected in the wing disc trachea (Gerlach et al., 2018).

In conclusion, the cellular behaviour is largely affected in the progression of the tumour, while the control is determined by cytoplasmic bridges, the building component of which is Kelch. Finally, the progression of mutant cells is accompanied by active ECM remodelling, characterised by the activity of Mmp1.

1.6 Summary

D. melanogaster provides a promising model to elucidate the mechanism of uncontrollable overgrowth, neoplasia formation, where its adult precursor structures, imaginal discs, are a platform to study these phenomena in epithelial tissue. Precisely, wing imaginal discs, due to their genetic plasticity and ease of dissection and staining against the protein of interest, deliver a relatively simple instrument to shed light on the aspects of incorrect epithelial development. With the help of advancements in genetic tools, one can control the development of wing imaginal discs, where GAL4/UAS+GAL80ts system provides the conditional expression of the protein of interest while RNAi system could be added to silence this protein. Therefore, the system of GAl4/UAS+GAL80ts together with RNAi provides an excellent opportunity to study conditional knockdown.

In this project, the polarity of epithelial tissue is disordered by inferring with Scrib, the apical-basal polarity determinant and tumour-suppressor. As the full loss of Scrib results in severe phenotypes, the conditional knockdown of Scrib by RNAi will be employed used. Both GAL4, Gal80ts and RNAi are placed under the *ptc* regulatory sequence resulting in tissue-specific silencing and temperature sensitivity. Therefore, the system of GAl4/UAS+GAL80ts together with RNAi provides an excellent opportunity to study the conditional knockdown of Scrib as a tumour suppressor gene at different time points of the larvae life cycle.

Based on previous fundings, the Scrib knockdown cells interact with healthy ones, altering their cell capabilities prior to neoplasia formation. To give light on how these tumorous cells interact, the immunohistochemistry analysis of the developmental and interactions markers, like apoptosis, engulfment, cytoplasmic bridges, and extracellular remodelling could be used. The developmental determinants Nubbin and Wingless can be chosen. Nubbin is the growth control and repressor of Wingless. Wingless is a morphogen which specifies the growth in a concentration-dependent manner. To give light on possible tools of cell interactions, cell-cell interactions could be studied. One of them is apoptosis, the induced cell death within mutant cells that can be caused by DNA damage and initially by down-regulation of Peanut. Both are related to cytokinesis failure, which allows cells to gain genetic instability, which is oncogenic in animals. However, mutant cells can overcome the apoptosis barrier. This results in the progression of the tumour, where the healthy cells now undergo apoptosis. The neighbouring cells' alteration was found to be related to Myc overexpression. On the other hand, there is engulfment, where the receptor Draper plays a crucial role. Moreover, the intercellular bridges that control cell behaviour

could affect the communication within tumorous tissue. Finally, the cell surrounding itself can be affected within mutant tissue, where the readout of ECM remodelling in wing imaginal discs is Mmp1.

2 THE AIMS OF THE THESIS

Based on previous studies, the existence of possible cell-cell interactions were highlighted prior to neoplasia formation, where the mutant cells alter the healthy cells. One of such interactions could be proposed to be apoptosis and engulfment, where cytoplasmic bridges and ECM remodelling could adjust the control over the behaviour of cells. Our host lab found that the progression of loss polarity caused by *scrib* RNAi exists, indicating that possible cell-cell interactions are affecting tissue homeostasis and later resulting in the neoplasia formation. Therefore, to illuminate the possible mechanism of such progression of polarity loss and later neoplasia formation, the following research questions could be set:

- **How does Scrib depletion affect the patterning of development determinants?**

To address this question, the antibody-based staining against Nubbin and Wingless will be used.

- **What is the effect of Scrib loss on the cell-cell communication: apoptosis, engulfment?**

To answer this question, we will address the rates of DNA damage, cytokinesis failure, and tumour progression rates. The immunohistochemistry analysis of phosphorylated histone two A gamma, Peanut and Myc, will be used to receive such data.

- **What happens with the intercellular communications: cytoplasmic bridges and ECM remodelling?**

To receive the answer, we will analyse the patterning of Kelch and Mmp1.

Therefore, the received information could help to illuminate the possible interactions between cells with no polarity and healthy cells resulting in the primary polarity loss and later neoplasia formation.

3 EXPERIMENTAL PART

To receive the conditional knockdown, *scrib* RNAi and RNAi control under UAS were used, where the former has a sequence of hairpin dsRNA complementary to *scrib*, but no sequence is included in the control. These lines were crossed with the driver line ptc-GAL4, UAS-GFP/CyO; GAL80ts, resulting in the final cross w; ptcGal4,UAS-GFP,ex-lacZ/CyO; UAS-*scrib* RNAi/Gal80ts or w; ptcGal4,UAS-GFP,ex-lacZ/CyO; UAS-RNAi control/Gal80ts. These systems have the expression of GFP in *ptc* region, but *scrib* RNAi is additionally expressed for the former cross. This conditional expression is achieved by GAL4, which is under the regulatory sequences of *ptc* gene. It allows the expression of GFP and RNAi within a stripe of cells at the anterior/posterior. GAL80ts, the repressor of GAL4 activity, is used to achieve the temporal control. Therefore, the conditional knockdown of Scrib is received in *ptc* region with GFP as a readout of cells with initial depletion of Scrib.

Two protocols for the induction of Scrib loss were created to visualise the progression of polarity loss and neoplasia formation. They are employed to investigate how Scrib loss affects the patterning of chosen candidates (Table 1). The third instar larvae start to wander around ~110h after the eggs laying at 25°C for a wild-type strain (Ashburner et al., 2004). The development of flies is faster at higher temperature (Petavy et al., 2001). To simplify the calculations, linear dependence was assumed, resulting in ~1.2 faster development at 29°C and ~0.76 – at 18°C. Experimentally, it was found that the mild neoplastic condition can be achieved within four days at room temperature and two days at 29°C, resulting in ~150h larvae stage at 25°C. On the other hand, the severe condition can be achieved with the maintenance at 18°C for two days and then for three days at 29°C, resulting ~120h larvae stage at 25°C. The ~30h gap is compensated by variable egg-laying time within the first 24h. Only wandering larvae were collected from both conditions (the distinguished behavioural pattern of third instar larvae (Sokolowski et al., 1984)), reducing the possible larvae's stage difference even more. Therefore, by establishing two distinct protocols of wing imaginal disc collection, two conditions could be compared based on the effect of Scrib loss on the patterning of chosen candidates.

The collected larvae were dissected, fixated, and stained based on discussed later protocol to receive the stained wing imaginal discs, where the red fluorescent dye (excitation 568 nm) is the readout of the candidate, GFP is the readout of Scrib knockdown cells and DAPI is the DNA stain. The images were then received either by fluorescent microscope (at least

five samples) and then modified by Fiji (Schindelin et al., 2012), or by confocal microscope and analysed by Imaris (for Nubbin).

Table 1: Experimental setup.

Phenotype	**Room temperature (18°C)**	**Temperature shift (29°C)**	**Total scheme**
Control	2 days	3 days	
2 days knockdown	4 days	2 days	
3 days knockdown	2 days	3 days	

Two conditions for ptcGal4, UAS-GFP,ex-lacZ/CyO; UAS-*scrib* RNAi/Gal80ts were set, where 2 days knockdown was achieved by the knockdown of Scrib for two days at 29°C, while 3 days knockdown – for three days. The control had an identical scheme to the final tumour condition.

The presenting figures were then created with the help of the Python matplotlib library (Hunter, 2007).

3.1 MATERIALS AND METHODS

3.1.1 Materials

3.1.1.1 Lines

RNAi lines: UAS-*scrib* RNAi on the third chromosome (BL35748) and UAS-RNAi control on the third chromosome (B36303) were received from Bloomington Drosophila Stock Center (BDSC). Fly stocks were raised at 18°C.
Driver line: The laboratory stock was w; ptcGal4,UAS-GFP,ex-lacZ/CyO;Gal80ts. The cross was constantly amplified, and the virgins were collected twice per day.

3.1.1.3 The cross

To receive the wing imaginal disc with the final genotype w; ptcGal4,UAS-GFP,ex-lacZ/CyO; UAS-*scrib* RNAi/Gal80ts for conditional knockdown of Scrib or w; ptcGal4,UAS-GFP,ex-lacZ/CyO; UAS- RNAi control/Gal80ts for control, the crosses were set at room temperature and kept at temperatures based on experimental setup (Table 1). Afterwards, GFP-positive third instar larvae were collected. To check for GFP, stereo microscope was used (*M165 FC - Inspection Stereo Microscope by Leica Microsystems GmbH | DirectIndustry*, n.d.). If the salivary glands were GFP-positive, then the larvae were collected, and wing imaginal discs were dissected.

3.1.2 Immunohistochemistry

The wandering larvae are collected to 1xPhosphate-buffered saline (1xPBS) and immobilized on ice. The larvae are then dissected to expose the wing imaginal discs and left in 1xPBS. The fixation of tissue is achieved with 3.7% formaldehyde solution in 1xPBS and 0.1% Twen20 (1xPBT) for 20 minutes of incubation at room temperature (RT) (Fig. 4.1). The next step is throughout washing with 1xPBT. Next, the samples are blocked in 10% Goat Serum 2 hours at RT. The primary antibody (Table 2) is added to the blocking solution with the concentration 1:50 and left overnight for 4°C. The samples are washed with 1xPBT and secondary antibody Goat Anti-Mouse Immunoglobulin G (IgG), targeting heavy and light chains of the IgG molecule (H&L) (Alexa Fluor® 568) (Abcam, ab175473) in the concentration of 1:500 in 1xPBT was added. The incubation was for 2 hours at RT. To visualise the nucleus, the DNA-specific dye, DAPI, was used (Thermofisher, D1306). The stock solution of 1mg/ml was used in the final concentration

of 1:500 for fluorescent imaging (Fig. 4.2). The samples are then washed with 1xPBT and mounted with 70% glycerol.

