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Primary screening of co-factors involving Scribble-mediated growth regulation in the Drosophila wing imaginal disc

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Primary screening of co-factors involving Scribble-mediated growth regulation in the Drosophila wing imaginal disc

Abstract:

The wing imaginal disc of *Drosophila melanogaster* has been used as a model to study tissue growth and morphogenesis. This disc is composed of epithelial cells exhibiting apical-basal polarity (APB), which is essential for tissue development and homeostasis. Disruption of APB can lead to a loss in cell polarity that may result in neoplasia formation. Scribble (Scrib) is one of the key components involved in maintenance of ABP. Recent studies indicate that the copy number of *scrib* in the tissue directly influences formation of neoplasia when *scrib* is conditionally knocked down using an RNAi approach. This leads to the hypothesis that there is a synergy between additional genes and Scrib in the Scrib-derived neoplasia formation. To test this hypothesis, gene screening was performed. Deficiency fly lines with a specific deleted region and a conditional knock-down of *scrib* initiated by RNAi were used to find genes of interest. Following the evaluation of two different protocols, possible candidates for further screening were found.

Keywords:

Drosophila; ABP; Scribble; gene screening; deficiency line; wing imaginal disc.

CERCS:

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

Scribble vahendatud äädikakärbse tiivadiski kasvu regulatsioonis osalevate ko-faktorite esmane sõeltest.

Lühikokkuvõte:

Äädikakärbse *Drosophila melanogaster* tiiva imaginaaldiski areng on hea mudel uurimaks kudede kasvu ja morfogeneesi. Tiivadisk koosneb apiko-basaalse polarisatsiooniga (ABP) epiteliaalsetest rakkudest. Korrektne ABP on ülioluline kudede arengus ja homöostaasi säilitamisel. Muutused ABP võivad viia kudede vohamiseni ja neoplaasia kujunemiseni. Sribble (Scrib) on üks võtmekomponente ABP säilitamisel. Hiljutised teadustööd on näidanud, et *scrib* koopianumber kudedes otseselt mõjutab neoplaasia kujunemist kui Scrib on konditsionaalselt alla surutud kasutades RNAi metoodikat. Sellest johtuvalt püstitati antud töös hüpotees, et eksisteerib sünergia teatud geenide ja Scribble vahel neoplaasia kujunemisel. Uute kandidaatgeenide leidmiseks, mis sünergiliselt osalevad koos Scribble`ga neoplaasia kujunemisel, kasutati uuringutes spetsiifilisi deleteeritud piirkondadega puudulikke kärbseliine, kus RNAi abil oli ka Scrib konditsionaalselt alla surutud. Kasutades kahte erinevat protokolli, leiti kärbseliinid, mis sisaldavad võimalikke kandidaatgeene edasisteks sõeluuringuteks.

Võtmesõnad:

Drosophila; ABP, Scribble; geeni sõeluuring; defitsiitne kärbseliin; tiiva imaginaaldisk.

CERCS:

B350 Arengubioloogia, loomade kasv, ontogenees, embrüoloogia.

TABLE OF CONTENTS

TERMS, ABBREVIATIONS AND NOTATIONS

ABP- apical-basal polarity

AJs- adherens junctions

APC- Adenomatous plyposis coli

BDSC- Bloomington Drosophila Stock Center

Cora- Coracle

Crb- Crumbs

D- day

Dfs- deficiency lines

Dlg1- Discs-large-1

GFP- green fluorescent protein

KD- knockdown

Lac- Lachesin

Lgl2- Lethal giant larvae 2

LRR- leucine-rich repeats

NrxIV- Neurexin IV

RFP- red fluorescent protein

SAC- spindle assembly checkpoint

Scrib- Scribble

SJs- septate junctions

TJs- tight junctions

UAS- upstream activation sequence

Wnt- wingless

Wts- Warts

WT- wildtype

Yki- Yorkie

INTRODUCTION

Apical-basal polarity (APB) is a necessary attribute for precise location in cell composition. It is required for correct tissue organization and organ formation, as well as cell differentiation (Riga et al., 2020). The loss of cell polarity can lead to tumors (Hariharan and Bilder, 2006; Dow and Humbert, 2007; Humbert et al., 2008).

Scribble (Scrib) complex is an important component in establishing APB. Scrib assembles and positions different multiprotein complexes involved in processes like planar polarity, adhesion, and oriented cell division (Bonello and Peifer, 2019). It has been shown that loss of Scribble affects the Hippo pathway. Studies done with *Drosophila* have provided a framework for understanding how the Hippo pathway changes the pattern of gene transcription inside the cell, like changes in the way cells divide (Verghese et al., 2012).

Drosophila melanogaster has been widely used as a model organism since the early 1900s (Kohler, 1994). The advantage of using the *Drosophila* as a model organism in experimental research is their relatively short life cycle and simple cultivation (Ashburner 1989). Another advantage is that they have only 4 chromosomes. First is a sex chromosome, second and third are larger metacentric autosomes, and fourth is a smaller heterochromatic autosome (Schwartz and Cavalli, 2017).

Stock lines of *Drosophila*, among them deletion stocks, which play a crucial role in gene mapping and the identification of genetic enhancers and suppressors of mutant phenotypes, are used for screening purposes. These are deficiency lines (Dfs) that allow comprehensive coverage of the genome (Cook et al, 2010).

Previous research has shown that surrounding cells are affected by conditional knock-down of *scrib*, leading to neoplasia. This showed the importance of cellular communication before the formation of neoplasia (Huang et al., 2023). A screening was done on the left arm of the third chromosome to find genes that work with Scrib and are involved in establishing tissue homeostasis (Fischbach, 2022).

This thesis aims to continue screening these gene regions and establishing a proper protocol for doing so, which is expected to result in the identification of novel genes involved in cell-cell communication during epithelial growth.

1 LITERATURE REVIEW

1.1 Drosophila melanogaster

1.1.1 Life cycle

Drosophila melanogaster, commonly referred to as fruit fly, has been widely used as a model organism since the early 1900s (Kohler, 1994). *Drosophila* has a compact genome and various orthologous genes related to diseases in humans. In addition, their genome is easy to manipulate, which proves useful in interdisciplinary research (Adoutte et al. 2000). Fruit flies have only 4 chromosomes. First is a sex chromosome, second and third are larger metacentric autosomes, and fourth is a smaller heterochromatic autosome (Schwartz and Cavalli, 2017).

Other advantages of using the *Drosophila* as a model organism in experimental research are their relatively short life cycle and simple cultivation (Ashburner 1989). In optimal conditions at 25℃ it takes around 10 days for a fertilized egg to develop into an adult fly (Figure 1). The speed of *Drosophila* development depends on temperature. At 18°C the same process may take up to 19 days or in some cases longer. After the egg is fertilized the embryogenesis takes around 24 hours (Figure 1.1). The following larval phase is further separated into 3 stages named first (Figure 1.2), second (Figure 1.3), and third (Figure 1.4) instar larval stages. The first and second stages last for 1 day each, while the third larval stage lasts 2 days. In the subsequent stage of the life cycle the larvae undergo metamorphosis into pupa (Figure 1.5). A hard case is formed from the outer cuticle of the fully developed larvae. During the pupal stage, which lasts approximately 4 to 5 days, larvae's tissues undergo breakdown while adult structures develop from imaginal discs within the pupal case. After emerging, the adult flies are sexually immature for the next 8 to 12h (Figure 1.6) (Hales *et al*., 2015).