Table 2: The primary antibodies of concentrations.

Candidate	Primary antibody	Epitope site	Host species	Link
Nubbin	Nub 2 days4	Homeodomain of protein Pdm1	Mouse	link
Wingless	4D4 (anti-wingless)	C-terminus a.a. 3-468	Mouse	link
Myc	P4C4-B10	Peptide sequence	Mouse	link
Peanut	4C9H4 anti-peanut (B1)	amino-terminal 116 aa	Mouse	link
Histone 2A gamma variant, phosphorylated	UNC93-5.2.1	C-terminus	Mouse	link
Draper	Draper 8A1	NPIVYNESLK	Mouse	link
Kelch	kel 1B-s	N terminus	Mouse	link
Mmp1	3A6B4 (Mmp1 catalytic domain)	Catalytic domain	Mouse	link

3.1.3 Imaging

3.1.3.1 Fluorescence microscopy

To receive images, the fluorescence microscope (*Olympus BX51 Fluorescence Microscope | Olympus LS*, n.d.) (Fig. 4.3). The main principle is to illuminate a specimen with fluorochromes with a specific wavelength to observe the emitted light. The light source is the mercury burner of Olympus U-RFL-T. Moreover, confocal microscope (*FV1000 | Olympus LS*, n.d., p. 100) was used to receive the Nubbin images. The difference from fluorescence microscope lies within the higher resolution of received images that is achieved by spatial filtering techniques eliminating out-of-focus light. This allowed to receive more precise information about Nubbin expressing cells.

3.1.3.1 Analysis

Two channels were merged in Fiji (Fig. 4.3) using the created macro plugin (see Appendix for more information). This program allows the batch merging of two channels and the addition of a scale bar. To receive the final presenting figures the Python code employing matplotlib library was created (see Appendix for more information). In the case of Nubbin images, the creation of composite image and the addition of scale bar was done in Imaris Viewer 9.7.

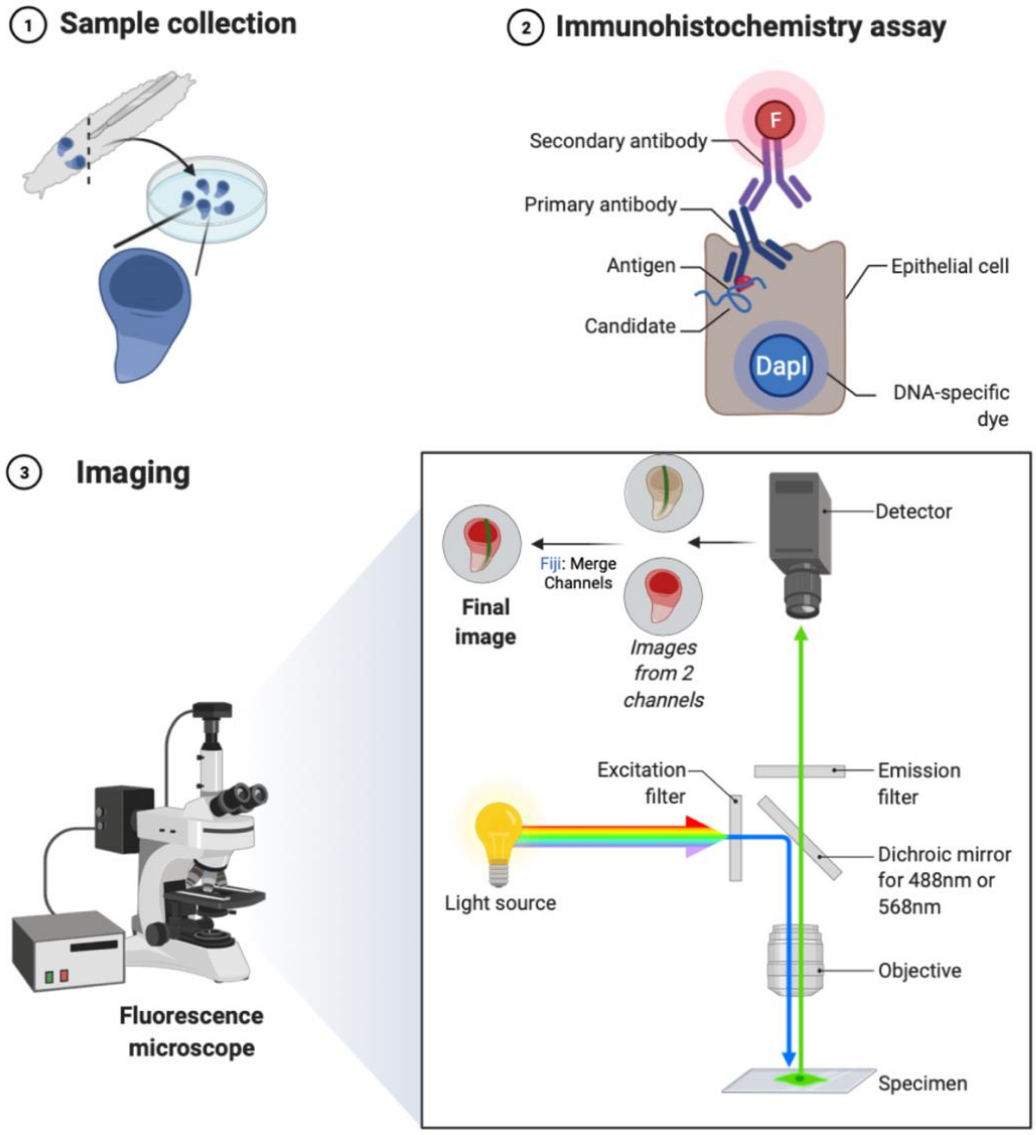


Figure 4: Summary of the procedure. 1. The first step is the collection of wing imaginal discs and their fixation. 2. Next, the immunohistochemistry essay is performed, where the primary antibody against the candidate is used, followed by a secondary antibody with the fluorescent conjugate. 3. Finally, the samples are mounted on the microscopic slide. The images of fluorescents dyes and GFP are received by fluorescent microscope, where the former is the readout of the candidate's patterning, while the latter is Scrib knockdown cells. Finally, the channels are merged, and scale is added with the Fiji macro plugin. The figure is created with BioRender.com.

3.2 RESULTS AND DISCUSSION

The received wing imaginal discs have the expression of GFP and RNAi in the *ptc* region. The three regions are crucial within wing disc: notum, hinge-pouch boundary, and pouch region (Fig. 5.1).

Scrib loss affects the development of wing imaginal discs largely, resulting in neoplasia formation with more prolonged knockdown (Fig. 5.2). The clear progression of polarity loss can be seen already in 2 days knockdown, supported by previous findings (Gui et al., 2021). This is visualised by comparing control and tumour conditions, expressing *scrib* RNAi in the *ptc* region, where the neoplastic conditions are disorganised and with longer knockdown more overgrown, forming neoplasm. The control is the largest, resembling a clear shape. The morphology of 2 days knockdown is variable, with one common phenotype having an outgrowth part, while another has a disorganised structure in the pouch region. On the other hand, 3 days knockdown is consistent: largely overgrown, neoplastic structure, resembling no similarities with control samples.

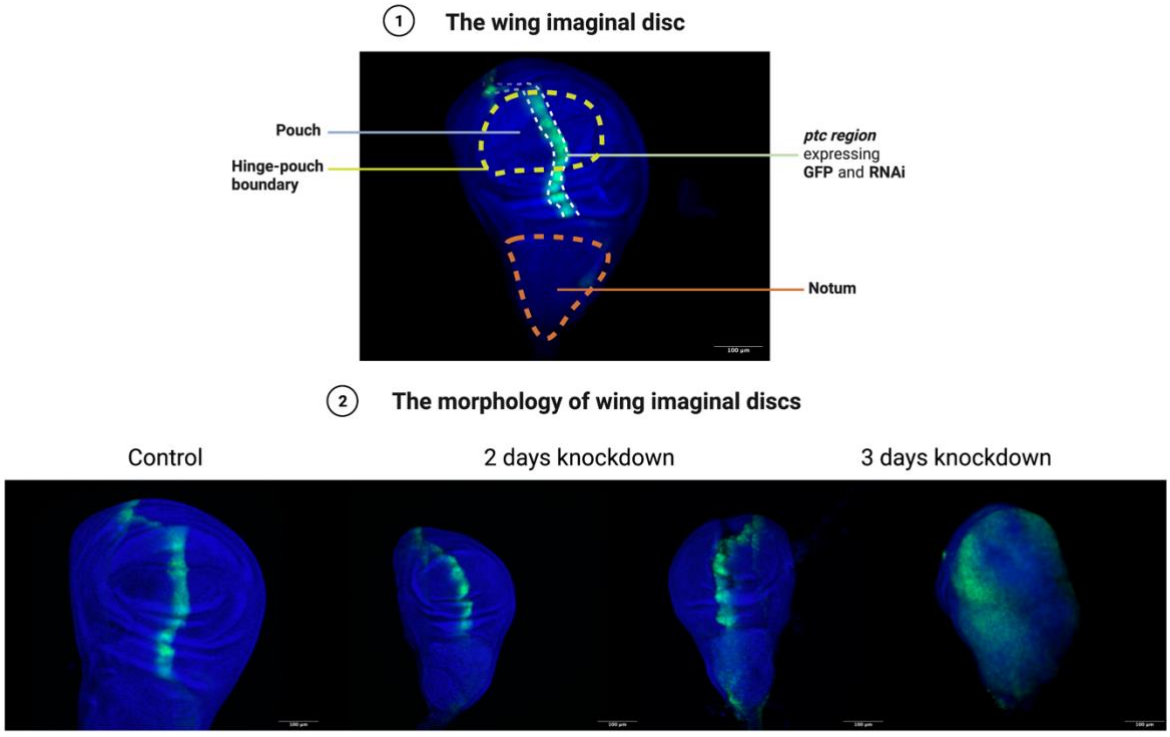


Figure 5. Morphology of samples. 1. The samples have the expression of RNAi in the *ptc* region (white dotted line) and GFP, that is the readout on the final images. Three regions are highlighted: notum (orange dotted line), hinge-pouch boundary (green dotted line) and pouch (blue line). 2. Three types of samples were collected: control, 2 days and 3 days knockdown. The DAPI staining is used to characterise the cell distribution within wing discs. The control expresses GFP control in the *ptc* region (green). The samples resemble a clear structure. On the other hand, neoplastic conditions have the expression of *scrib* RNAi and GFP in the *ptc* region (green). Noticeably, the morphology of 2 days Scrib knockdown is already altered. The

outgrowth part is present, or the pouch is disorganised. Finally, the condition with 3 days Scrib knockdown is overgrown and disorganised. Scale bar is 100 μm.

3.2.1 Developmental markers lose control over growth with the severity of neoplasia

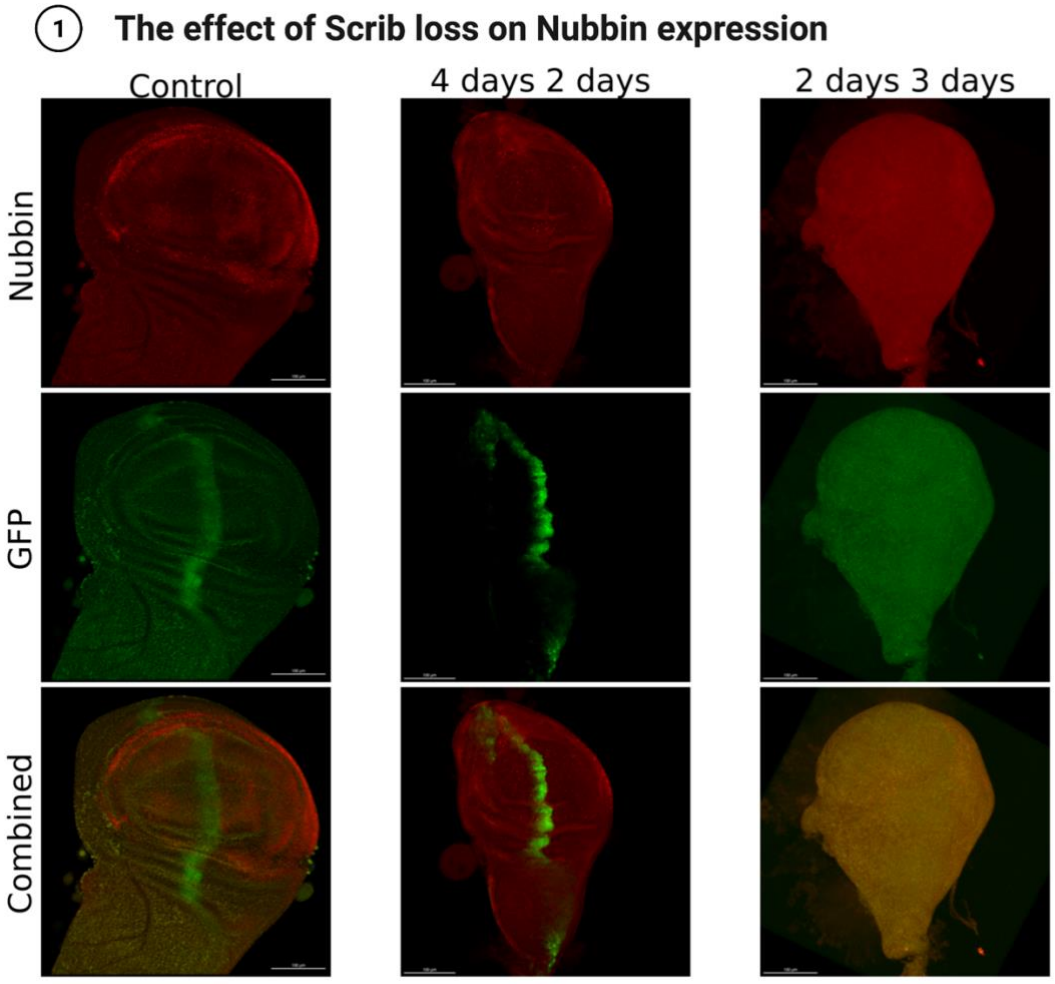
3.2.1.1 Nubbin is up-regulated in the Scrib knockdown and flanking cells of 2 days knockdown

The levels of Nubbin expression experience a reduction with prolonged Scrib depletion, starting from the high levels in the primordium, few cells in the pouch-hinge region and almost none in the neoplasm (Fig. 6.1). The heat map was created to illustrate the drastic expression change between control and 2 days knockdown (Fig. 6.2). In 2 days knockdown, mutant cells and flanking cells experience up-regulation of Nubbin. This indicates that the growth control function of Nubbin is activated in the 2 days knockdown only in mutant and flanking cells in the hinge-pouch boundary. However, Nubbin lost control over the growth in the final tumour stage with 3 days knockdown.

3.2.1.2 Wingless present at the intermediate abundance in the pouch and high abundance in Scrib knockdown cells of 2 days knockdown

A similar trend can be observed with Wingless. The clear D/V expression line turns to expression in the whole pouch region with a higher signal in both D/V and Scrib knockdown cells and later to just a cluster of cells in the final tumour condition (Fig. 7.1). The heat map to visualise the difference between the control and 2 days knockdown condition, where the expression of Wingless is in the whole pouch region, with similar levels in D/V and Scrib knockdown cells can be observed (Fig. 7.2).
To summarise, both developmental markers experience less expression with more prolonged Scrib loss, with only a cluster of cells expressing them in tumour conditions, indicating no control over growth and proliferation with the severity of neoplasm. In the case of Nubbin, as growth control, few cells expressing it can be seen in 2 days knockdown condition, indicating possible up-regulation. While Wingless expression changed from just D/V boundary to the whole pouch, with up-regulation in cells with Scrib loss. This agrees with the mutant for *nubbin*, where Wingless D/V stripe was expanded (Neumann & Cohen, 1998). Moreover, the control of proliferation of Wingless in a concentration-dependent manner agrees with the results, where Wingless's high abundance in Scrib knockdown cells could compensate high proliferation rate of mutant cells (Baena-Lopez et al., 2009). In contrast, low levels in more severe conditions can reflect the faster proliferation. However,

the previous studies can give an inside on the possible dependencies of these markers' patterns, but more research should be done to explain the link between loss of polarity and change in their expression patterns.



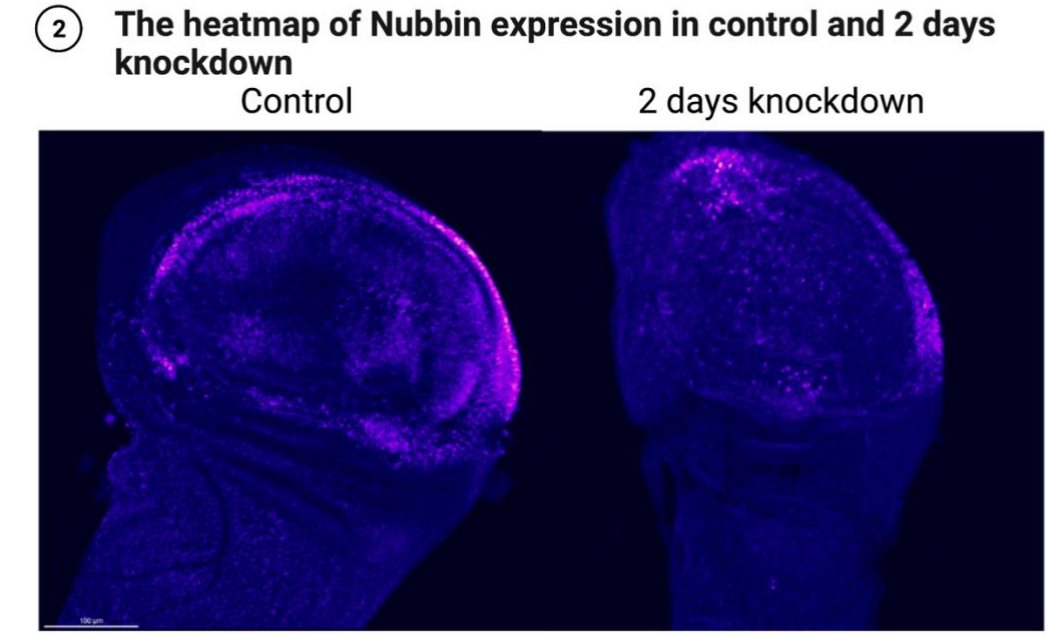


Figure 6: Nubbin patterning. 1. GFP indicates the cells within *ptc* region, where knockdown samples experience Scrib loss (green). The patterning of Nubbin is in red. In control, clear expression in the primordium can be observed, while in 2 days knockdown, expressing *scrib* RNAi, mutant and flanking cells in the hinge-pouch boundary have a higher signal. In contrast, low levels of the signal are in 3 days knockdown. 2. The clear expression pattern is in the primordium of the control condition. However, only mutant and flanking cells have a high signal of Nubbin in 2 days knockdown. The intensity of red fluorescent dye was converted to the fire look-up table, where the lowest intensity corresponds to blue colour, while the highest is white. Scale bar is 100 µm.