In conditions provided in the laboratory the adult lifespan of the fruit fly is around 30 to 40 days for females and 5 to 10 days longer for males (Pearl and Parker 1924). A female that has reached sexual maturity peak can produce around 100 eggs a day and up to 3000 in her lifetime (Shapiro 1932).

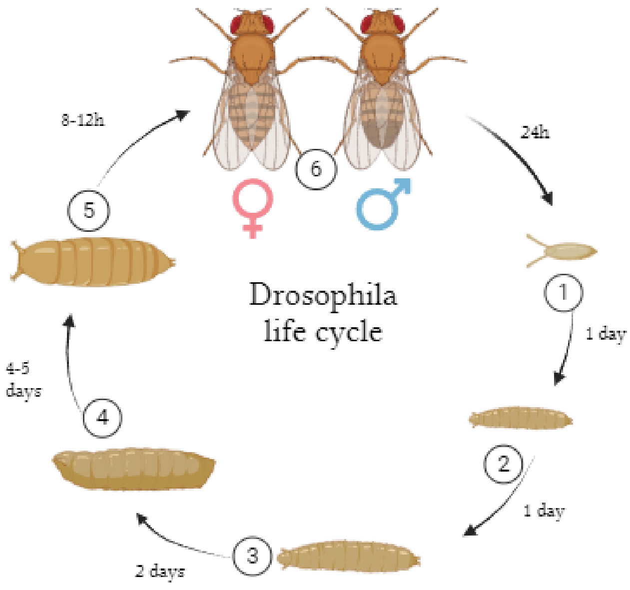


Figure 1. Life cycle of *Drosophila melanogaster*. The 6 main stages of *Drosophila melanogaster*'s life cycle include: embryonic stage (1), larval stages: first (2), second (3), and third instar larva (4), pupal stage (5), and adult stage (6). Figure created with https://biorender.com.

1.1.2 The wing imaginal disc

Many adult *Drosophila* structures develop from 19 imaginal discs. These discs are bundles of tissue-specific parental cells. These cells develop in the first stages of the fly's life cycle and produce most structures for adult flies while in the pupa stage (Hales *et al*., 2015).

The simple structure of the wing imaginal disc, a monolayer of epithelial cells, combined with genetic tools in *Drosophila* has made it a well known and widely used system for researching genes and mechanisms necessary in developmental biology and more (Tripathi and Irvine, 2022). Due to the simple and flat structure of the wing disc it is useful for wing disc imaging. The wing disc expands by increasing the number of cells as it remains diploid (Tripathi and Irvine, 2022). The larvae stage is an intensive growing period for the imaginal discs. For example, the wing imaginal disc, initially composed of around 30 primordial cells, undergoes an increase of 1000 times in size during the larval stage (Martín et al. 2009).

The structure of the wing disc can be divided into three primary segments (Figure 2). Firstly, the notum, which will attach the wing to the thorax. Secondly, the hinge, which acts as an intermediary link between the wing and notum on adult fly. Lastly, the wing pouch, which develops into the wing itself (Tripathi and Irvine, 2022).

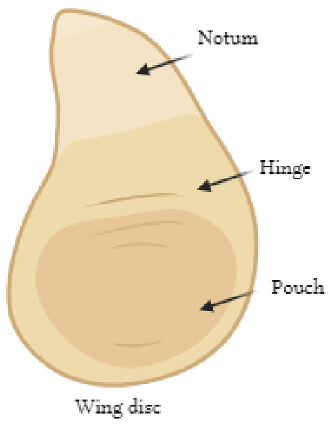


Figure 2. *Drosophila* wing imaginal disc segments. The wing disc comprises three main components: the notum, the hinge, and the pouch. Figure created with https://biorender.com.

1.2 Epithelial cells and mechanisms within them

1.2.1 Epithelial cells

Epithelial tissue is a sheet of closely packed cells that act as a lining of the external surface of tissues and organs, forming a barrier between the internal and external domains. Epithelial tissue properties are maintained by many types of cell to cell junctions that bind the cells together in tight structures (Helander and Fändriks, 2014). These cells are preserved by stem cells that maintain the self renewal and differentiation to replace dying cells or repair injured tissue (Blanpain et al., 2007).

Epithelial cells act like guards that can pick up stress in their microenvironment. Epithelia is supplied with many pattern recognising receptors that allow them to respond to many environmental cues. These cues play different roles in epithelial stem cell responses like strengthening barrier integrity, making changes to neighboring cells, recruiting immune cells, and improving damaged tissue. The communication between epithelial parent cell and immune cell is well calibrated to coordinate response in order to preserve homeostasis in the host (Naik et al., 2018).

1.2.2 Apical-basal polarity

Epithelial cells need apical-basal polarity (ABP) to form semi-permeable barriers. For that epithelial cells polarize down the apical-basal axis, which establishes molecularly and functionally different domains. To maintain the polarity many factors are needed. One of them are junctions like septate junctions (SJs) (tight junctions (TJs) in vertebrates) and adhesion junctions (AJs). Cytoskeleton, particular proteins, and membrane lipid regulators all play a role in epithelial polarization (Roignot et al., 2013).

There are three main protein complexes that contribute to ABP (Figure 3). On the apical domain there are Crumbs (Crb) and PAR complexes, and on the basolateral domain, Scribble (Scrib) complex. However new research indicates that not the same polarization mechanisms can be applied to all epithelial cells (Buckley and St Johnston, 2022).

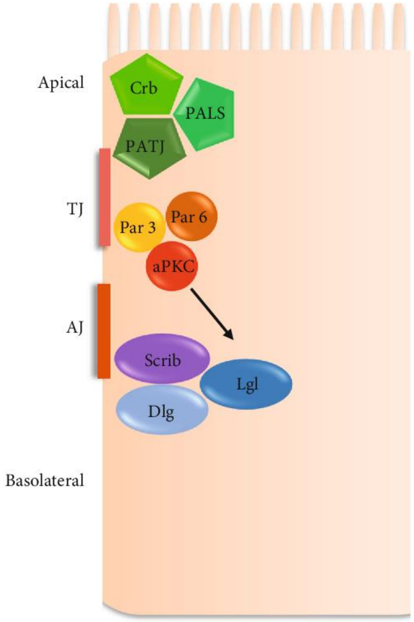


Figure 3. Apical basal polarity in epithelial cells. Three main protein complexes involved in establishing and maintaining epithelial cell polarity include Crumbs and Par complexes which are located on the apical side near tight junctions (TJs), and Scribble complex, located at the basolateral region near adherens junctions (AJs). Figure adapted from Barreda, 2020.

The loss of cell polarity can lead to diseases like cancer. For example altering the gene expression in polarity complexes responsible for establishing and maintaining the ABP often result in neoplastic tumors in *Drosophila* imaginal disc (Hariharan and Bilder, 2006; Dow and Humbert, 2007; Humbert et al., 2008). Research has shown that homozygous mutant wing discs in *scrib, crb,* and *sdt* result in severe overgrowth and loss of ABP. However the mechanisms behind it are still poorly understood (Tepass *et al*., 2001; Dow and Humbert, 2007; Assémat *et al.*, 2008; Humbert *et al.*, 2008).