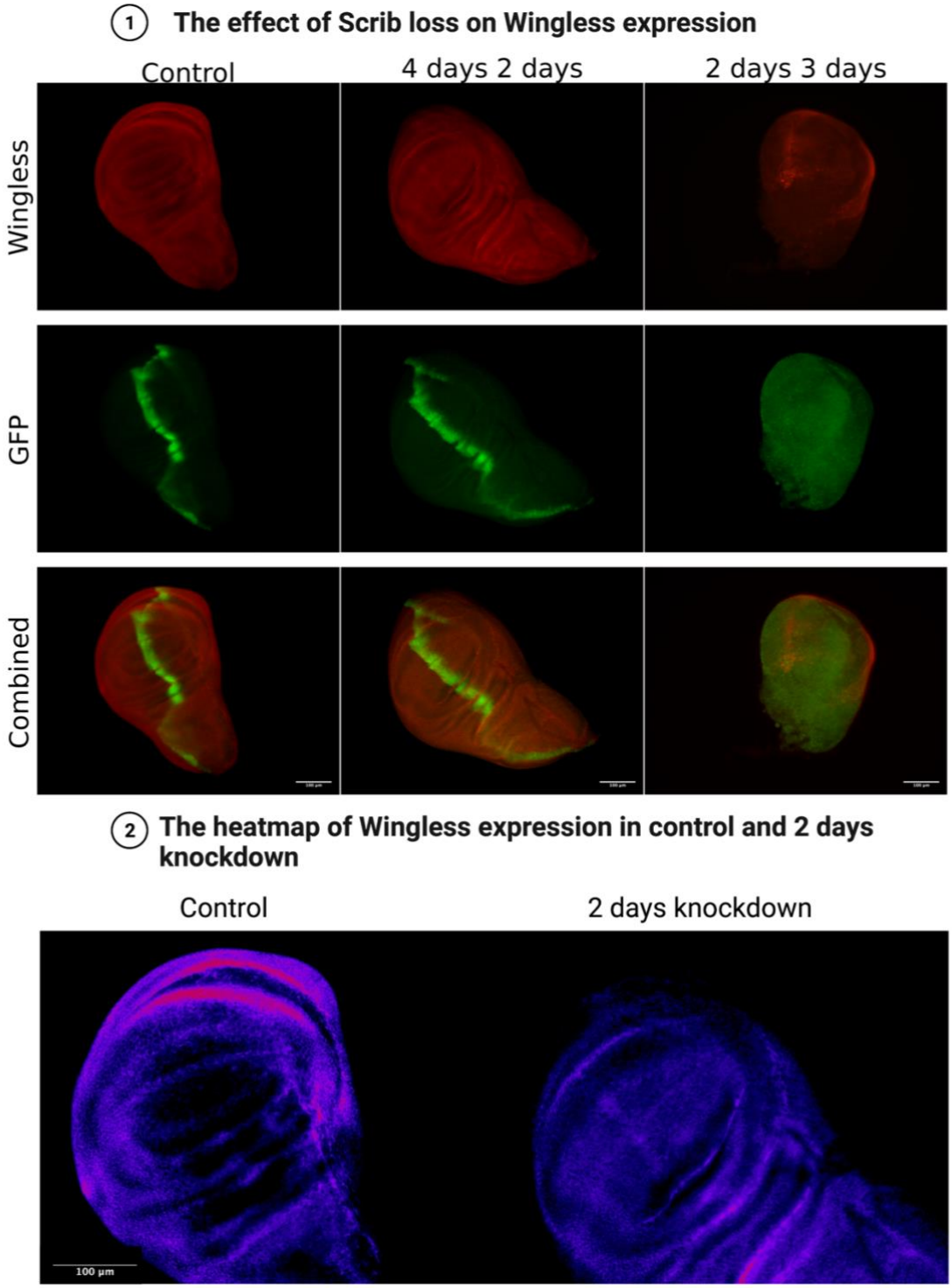


Figure 7. Wingless patterning. 1. GFP indicates the cells within *ptc* region, where neoplastic samples experience Scrib loss (green). The patterning of Wingless is in red. In control, Wingless is expressed in the D/V boundary, while intermediate levels are in the whole pouch region with a similar signal in the D/V border and Scrib knockdown cells. Finally, only a cluster of cells with signal in 3 days knockdown. 2. The control has D/V boundary expression, while Wingless's intermediate expression is within the whole pouch in 2 days knockdown. Moreover, Wingless is present at high abundance not only in the D/V boundary but also in Scrib knockdown cells. The intensity is represented by the fire lookup table, with the lowest intensity being in blue, and the highest - in white. Scale bar is 100 μm.

3.2.2 Dual pattern in the rates of DNA damage, cytokinesis failure and Myc overexpression

3.2.2.1 Initial DNA damage in mutant cells altered to the damage in healthy cells

The phosphorylation of histone two gamma A experiences a contrasting pattern with the longer Scrib knockdown (Fig. 8). A higher signal is within a few cells of notum in control wing imaginal discs. 2 days knockdown is not following a consistent pattern. Out of five collected discs, four had a higher signal in Scrib knockdown cells (See Supplemental Fig. A1). The higher expression pattern could be explained by previous hypothesis, where the induced DNA damage prevents the tumorigenesis (Dalton & Yang, 2009; Hayashi & Karlseder, 2013). On the contrary, 3 days knockdown wing imaginal discs have a higher signal in the flanking non-mutant cells, indicating more frequent DNA damage.

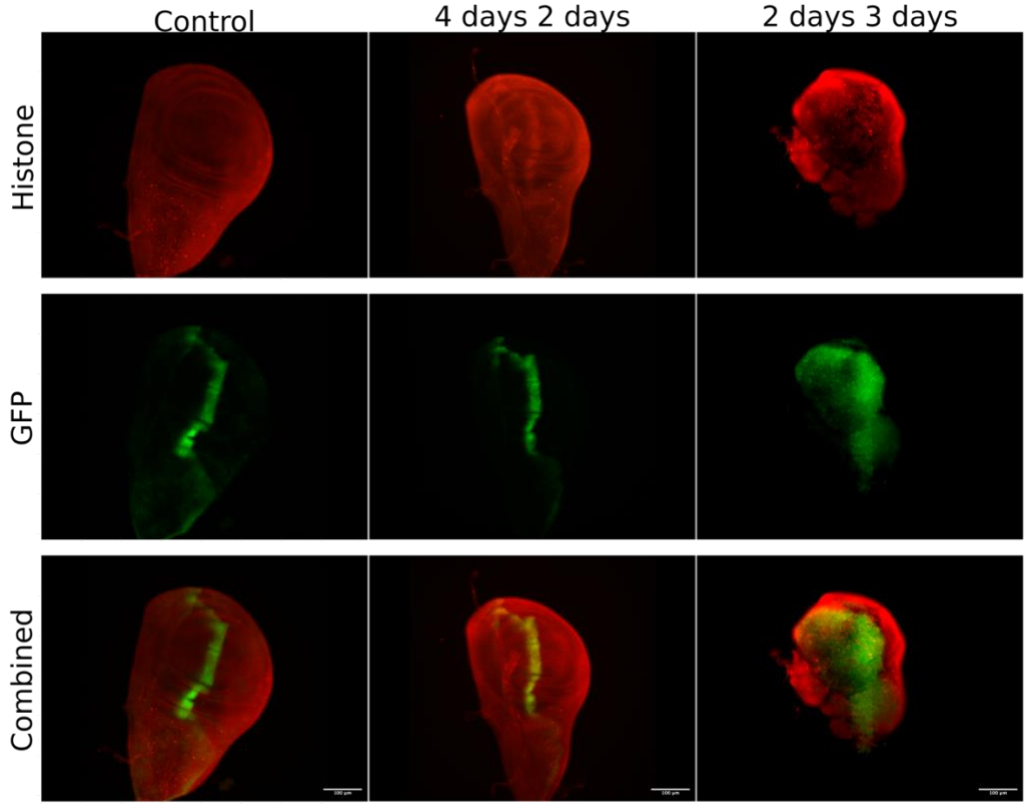


Figure 8. Effect of Scrib loss on the rate of DNA damage: the staining against phosphorylated histone two gamma A. GFP indicates the cells within *ptc* region, where knockdown samples experience Scrib loss (green). The patterning of histone two gamma A is in red. The control samples have a high signal of histone two gamma A phosphorylation in the notum region. However, 2 days knockdown experiences higher phosphorylation rates in Scrib loss cells as well. In contrast, 3 days knockdown condition experiences higher rates in the flanking cells.

3.2.2.2 Down-regulation of Peanut progresses from mutant cells to only healthy cells

The patterning for Peanut gave inconsistent results for control staining (See Supplemental Fig. A2). Similarly, the staining of 2 days knockdown conditions showed two distinct phenotypes, where two out of five samples have expressions similar to the control (See Supplemental Fig. A3). In contrast, three of them had down-regulation of Peanut in mutant cells and flanking cells. This pattern can be consistent with previous fundings where the mutant cells initially downregulate Peanut (Eichenlaub et al., 2016). Interestingly, the mutant cells were already able to alter the healthy cells, as the flanking cells have depleted Peanut as well. It could indicate the primary effect of Scrib loss on Peanut expression. As for 3 days knockdown, the flanking cells experience down-regulation, while the higher levels of Peanut can be observed in the edges of tissue (Fig. 9). The former could be explained by the progress of tumour by the depletion of Peanut in flanking cells. However, the latter can be explained only if more research on peanut overexpression in wing imaginal discs could be conducted.