1.2.3 Scribble complex

Scribble (Scrib) complex is identified as an important component in establishing and maintaining ABP as well as epithelial integrity. It is shown that Scrib assembles and positions different multiprotein complexes that are included in processes like planar polarity, adhesion, and oriented cell division (Bonello and Peifer, 2019). Scrib is known to play an important role in many essential early stages in cancer development (Santoni *et al*., 2020).

The Scrib protein was first found in *Drosophila melanogaster* in 2000 and later in humans as well (Bilder and Perrimon, 2000). The studies have shown that if there is loss of function of *scrib* it results in mislocalization in apical proteins and AJs. It also disrupts the organization of the monolayer of embryonic epithelia (Santoni *et al*., 2020).

Scribble functions as an adapter protein by making it easier for key molecular interactions at specific subcellular locations. It occurs by virtue of the domain structure and structurally restricted location pattern. Scribble is part of the leucine-rich repeats (LRR) and postsynaptic density-95/Disc-large/Z0-1 (PDZ;LAP) family. Its characteristics include the 16 N-terminal LRR, two LAP domains, and four PDZ domains (Bonello and Peifer, 2019).

LRRs are structural repeats that consist of 20 to 30 amino acids, forming α/β horseshoe-like ternary structures (Figure 4A). Essential for the functionality of LAP proteins, LRRs precede two LAP domains, LAPSD a and b. Scribble's PDZ domains adhere to the canonical class I PDZ formation. The tumor suppressor proteins Scribble and Lethal giant larvae 2 (Lgl2) exhibit interactions through their LRR domains (Santoni *et al*., 2020). In certain biological contexts, the LRR region demonstrated the capacity to restoreScribble functions (Bonello and Peifer, 2019).

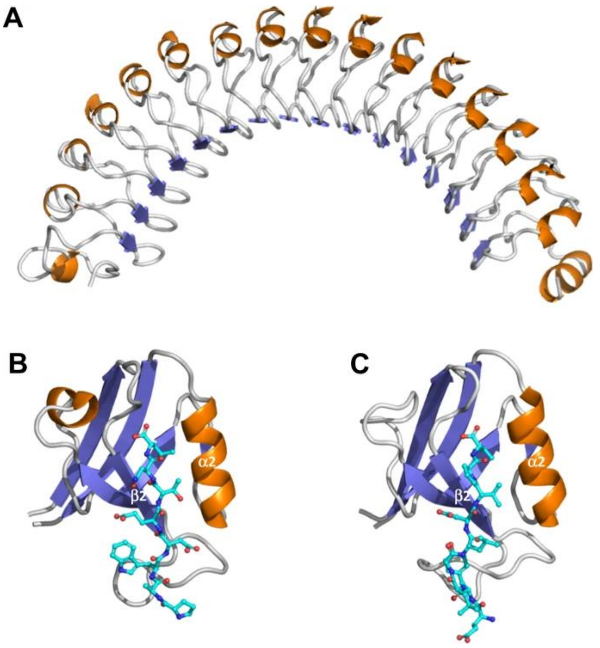


Figure 4. Structure of LAP domains. (A) The α-helices are shown in orange and in blue are β-sheets. (B) The Scribble PDZ1 domain is bound to β-PIX C-term peptide shown in turquoise. (C) The Erbin PDZ domain is bound to ERBB2 C-terminal peptide. Figure adapted from Santoni et al., 2020.

Several studies have demonstrated the multifaceted role of Scribble in various stages of tumor development through its PDZ domains. It has been observed that tumor suppressors such as Adenomatous plyposis coli (APC), a key player in the Wingless (Wnt) signaling pathway, and ZO-2, a junctional protein, undergo mutations as cancer progresses. Notably, Scribble PDZ domains interact with the PDZBM of GEF β-PIX/ARHGEF7, promoting cancer cell migration (Santoni *et al*., 2020).

1.2.4 Cell to cell junctions

Cell to cell junctions are links between cells inside a tissue. They are responsible for tissue homeostasis in processes like cell migration, barrier function, and cell proliferation. If these junctions are disrupted, it can result in tissue abnormalities and diseases like cancer (Humar and Guilford, 2009). In epithelial cells junctions involved in cell signaling and adhesion are especially important as they form extracellular and intercellular connections with cytoskeleton to maintain tissue architecture (Garcia *et al*., 2018).

Septate junctions (SJs) show similarities to tight junctions (TJs) (Figure 4) found in vertebrate cells. Both hold claudin-family proteins as well as provide a diffusion barrier between epithelial cells (Nelson and Beitel, 2009). SJs are located in the apical region of the cell and consist of transmembrane and adapter proteins (Garcia *et al*., 2018). They form a fence that is in control of managing the paracellular pathway. This fence is a proteinaceous seal that manages diffusion of ions and molecules between cells (Zihni et al. 2016). SJs also include components like Lachesin (Lac), Neurexin IV (NrxIV), Coracle (Cora), and Discs-large-1 (Dlg1) (Woods *et al.*, 1996; Banerjee *et al.*, 2006). These junctions are a necessary aspect in the development of tissue architecture and later function. For example, loss of *dlg1* will result in abnormal fusion and growth in the *Drosophila* imaginal discs (Woods and Bryant, 1989). That would indicate that SJs not only form a diffusion barrier but are also a part of cell signaling needed for maintaining epithelial integrity (Dubey *et al*., 2019).

Another type of junctions in *Drosophila* are adherens junctions (AJs) (Figure 4). They have many vital functions within the cell, including the maintenance of cell-cell adhesions, regulation of the actin cytoskeleton and its organization, and acting as a central hub for cell signaling and regulating the gene transcription. The primary transmembrane proteins constituting AJs are classical cadherins, such as E-cadherins. Classical cadherins consist of five cadherin repeat domains. These domains facilitate trans binding with cadherins on adjacent cells (Shapiro and Weis, 2009). The AJs form a continuous belt-like structure encircling the cell, known as zonula adherens (Takeichi, 2014).

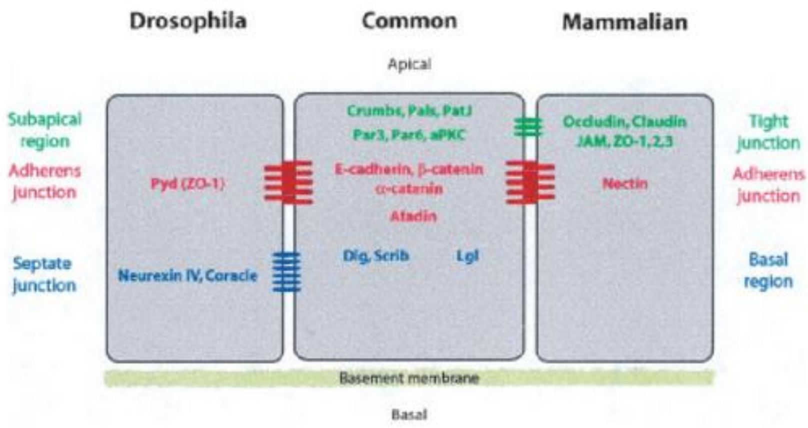


Figure 4. Cell-cell junctions. *Drosophila* (left) and mammalian (right) junctions are compared with common proteins in the middle. *Drosophila* have SJs where mammals have TJs, and AJs are present in both. *Drosophila* have SJs as well. Figure adapted from Humbert et al., 2003.

1.2.5 Proliferation of epithelial cells

In the case of the inactive Hippo pathway the cells continue to proliferate and regulation of apoptosis is lost leading to tissue overgrowth and tumorous organs. Hippo signaling pathway is a highly conserved pathway, which is necessary for cell proliferation, apoptosis, and differentiation (Kango-Singh and Singh, 2009; Halder and Johnson, 2011; Staley and Irvine, 2012; Boggiano and Fehon, 2012). Studies done with *Drosophila* have provided a framework for understanding how the Hippo pathway changes the pattern of gene transcription inside the cell, like changes in the way cells divide. It has been shown that loss of Scribble affects the Hippo pathway (Verghese *et al*., 2012). An overgrowth in *scrib* mutant clones in fly tissue is dependent on Yorkie (Yki) (Grzeschik *et al*., 2010; Doggett *et al*., 2011). It is shown by the fact that *scrib* mutant clone size is suppressed by heterozygosity of yki. When testing Yki and Scrib interactions by overexpressing *yki* in nub-Gal4 UAS-*scrib*RNAi wing imaginal discs, the results showed an overgrown wing pouch close to those discs that overexpressed only UAS-*yki* (Verghese *et al*., 2012).

Scribble interaction with Warts (Wts) was tested by examining the sizes of *wts* mutant clones compared to *scrib*-*wts* double knockdown (KD) mutant clones (Verghese *et al*., 2012). Both sets of clones exhibited significant overgrowth, contrasting with the small and slow-growing nature of *scrib* mutant clones alone . Analysis of clone size and DIAP1 expression suggests that *wts* is acting downstream of *scrib* since the phenotypes of *wts*-*scrib* mutant clones closely resemble those of *wts* mutants (Verghese *et al*., 2012).

1.3 Genetic tools

1.3.1 The UAS/GAL4/GAL80^{ts}system

The UAS/GAL4 system represents a versatile tool for gene expression analysis that is widely used not only in *Drosophila* research but also in other model organisms such as zebrafish (Fischer et al., 1988; Brand and Perrimon, 1993; Hales *et al*., 2015). GAL4, a transcription factor, was initially isolated from yeast. It comprises distinct functional domains, including a DNA-binding domain and a transcription activation domain. Through its recognition of a specific DNA sequence upstream activation sequence (UAS), GAL4 mediates the activation of transcription from the downstream promoter region (Yamada *et al*., 2020). This system enables researchers to selectively modulate the expression of target genes, facilitating detailed investigations into their biological functions and regulatory mechanisms.

The UAS/GAL4 system is a very useful tool apart from the fact that it does not provide the ability to regulate temporal activity, as it remains active across various stages of the development (Osterwalder *et al.*, 2001; Roman *et al.*, 2001). To address this constraint, GAL80^{ts} is introduced (Figure 5). The GAL80^{ts} functions by binding to GAL4, thereby inhibiting the transcription of the target gene through its role as a GAL4 repressor. Notably, GAL80^{ts} exhibits temperature sensitivity, allowing for its deactivation with relative ease. By adjusting the temperature to appropriate conditions, GAL80^{ts} dissociates from GAL4, allowing the activation of the gene of interest. Specifically, at 18°C, the GAL80^{ts} protein effectively binds to GAL4, preventing the expression of the downstream gene (Figure 5II). Conversely, at 29°C, GAL80^{ts} disassociates from GAL4, enabling the expression of the downstream gene (Figure 5IV) (Barwell *et al.*, 2017).

To visualize the outcomes facilitated by this system, researchers can employ a green fluorescent protein (GFP) marker to pinpoint the protein of interest. Additionally, alternative markers such as red fluorescent protein (RFP) can also be utilized for this purpose (Morimot and Tamori, 2017).

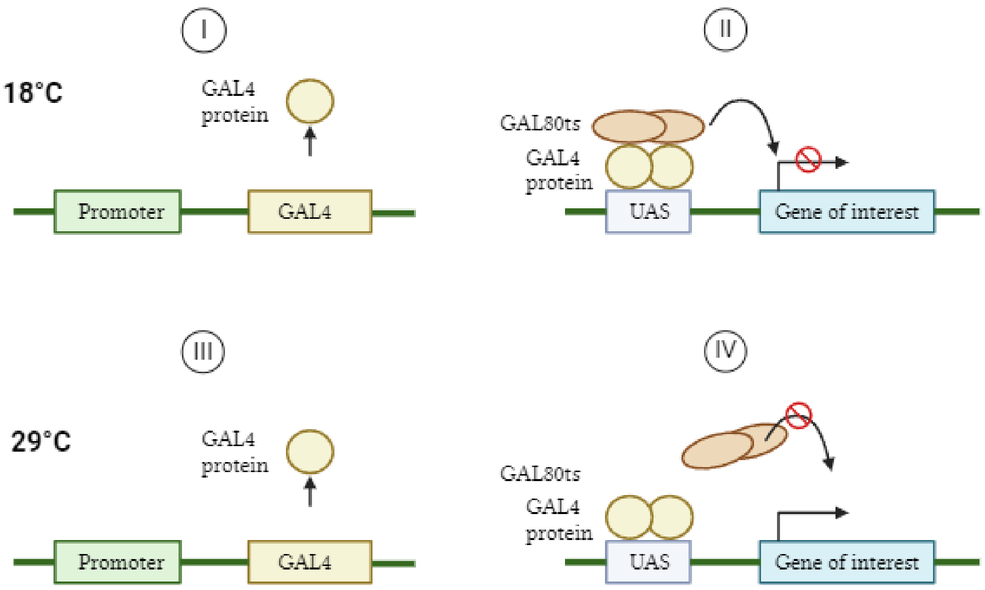


Figure 5. UAS/GAL4/GAL80^{ts} system. GAL4 is a gene expression activator, producing a protein (I, III) that binds to regulatory sequence UAS and results in the expression of genes of interest. GAL80^{ts} is a temperature sensitive protein that is able to bind to GAL4 and inhibit the gene expression of the target gene. At 18°C, GAL80^{ts} to GAL4 and the downstream gene is not expressed (II). At 29°C, GAL80^{ts} is not able to bind to GAL4, leading to its dissociation and subsequent expression of the downstream gene (IV). This system allows temporal regulation of gene expression. Figure created with https://biorender.com.

1.3.2 In vivo conditional RNAi and deficiency lines

RNA interference (RNAi) has transformed the creation of genome-wide libraries for investigating gene function. In fly genetics the GAL4/UAS enables the expression of short, inverted repeat RNA hairpins designed to target and knocks-down specific genes' mRNA

(Kennerdell and Carthew 2000). To better understand the functions of target genes in cellular and developmental processes, researchers utilize characterized GAL4 driver lines that exhibit activity in specific developmental stages or cell types. This approach involves expressing RNAi hairpins in particular cells or during specific developmental timeframes (Boutros et al. 2004; Armknecht et al. 2005; Mathey-Prevot and Perrimon 2006; Dietzl et al. 2007). GFP distinctive green fluorescence renders it highly visible, making it a good choice as a reporter in studies focusing on gene expression or protein localization (Chalfie et al, 1994; Marshall et al, 1995; Chalfie M., 1995; Cubitt et al., 1995). Notably, its utility is augmented by the fact that GFP does not require substrates or cofactors for visualization (Heim et al, 1994).