3.2.2.3 Myc overexpression indicates progression in the 2 days knockdown

Finally, Myc expression pattern is increasing with the severity of neoplastic phenotype (Fig. 10). The control samples have low expression, mainly in the hinge and pouch regions. This is partially supported by *myc* mRNA hybridisation staining, where mRNA was found mostly in wing pouch (Johnston et al., 1999). In 2 days knockdown condition, the upregulation of Myc in Scrib knockdown cells can be observed. This pattern is similar to Wingless up-regulation in mutant cells. Therefore, the data could support previous finding of Wingless promoting role on Myc levels (Herranz et al., 2008). Moreover, the overexpression of Myc in some cells was found to lead to the death of surrounding cells (de la Cova et al., 2004; Moreno & Basler, 2004). This could explain the possible expansion of Scrib depletion in the flanking cells from *ptc* region (Gui et al., 2021), as now

the mutant cells alter the flanking ones. In contrast, in 3 days knockdown condition, the expression of Myc protein in the mutant cells is moderate, while few flanking cells experience higher levels of Myc. It could be a compensating mechanism where the wild-type cells up-regulate the levels of Myc to counteract the mutant cells. However, based on the data, only few cells try to affect mutant ones.

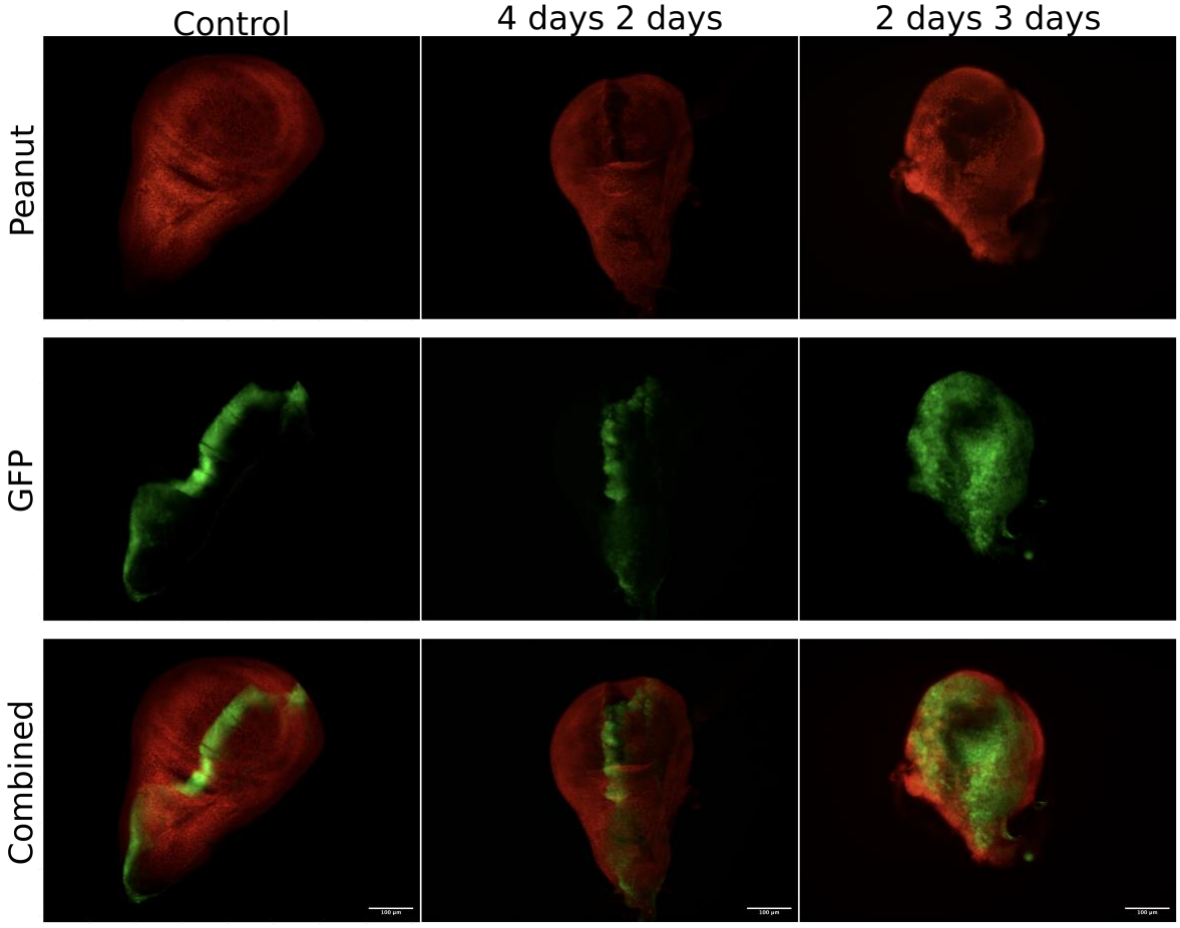


Figure 9: Effect of Scrib loss on Peanut. GFP indicates the cells within *ptc* region, where knockdown samples experience Scrib loss (green). The patterning of Peanut is in red. The control experience intermediate expression, while the sample with 2 days knockdown has down-regulation of Peanut in mutant and flanking cells. On the contrary, 3 days knockdown has down-regulation in only flanking cells, with up-regulation in the edges of tissue. Scale bar is 100 µm.

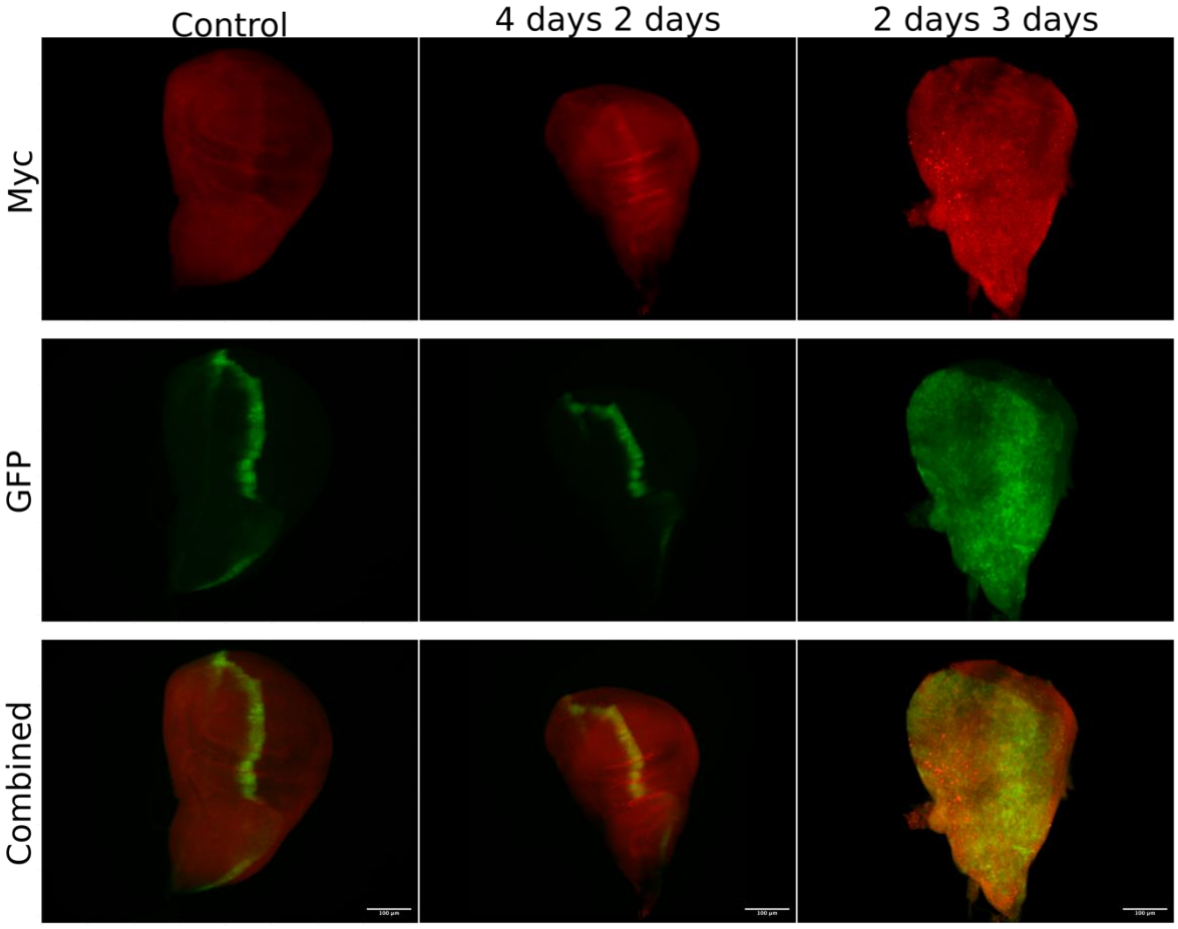


Figure 10: Effect of Scrib loss on Myc levels. GFP indicates the cells within *ptc* region, where knockdown samples experience Scrib loss (green). The patterning of Myc is in red. The control samples have moderate levels within the whole tissue, with a brighter signal in the pouch. However, 2 days knockdown conditions experience up-regulation in mutant cells. Finally, 3 days knockdown discs have only a few flanking healthy cells with up-regulation of Myc. Scale bar is 100 µm.

To conclude, the control samples were consistent for Myc and histone two gamma A, while the samples for Peanut could be recollected to ensure the feasibility of received patterning. In the case of 2 days knockdown conditions, Myc overexpression and down-regulation of Peanut can explain Scrib depletion in the flanking cells (Gui et al., 2021). On the contrary, a high level of DNA damage in *ptc* was proposed to indicate the apoptosis of mutant cells. One can propose that mutant cells have mitotic failures, thus, DNA damage, that could cause the apoptosis of mutant cells. However, the mutant cells were able to overcome the barrier of elimination, as the overexpression of Myc and down-regulation of Peanut in flanking cells indicates the progression of tumour. However, opposite is observed with the final tumour stage, where non-mutant cells are eliminated. Mutant cells do not experience high rate of DNA damage and no down-regulation by Peanut, both

causing apoptosis. In contrast, non-mutant cells are proposed to undergo apoptosis, as the levels of DNA damage high and cytokinesis failure is present. Interestingly, few healthy cells have overexpression of Myc, causing cell death of surrounding cells. However, this is insufficient to stop the progression of mutant cells. To proof the hypothesis, the apoptotic marker could be used. For example, cleaved caspases 3 (Fan & Bergmann, 2010).