The Bloomington Drosophila Stock Center (BDSC) is a center that creates and disseminates genetically defined stock lines of *Drosophila*. Among them are deletion stocks, which play a crucial role in gene mapping and the identification of genetic enhancers and suppressors of mutant phenotypes. The overarching aim of the project is to achieve comprehensive coverage of the genome through the generation of deletions with breakpoints spaced no more than dozen genes apart. Particularly beneficial for screening purposes are the Deficiency Kits, as the deletions within them have been generated within a uniform genetic background (Cook et al, 2010).

2 THE AIMS OF THE THESIS

One of the ongoing projects in the host lab is the identification of genes that interact with Scribble to maintain cellular homeostasis. Understanding these mechanisms is necessary for elucidating intercellular communication and cooperation. We hypothesize that synergy exists between the loss of Scribble and other components that contribute to the progression of neoplasia.

An optimized protocol is needed for obtaining viable results as incorrect time or temperature settings can lead to inaccurate results.

The aims of this thesis are:

- To establish a working protocol that contributes viable results of the screenings.
- To perform a systematic primary screening of the left arm of the third chromosome in *Drosophila* using Dfs.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Deficiency lines

Variety of fly lines were used for the deficiency screening (Table 1). These lines were obtained from a deficiency toolkit collected by the Bloomington *Drosophila* Stock Center (BDSC; https://bdsc.indiana.edu/index.html). These kits cover 2845 genes, which is around 97% of the genes located on the left arm of the 3rd chromosome. A kit consists of 77 stocks, with each stock containing deletions spanning 20 to 30 genes.

Table 1. Fly lines used in the thesis.

Stock number	Genotype	Purpose
9482	w[1118]; Df(3R)ED10642, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED10642/TM6C, cu[1] Sb[1]	Df, primary screening
9347	w[1118]; Df(3R)ED6187, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED6187/TM2	Df, primary screening
27580	w[1118]; Df(3R)BSC819, P+PBac{w[+mC]=XP3.RB5}BSC819/TM6C, Sb[1] cu[1]	Df, primary screening
25075	w[1118]; Df(3R)BSC547/TM6C, Sb[1]	Df, primary screening
25011	w[1118]; Df(3R)BSC507/TM6C, Sb[1] cu[1]	Df, primary screening
9210	w[1118]; Df(3R)ED6255, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED6255/TM6C, cu[1] Sb[1]	Df, primary screening
8957	w[1118]; Df(3R)ED5514, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED5514/TM6C, cu[1] Sb[1]	Df, primary screening

26529	w[1118]; Df(3R)BSC677, P+PBac{w[+mC]=XP3.WH3}BSC677/TM6C, Sb[1] cu[1]	Df, primary screening
94971	Df(1)CRIMIC-CR70104, y[1] TI{KozakGAL4}CG14795[CR70104-KO-kG4] w[*]	Df, primary screening
24971	w[1118]; Df(3R)BSC467/TM6C, Sb[1] cu[1]	Df, primary screening
8105	w[1118]; Df(3R)ED6232, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED6232/TM6C, cu[1] Sb[1]	+/+ control
Host stock	+/+; ptc-Gal4, UAS-GFP, ex-LacZ/CyO;Scrib RNAi, Gal80ts/Scrib RNAi, Gal80ts	+/- control
25006	w[1118]; Df(3R)BSC502/TM6C, Sb[1] cu[1]	Df, primary screening
26848	w[1118]; Df(3R)BSC750/TM6C, Sb[1] cu[1]	Df, primary screening
8103	w[1118]; Df(3R)ED5177, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED5177/TM6C, cu[1] Sb[1]	Df, primary screening
9204	w[1118]; Df(3R)ED5339, P{w[+mW.Scer\FRT.hs3]=3'.RS5+3.3'}ED5339/TM6C, cu[1] Sb[1]	Df, primary screening
23232	w[*]; ry[506] Dr[1]/TM6B, P{Dfd-GMR-nvYFP}4, Sb[1] Tb[1] ca[1]	Balancer for Dfs

The host stock utilized in the thesis was specially designed by our lab (Fischbach, 2022). In the host stock, the *scrib* gene was selectively knocked down, allowing for visualization through GFP in the Ptc domain. Notably, Ptc expression is located in the middle region of the wing imaginal disc (Huang et al., 2023).

3.1.2 Fly crosses

In this thesis, crosses were performed with Dfs and *scrib*-RNAi host stock (Figure 6). Balancers are mutations on specific chromosomes engineered with multiple inversions and recessive lethal mutations. These modifications prevent recombination and maintain the desired genotype (Hales et al., 2015). One of these balancers, Tm6B, was integrated within the *scrib*-RNAi host stock to determine the genotype during the third instar larval based on phenotype, facilitating sample collection. The genotype of interest consists of the *scrib*-RNAi-GAL80^{ts}-GFP and the selected Df on the 3rd chromosome (Figure 6). The correct genotype was determined by observing GFP expression in the wing disc under a fluorescent stereo microscope.

For genotypic identification a dominant marker Tb was employed. This dominant marker has distinct phenotypic characteristics such as a shorter and rounder body during larval and pupa stages, aiding in the selection of appropriate specimens for dissection. If the deficiency line of interest was under a different balancer, it must be switched to Tb, as all Dfs must be under the same balancer. This was achieved by crossing Dfs with Dr/Tm6B Tb Sb (Table 1) fly line, which are subsequently allowed to mate within the population to ensure the presence of the necessary balancer in all stocks.

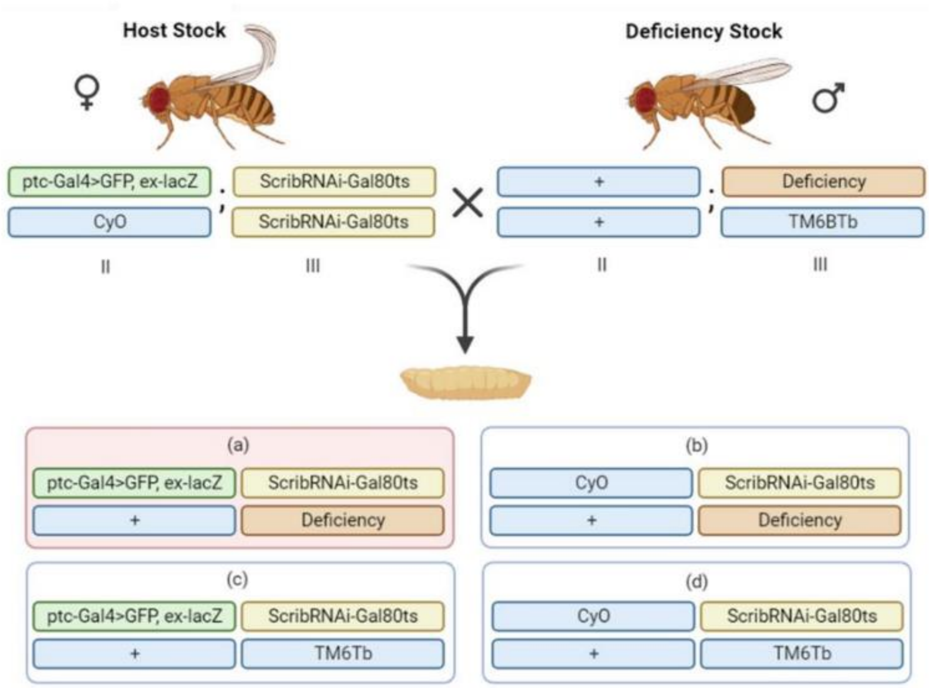


Figure 6. Expected offspring of fly line crosses. Genotype (a) is the desired progeny since it has both ptc-GAL4>GFP on the 2nd chromosome and *scrib*-RNAi-GAL80ts the chosen Df on the 3rd chromosome. Genotype (b) will show no GFP signal therefore it can be excluded. Genotype (c) does not have the Df present and neither does (d). They can be excluded by observing the Tb balancer in larvae. Figure adapted from Fischbach, 2022.

To ensure experimental integrity, only juvenile virgin females from the host stock were selected for fly crosses. Contamination could result if females have previously mated with male flies. The juvenility of female flies involves adhering to specific principles. Juvenile flies typically exhibit a dark spot on the abdomen and unfolded wings. Moreover, mature male and female flies are generally smaller and darker compared to the juvenile flies. It is important to accurately distinguish male and female flies, as the aforementioned criteria can be applicable to both sexes. Males can be identified by their flat, rounded, and dark anal plate, distinguishing them from female flies.

3.1.3 Conditional knockdown

Once the *scrib* RNAi host stock and Df cross were established, a small amount of dry yeast was added, and the flies were left to mate for a minimum of 2 days. Following this period, egg laying was started by transferring the flies to a new vial and adding yeast. The vial was put to 18°C for 12h to synchronize larval development, ensuring uniformity in larval age at the time of sample collection. After 12h, the adult flies were removed, and the vial containing embryos was left at 18°C for 3 or 4 days, depending on the chosen protocol. Subsequently, a temperature shift was initiated by transferring the vial to 29°C for 3 days. This temperature shift was necessary for the conditional knockdown of *scrib*, as facilitated by the UAS/GAL4/GAL80ts system (Figure 5). Finally, 3rd instar larvae were collected, and dissection can commence.

3.2 Sample preparation and analysis

3.2.1 First dissection

The dissection begins when larvae reach the late 3rd instar stage and are observed actively moving along the walls of the vial. Using a brush, non-Tb larvae, collected for Df screening, and Tb larvae, for the +/- control, are carefully collected and transferred to a plastic dish containing 1xPBS (phosphate-buffered saline). The dish was then placed on ice for a couple of minutes to immobilize the larvae. Ideally, a minimum of 10 larvae were obtained for dissection.

In this context, dissection refers to the extraction of the wing imaginal disc from the larvae. After removing the larvae from ice, they were in a 1xPBS puddle on a silicone plate under a stereomicroscope (Zeiss Stemi 305). Using forceps, approximately 2/3 of the larva's posterior end was removed and an opening at the anterior end was made. At this point, the wings should protrude and become visible laterally. The anterior end of the larva, with exposed wings, was then transferred into a 1.5ml Eppendorf tube containing 1xPBT (buffer with

1xPBS and non-ionic detergent Tween 20 (Roche®)) solution. This process was repeated until all larvae were dissected.

3.2.2 Fixation

Fixation of the sample was necessary for preserving the structural integrity of cells. The fixation solution consists of PBT and formaldehyde (FA) (Sigma-Aldrich®) with a final concentration of 3.7%. Following removal of the PBT, the wings with attached tissue were submerged in the fixation solution and left for 20 minutes at room temperature. Subsequently, they were washed with 1xPBT 3 times to remove excess fixatives and prepare them for further dissection.

3.2.3 Second dissection

Once the tissue was fixed, wings with attached tissue were removed from the 1.5 ml Eppendorf tube using a brush and placed inside a 1xPBS puddle on the silicone plate. Subsequently, each individual head was isolated using forceps and transferred into a fresh puddle Carefully, any excess tissue was removed to retain only the imaginal wing disc. The cleaned disc was then transferred into a new 1.5 ml Eppendorf tube filled with PBT solution and placed on ice. This process was repeated until all wings had been cleaned and prepared for further analysis.

3.2.4 Staining

In the experiments, DAPI (Thermofisher®, D1306) was used for staining purposes. This staining enables clear visualization of the wing disc under a fluorescent microscope (Olympus BX51). Cleaned wings were carefully washed and drained from 1xPBT solution. Subsequently a mixture of 0.34ul of DAPI (1:300) and 100ul 1xPBT solution was added. The wings were then incubated for 2 hours at room temperature (RT) or overnight at 4℃. It is important to conduct this incubation step in the dark, as DAPI is sensitive to light. Following incubation, the sample was washed 3 times with 1xPBT to remove excess dye.

3.2.5 Mounting

During the mounting process, the wing discs were prepared for microscopy examination. They were first prepared by adding a piece of tape onto the slide and cutting out a central strip using a scalpel. This ensures when the cover is placed, the sample remains protected from being damaged. Using a 200 μl pipette, the wings were gently lifted with minimal liquid and positioned onto the microscopy slide between the tape. The slide was then carefully tilted to remove any excess liquid, using a piece of paper towel. Once liquid has been removed, a few drops of 70% glycerol solution were added, and coverslip carefully placed on top. To secure the coverslip in place, a thin layer of transparent nail polish was applied.

3.2.6 Microscopy

The imaging was ideally conducted within the next 3 days after mounting, as GFP is known to be pH sensitive and may deteriorate over time. For imaging purposes, an Olympus BX51 Fluorescence Microscope equipped with a 20x magnification lens and CellB program was employed. Two images of each wing were captured, one with DAPI staining to visualize the nuclei and one with GFP exposure to detect GFP expression.

3.2.7 Image analysis

Subsequently, the ImageJ program was utilized and the two obtained images (DAPI and GFP) were merged into a single composite image for visualization.

Thereafter the merged images were analyzed using a scale introduced by Fischbach, 2022 (Fischbach, 2022) (Figure 7). This scale categorizes the degrees of overgrowth into five distinct categories, providing a standardized framework for assessing and quantifying the observed phenotypic changes.