3.2.3 Intercellular bridges are increasing in number with longer Scrib loss

The expression of Kelch increases with more severe phenotype (Fig. 11). The control showed a consistent low expression, while 2 days knockdown wing imaginal discs have moderate levels throughout the whole tissue, with a higher signal in hinge and notum. This can indicate higher number of cytoplasm channels. Finally, the high levels of Kelch appear to overlap with Scrib knockdown cells in 3 days knockdown, indicating that mutant cells have high number of cytoplasmic bridges.

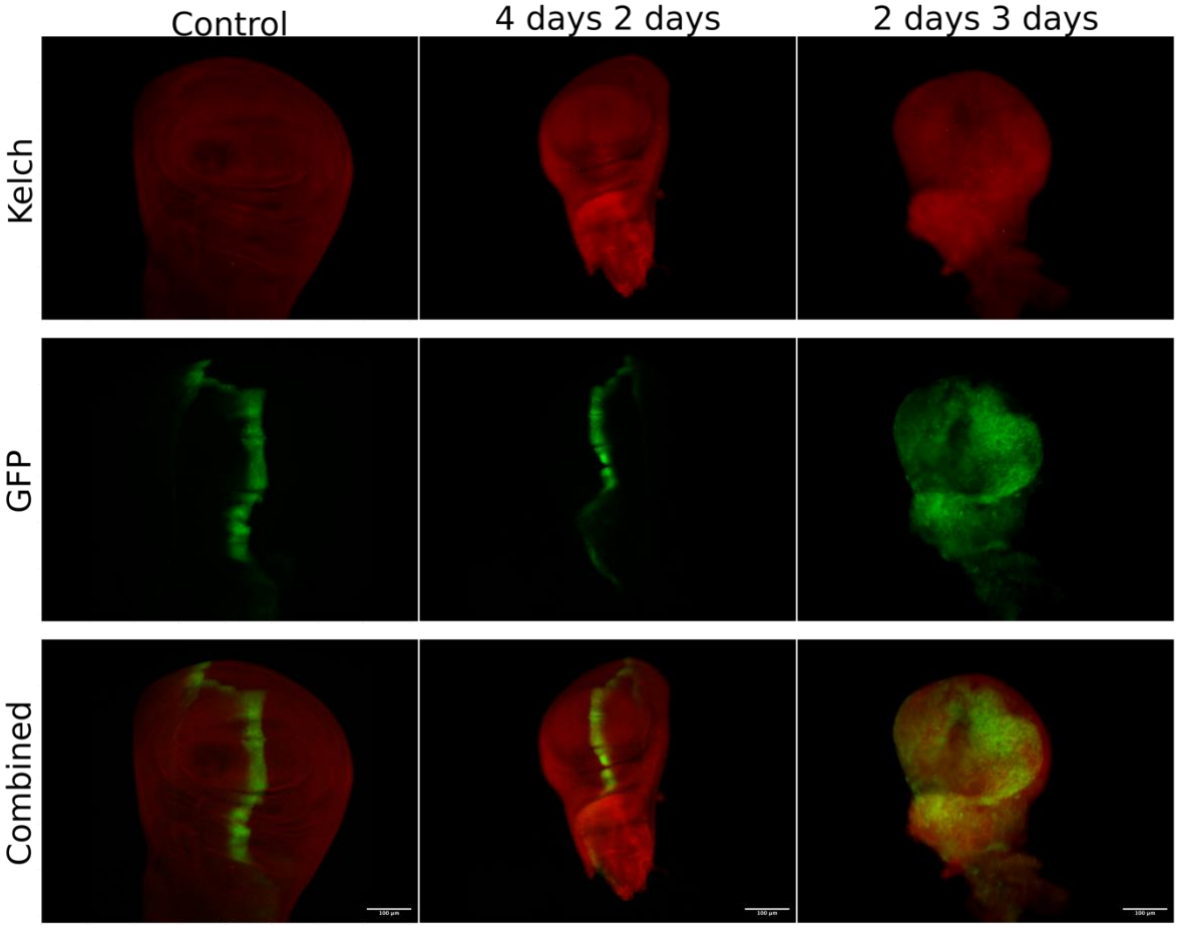


Figure 11: Effect of Scrib loss on Kelch expression. GFP indicates the cells within *ptc* region, where knockdown samples experience Scrib loss (green). The patterning of Kelch is in red. The control samples have low levels within the whole tissue. However, the 2 days knockdown conditions experience higher signal in hinge and notum. Finally, the tumorous discs have higher signal in mutant cells. Scale bar is 100 µm.

3.2.4 The ECM remodelling is at the higher rates with the severity of neoplasia

The expression of Mmp1 in control samples is low, with a high signal in the trachea (Fig. 12, Control, indicated by white arrow). The trachea delivers oxygen to the tissue, where the expression of Mmp1 is limited to it in the wild type tissue (Gerlach et al., 2018). Conversely, 2 days knockdown condition has a high signal in Scrib knockdown and flanking cells of the outgrowth part and hinge (Fig. 12, 4 days 2 days). It could indicate high proliferation rates within these regions. Intriguingly, compared to the down-regulation of Peanut within mutant and flanking cells, one can hypothesise the primary effect of Scrib is on Peanut, as only the outgrowth and hinge experience remodelling yet. Peanut regulation could be followed by high proliferation rates and consequent ECM remodelling. Finally, 3 days knockdown condition has a high signal in mutant cells compared to non-mutant ones (Fig. 12, 2 days 3 days).

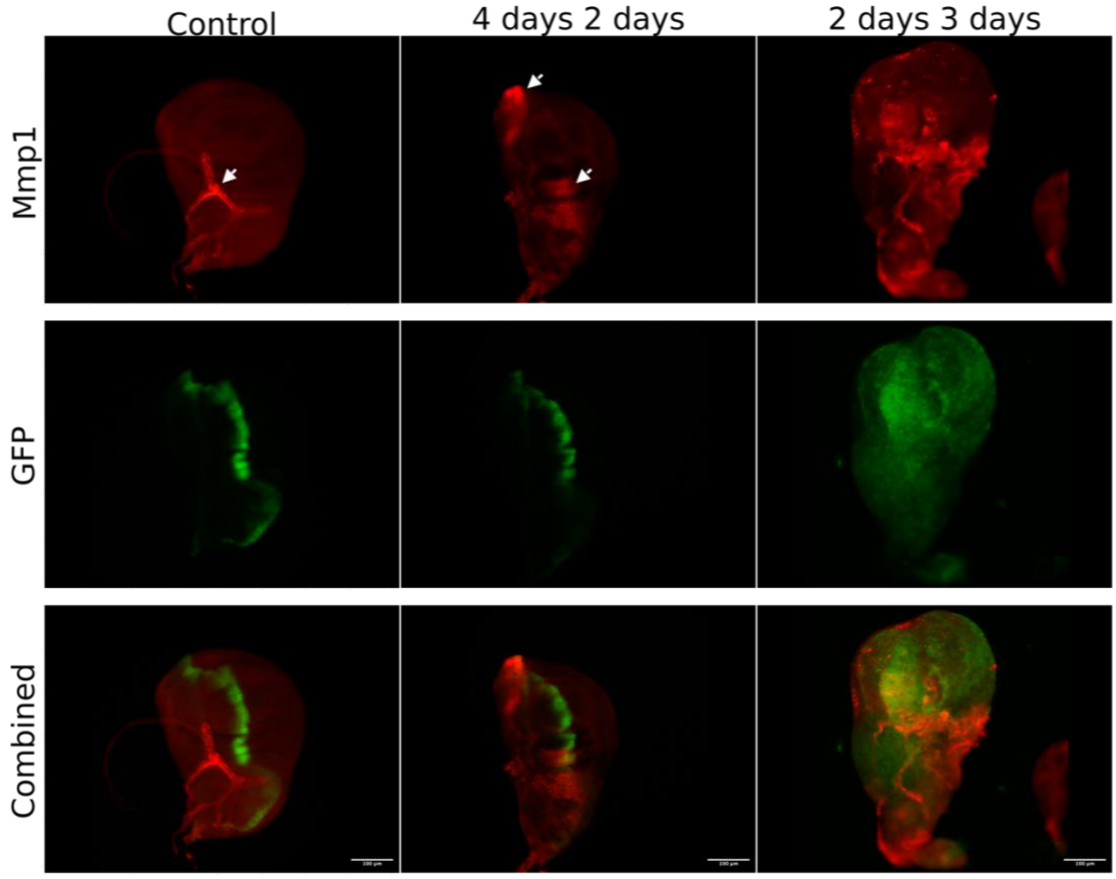


Figure 12: Effect of Scrib loss on ECM remodelling. GFP indicates the cells within *ptc* region, where knockdown samples experience Scrib loss (green). The patterning of Mmp1 is in red. The control samples have high levels of Mmp1 within in the trachea (indicated by white arrow), but low in whole tissue. On the contrary, knockdown conditions experience high remodelling rates in the tissue. 2 days knockdown experience higher signal in outgrowth part and hinge (indicated by white arrows). Finally, 3 days knockdown discs have higher signal in mutant cells.

3.2.5 Engulfment between Scrib loss cells and healthy cells is not coordinated by Draper

Immunohistochemistry against Draper did not give a specific pattern. All three conditions appeared to have similar readout throughout the whole tissue (See Supplemental Fig. A4). Therefore, in the case of cell-cell interactions between Scrib knockdown and healthy cells Draper appeared to play a non-vital role as it was observed with adult wing (Fullard & Baker, 2015).

3.2.6 Conclusions and future work

The morphology of wing imaginal discs is altered with more prolonged Scrib loss, where the initial progression of polarity loss results in neoplasia formation. The control of growth and proliferation by Nubbin and Wingless is lost with longer Scrib knockdown, while the compensating mechanism exists in 2 days knockdown. The apparent dependence of Nubbin and Wingless exists. The up-regulation of Nubbin in mutant and flanking cells of hinge-pouch boundary results in the intermediate abundance of Wingless in the pouch. Such abundance is suggested to induce the proliferation rate (Baena-Lopez et al., 2009). In contrast, the same expression of Wingless in Scrib loss cells as in the D/V boundary can be hypothesised to suppress the proliferation rate of tumorous cells. Therefore, the developmental markers are compensating initially for high proliferating rates of mutant cells, but losing control with longer Scrib knockdown.

In the case of cell interactions, DNA damage was found to proceed at higher rates in the mutant cells of 2 days knockdown conditions. In contrast, the opposite is observed with the neoplasm with 3 days knockdown, where healthy cells experience DNA damage. For Peanut, down-regulation was found to play a dual role, where firstly, mutant cells experience down-regulation, but later it turns to healthy cells. Interestingly, in 2 days knockdown, Peanut was found to be depleted in the flanking cells as well, indicating the possible progression of tumour. Same conclusion is supported by the overexpression of Myc in mutant cells of 2 days knockdown. Intriguingly, the compensating mechanism was found in the stage with 3 days knockdown, where few healthy cells experience high levels of Myc.

Based on the received results of DNA damage, down-regulation of Peanut and overexpression of Myc, the loss of polarity and neoplasia formation could be suggested to be accompanied by apoptosis. It is hypothesized to play a double role.

In 2 days knockdown conditions, mutant cells are suggested to be eliminated by apoptosis (Fig. 13, 2 days knockdown). It is supported by the down-regulation of Peanut, causing cytokinesis failure (Eichenlaub et al., 2016). The cells with Scrib loss are primarily affected by down-regulation of Peanut, as the flanking cells experience down-regulation of Peanut, but no DNA damage yet. Based on previous fundings, cytokinesis failure was found to cause DNA damage, which is observed in mutant cells, and consequently, apoptosis (Dalton & Yang, 2009; Hayashi & Karlseder, 2013). However, the progression exists already in 2 days knockdown, supported by previous fundings (Gui et al., 2021), down-regulation of Peanut in the flanking cells and Myc overexpression in the mutant cells. Overexpression of Myc could be promoted by up-regulation of Wingless in mutant cells, where up-regulation of both proteins is observed in mutant cells. In contrast, healthy cells undergo death in 3 days knockdown caused by DNA damage and cytokinesis failure (Fig. 13, 3 days knockdown).

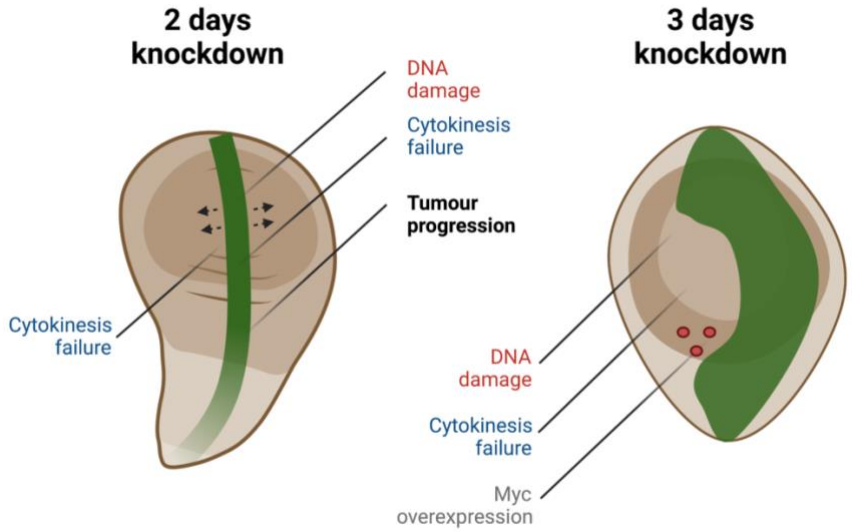


Figure 13. Effect of Scrib loss on DNA damage, cytokinesis failure and tumour progression. The dual pattern was received in 2 days knockdown and 3 days knockdown. In 2 days of knockdown, mutant cells with induced loss of Scrib were suggested to primarily down-regulate Peanut. It causes cytokinesis failure and DNA damage. However, the mutant cells were found to progress, as the flanking cells experienced possible cytokinesis failure. In contrast, healthy cells of 3 days of knockdown experience cytokinesis failure and DNA damage. A few cells were found to share Myc overexpression.

To verify the hypothesis, the following steps could be taken:

- The data supports the primary effect of Scrib on Peanut, where the latter is down-regulated, which could cause cytokinesis failure. To illuminate the interactions between Scrib and Peanut, the conditional knockdown of Scrib and the overexpression of Peanut could be studied.

- The mitotic failure was found to result in DNA damage, causing apoptosis. To receive the information about apoptosis, antibody cleaved caspases 3 could be used (Fan & Bergmann, 2010).
- The mutant cells can overcome the apoptosis barrier by *yorkie* overexpression (Gerlach et al., 2018). The staining for Hippo pathway could be used to test the hypothesis.

As for the engulfment, the chosen candidate, Draper, was found to play a non-vital role. Therefore, the role of engulfment is not clear, providing an open field for research, where one could start with the identification of the candidates that are modified by Scrib, concentrating on the engulfment between epithelial cells.

Finally, Scrib was found to affect the intercellular bridges, where mutant cells tend to increase the number of cytoplasmic bridges with longer Scrib loss. It indicates the possible alteration in the control of cellular behaviour. Moreover, ECM remodelling was found to be increased with more prolonged Scrib knockdown, where the data supports primarily down-regulation of Peanut followed by remodelling. In the 2 days knockdown condition, the flanking cells experienced down-regulation of Peanut, but no ECM remodelling, indicating rapid proliferation was not observed yet.

Therefore, the study provides inside into the effect of Scrib loss on tissue homeostasis based on two developmental markers, Nubbin and Wingless. Moreover, the data on the rates of DNA damage, cytokinesis failure and progression by Myc overexpression were collected. Based on the received data, we proposed the dual role of apoptosis. Moreover, the study highlights intercellular alterations within ECM and cytoplasmic bridges, controlling cellular behaviour. Finally, our results showed that Draper, the receptor for engulfment, was found to play a non-vital role in the progression of Scrib loss within wing imaginal discs.

SUMMARY

The thesis results are summarised in the order of stated research questions in the aims.

- **How does Scrib depletion affect the patterning of development determinants, like Nubbin and Wingless?**

The expression of developmental determinants decreases with the severity of the neoplasia. Nubbin was up-regulated in the mutant and flanking cells in the hinge-pouch boundary, compensating for high proliferation rate. However, the growth control by Nubbin was later reduced to a few cells. As Nubbin – the repressor of Wingless, the absence of Nubbin in the pouch resulted in the intermediate abundance of Wingless throughout the whole region. However, D/V expression level was at the stated boundary and in Scrib loss cells, indicating up-regulation that could compensate for faster growth of mutant cells. As the high level of Wingless was found to reduce the proliferation rate.

- **What is the effect of Scrib loss on the cell-cell communication: apoptosis, engulfment?**

The markers used to elucidate the effect of Scrib depletion on cell-cell communication were DNA damage, illustrated by the phosphorylation of histone two A gamma, cytokinesis failure, caused by down-regulation of Peanut, and progression of tumour, highlighted by Myc overexpression. Both were found to have a complementary expression in 2 days and 3 days knockdown. DNA damage rates were found to be high firstly in mutant cells but with the severity of neoplasia in healthy cells. However, the data supports the primary effect of Scrib loss on Peanut, both in mutant cells and healthy cells, indicating the progression already in 2 days knockdown condition. This conclusion is supported by Myc overexpression in mutant cells as well.

Therefore, apoptosis was hypothesised to play a bimodal role. Initially, it is present in the mutant cells, where the Scrib loss primarily affects Peanut, resulting possibly in cytokinesis failure and DNA damage. However, cells overcome the eliminating barrier and progress by Myc overexpression, resulting in the apoptosis of flanking healthy cells. It could be caused by down-regulation of Peanut and DNA damage.

As for engulfment, it was not found to be mediated by Draper between Scrib loss and healthy cells.

- **What happens with the intercellular communications: cytoplasmic bridges and ECM remodelling?**

The cytoplasmic bridges and ECM remodelling were largely affected by Scrib loss, indicating drastic alterations in cellular behaviour.

APPENDIX

Codes for macro plugin and creating the presenting figures – https://github.com/alexandrasgit/Bachelor_thesis.git .