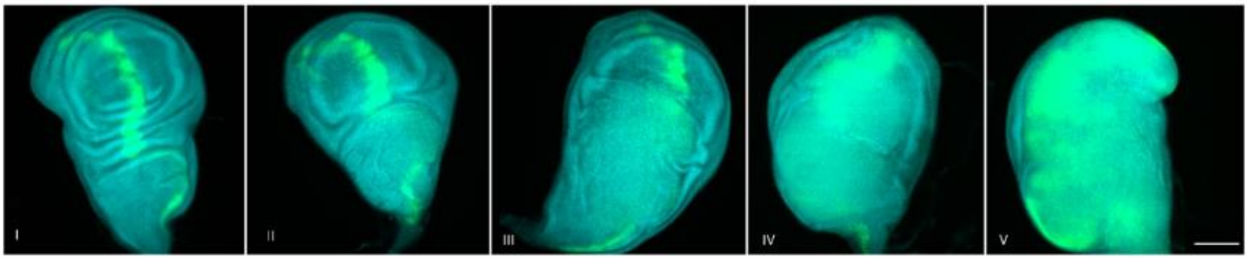


Figure 7. Categories of overgrowth phenotypes. There are five proposed categories starting from left to right. I - no overgrowth, II - mild overgrowth, III - medium overgrowth, IV - strong overgrowth V - tumor. Scale bar is 100 μm. Adapted from Fischbach, 2022.

3.3 RESULTS

3.3.1 Implemented protocols

The thesis employed two distinct protocols differing in the duration of time flies were maintained at 18°C before shifting to 29°C for 3 days (D). The initial protocol entailed 3 days at 18°C, while the second protocol extended this period to 4 days (Figure 8). In each protocol two controls were used. +/+ control contains one copy of *scrib* gene and since it is deficient in *scrib* locus it is expected to show severe neoplasia. +/- control has two copies of *scrib* gene and because of that is expected to show no neoplasia. In the 3D/3D protocol, the +/+ control of line 8105 crossed with *scrib* RNAi host stock exhibited widespread overgrowth across most wings, although some wings displayed mild to no overgrowth (Figure 8A). In this protocol, the *scrib* RNAi host stock served as +/- control and showed diverse results (Figure 8C). *Scrib* RNAi host stock and cross with line 8105 were used for the other experiments therefore they have the most amount of wing samples (Figure 9). In the 4D/3D protocol +/+ control was a cross of 26525 with *scrib* RNAi host stock and a +/- control. In +/+ control the stock exhibited no overgrowth (Figure 12A). This contradicts expectations, as the +/+ control, containing only one copy of *scrib* gene, should display tumor phenotypes. A +/- control was used and exhibited no overgrowth (Figure 12B). This aligns with expectations that +/- , containing two copies of the *scrib* gene, should not express tumors.

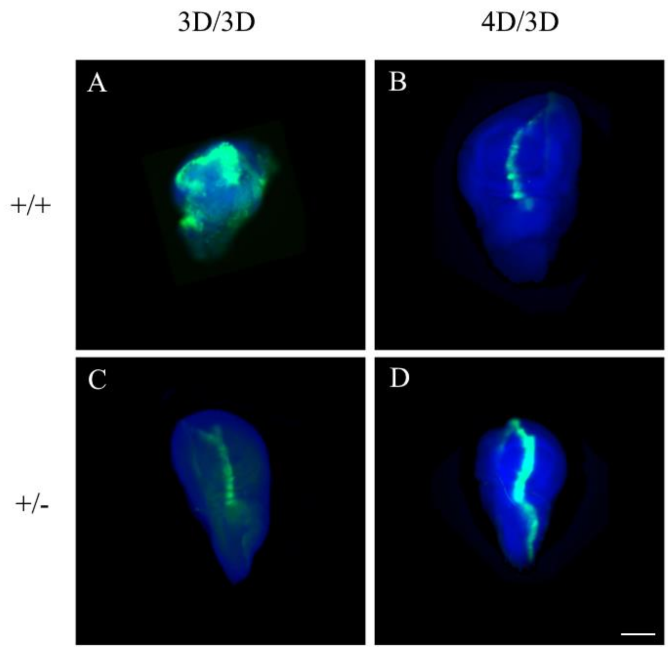


Figure 8. Difference in controls used in the 3D/3D and 4D/3D protocols. In the 3D/3D protocol, the +/+ control showed neoplasia (A), while in the 4D/3D protocol, this phenomenon is absent (B). Under the 3D/3D protocol, the +/- control displayed medium overgrowth (C), whereas under the 4D/3D protocol, the +/- control showed no overgrowth (D). Scale bar is 100 um.

3.3.2 Df screening using 3D/3D protocol

The 3D/3D protocol was used for 7 Deficiency lines (Dfs). Additional Dfs investigated included Df 9204, 8103, 26848, 25006, 24971, 9347, and 9482. The percentage distribution of each category for every line analyzed is illustrated in Figure 9.

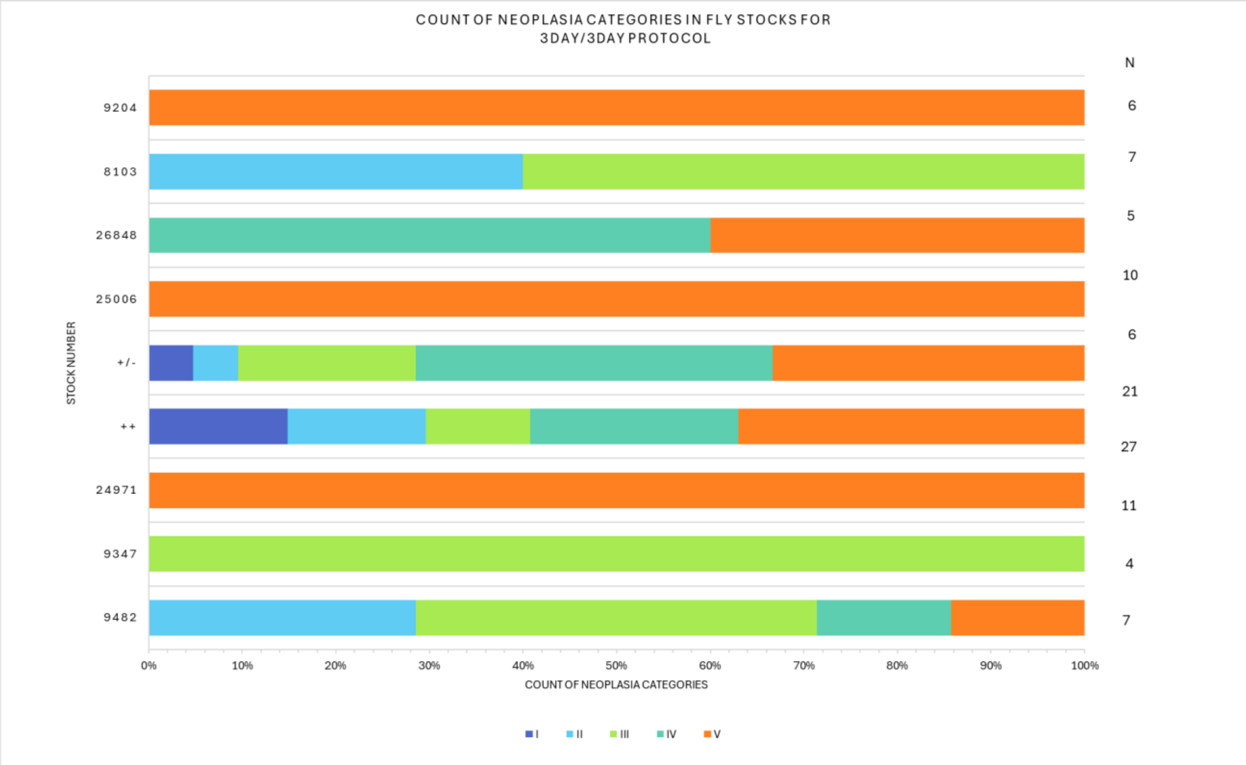


Figure 9. Neoplasia category distribution in fly stocks under 3D/3D protocol. The graph shows +/- control and +/+ control, and screened Dfs used in the experiment. Five categories of stages of neoplasia are shown: dark blue - no overgrowth (I), light blue - mild overgrowth (II), green - medium overgrowth (III), turquoise - strong overgrowth (IV), and orange - tumor (V).