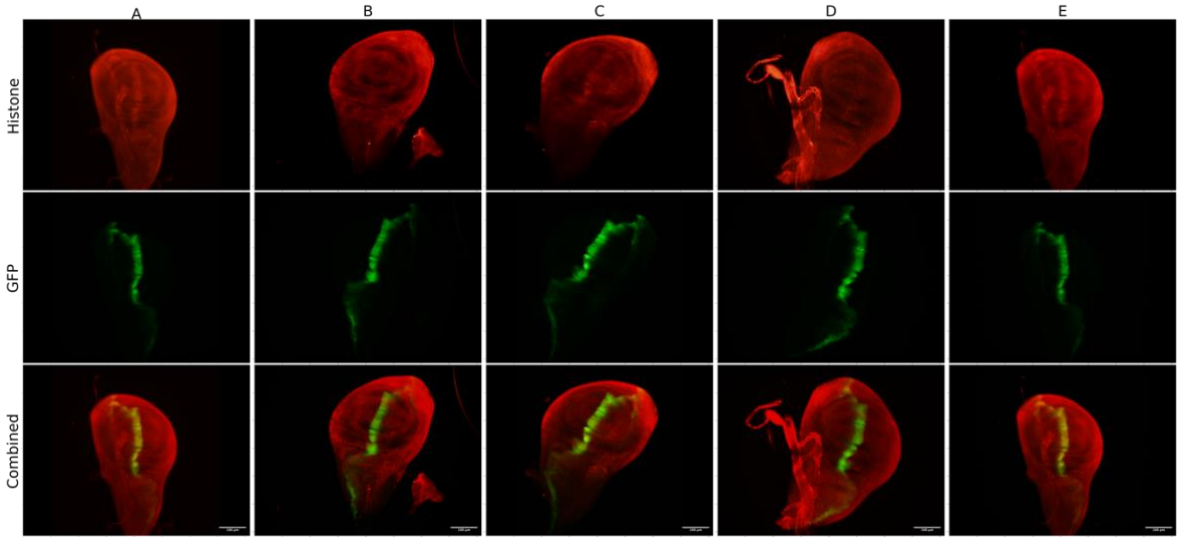


Figure A1: **Effect of Scrib loss on DNA damage rates in 2 days knockdown samples.** The five samples of 2 days knockdown condition (2 days induction of Scrib loss in *ptc* (green)) stained against phosphorylated histone two gamma A (red). It is the readout of DNA damage rates.

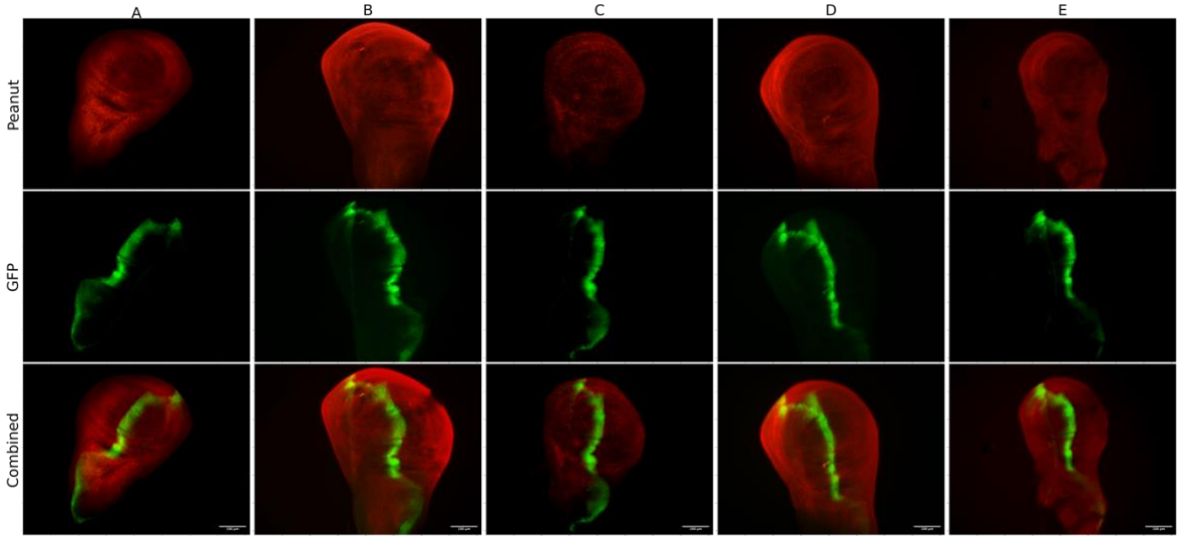


Figure A2: **Effect of Scrib loss on Peanut in control samples.** Control samples express ing GFP (green) in *ptc* region stained against Peanut (red).

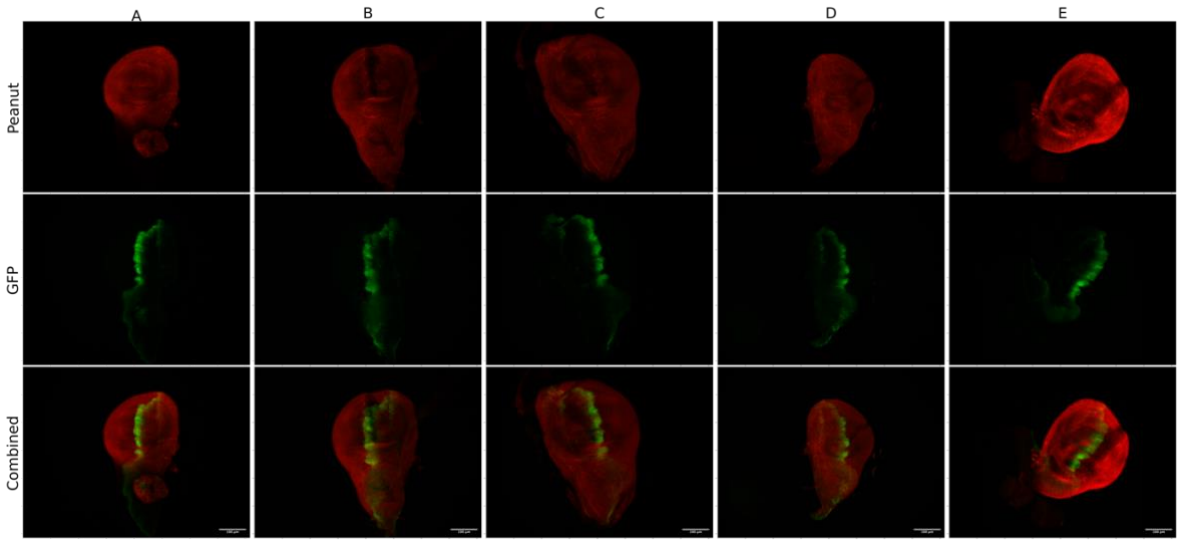


Figure A3: **Effect of Scrib loss on Peanut in 2 days knockdown samples.** 2 days knockdown samples with 2 days induction of Scrib loss in the *ptc* (green) stained against Peanut (red).

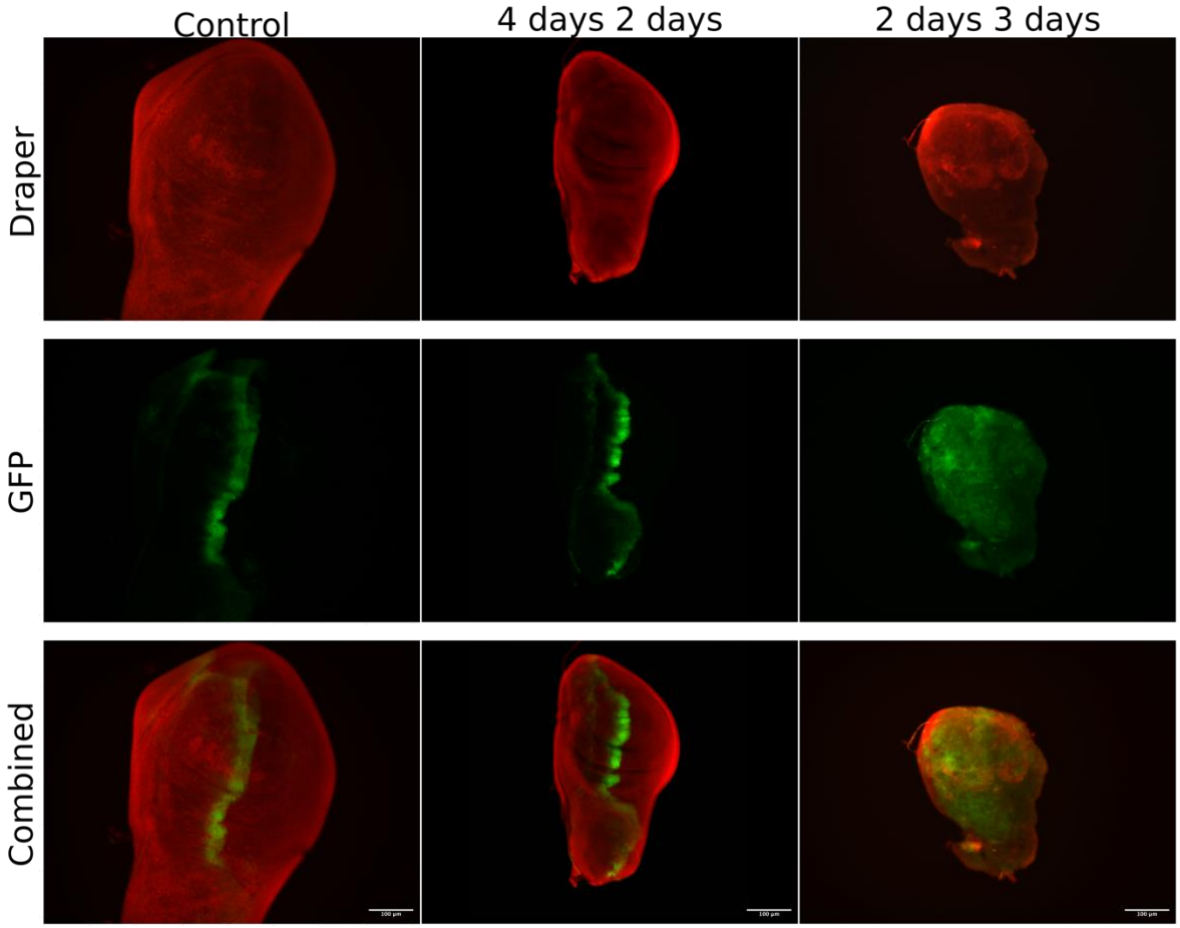


Figure A4: **Effect of Scrib loss on Peanut in 2 days knockdown samples.** The samples stained against Draper, the engulfment receptor (red). The control expresses GFP in *ptc* region, while both 2 days and 3 days knockdown samples express GFP and scrib RNAi in *ptc* (green).

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