For the 3D/3D protocol, +/+ control (one copy of *scrib*) fly line 8105 crossed with *scrib* RNAi host stock was used. It displayed mixed results with most wings still expressing tumors. It has 5% of I, 5% of II, 19% of II, 38% of IV and 33% of V category overgrowth. This is expected for the +/+ control as it is deficient in *scrib* locus and is expected to show severe neoplasia phenotype (Figure 10A). However, there are also the I category of 15%, II category of 15% and III category of 11% present (Figure 9). While the majority of analyzed wings have severe overgrowth, a significant portion of the samples do not yield ideal results.

Scrib RNAi host stock was used as a +/- control (two copies of *scrib*). This line should show no neoplasia since it has two copies of the *scrib* gene. However, with the 3D/3D protocol, the results contradict that. Most of the wings analyzed show overgrowth (Figure 10B). 53% of IV, 27% of III, and 7% V category are present (Figure 9). Wings that showed no or little overgrowth (I and II category) consist of 13%. As stated above, host stock containing two copies of *scrib* should not show neoplasia therefore in 3D/3D protocol host stock is not an appropriate +/- control.

Line 9204 and +/- control showed identical results for their respective experiment. All wings for Df 9204 showed V category overgrowth (Figure 10C). Either a mistake was made while making this cross of host stock and Df 9204, therefore the cross itself failed, or the 3D/3D protocol does not work. The experiment should be repeated with the same cross once again.

Df 8103 exhibited mild to medium overgrowth, and an absence of severe overgrowth (Figure 10D). The wings displayed a distribution of 40% in the II category and 60% in the III (Figure 9), indicative of mild to medium overgrowth.

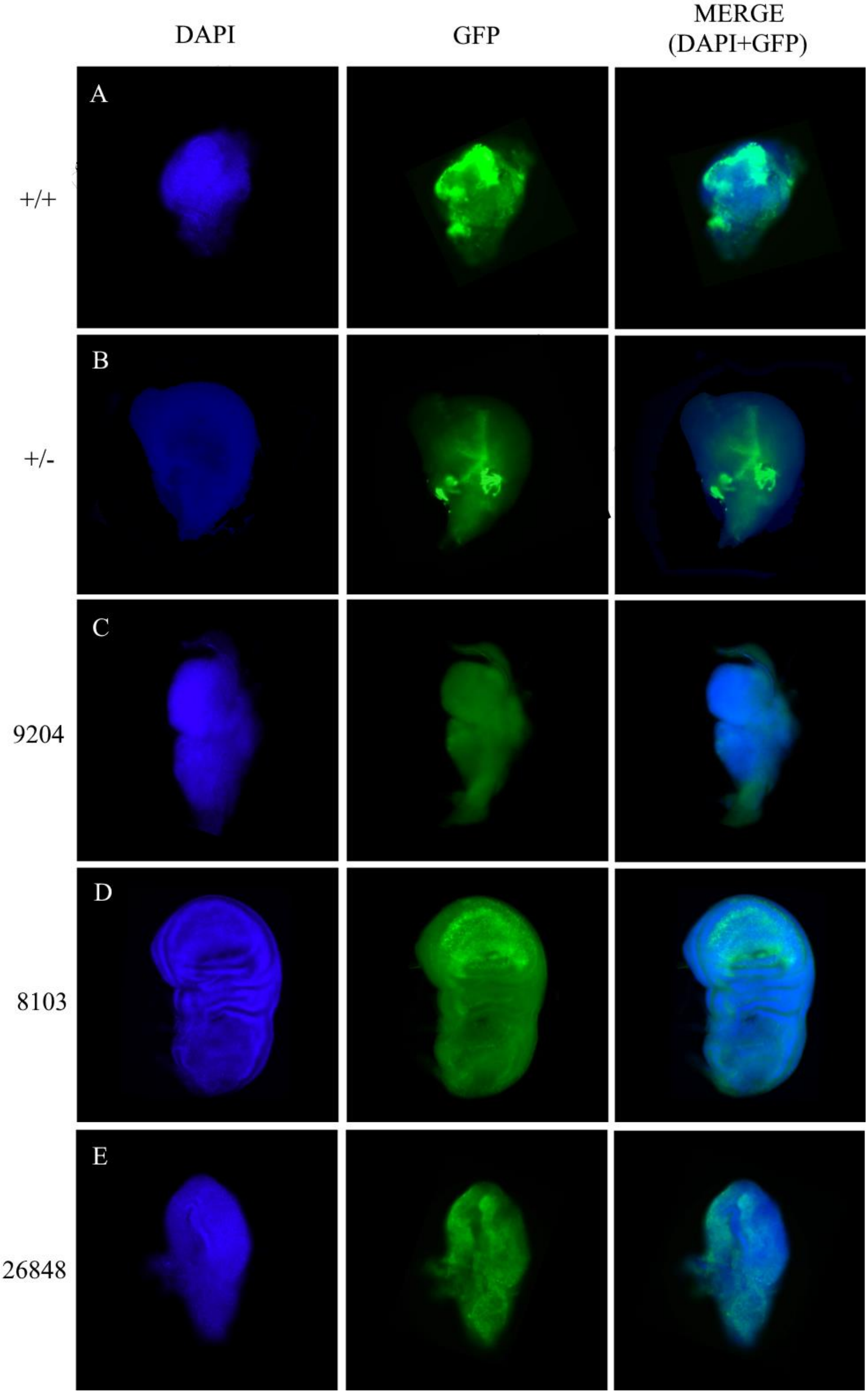
In contrast, Df 26848 showed strong overgrowth, with 60% falling into IV category and 40% into the V category (Figure 9, Figure 10E). Given this observation, this line emerges as a promising candidate for further screenings.

Similarly, Df 25006 presented a distinct phenotype, with wings exclusively displaying the tumor phenotype and belonging to the V category (Figure 9, Figure 10F), suggesting its potential significance for future screenings.

Likewise, Df 24971 mirrored the results of line Df 25006, with wings categorized under the tumor phenotype (Figure 9, Figure 10G). This line is also a viable candidate for subsequent screening endeavors.

Conversely, Df 9347 exhibited solely medium overgrowth, with all wings falling into the III category (Figure 9, Figure 10H). This suggests that the genes located within this region may not exhibit as strong of a synergetic relationship with Scribble compared to other mentioned deficiency lines.

Finally, Df 9482 has very diverse results (Figure 10I), with a distribution of 29% in the II category, 43% in the III category, 14% in the IV category, and 14% in the V category (Figure 9).



DAPI
GFP
MERGE
(DAPI+GFP)
A
+/+
B
+/-
C
9204
D
8103
E
26848

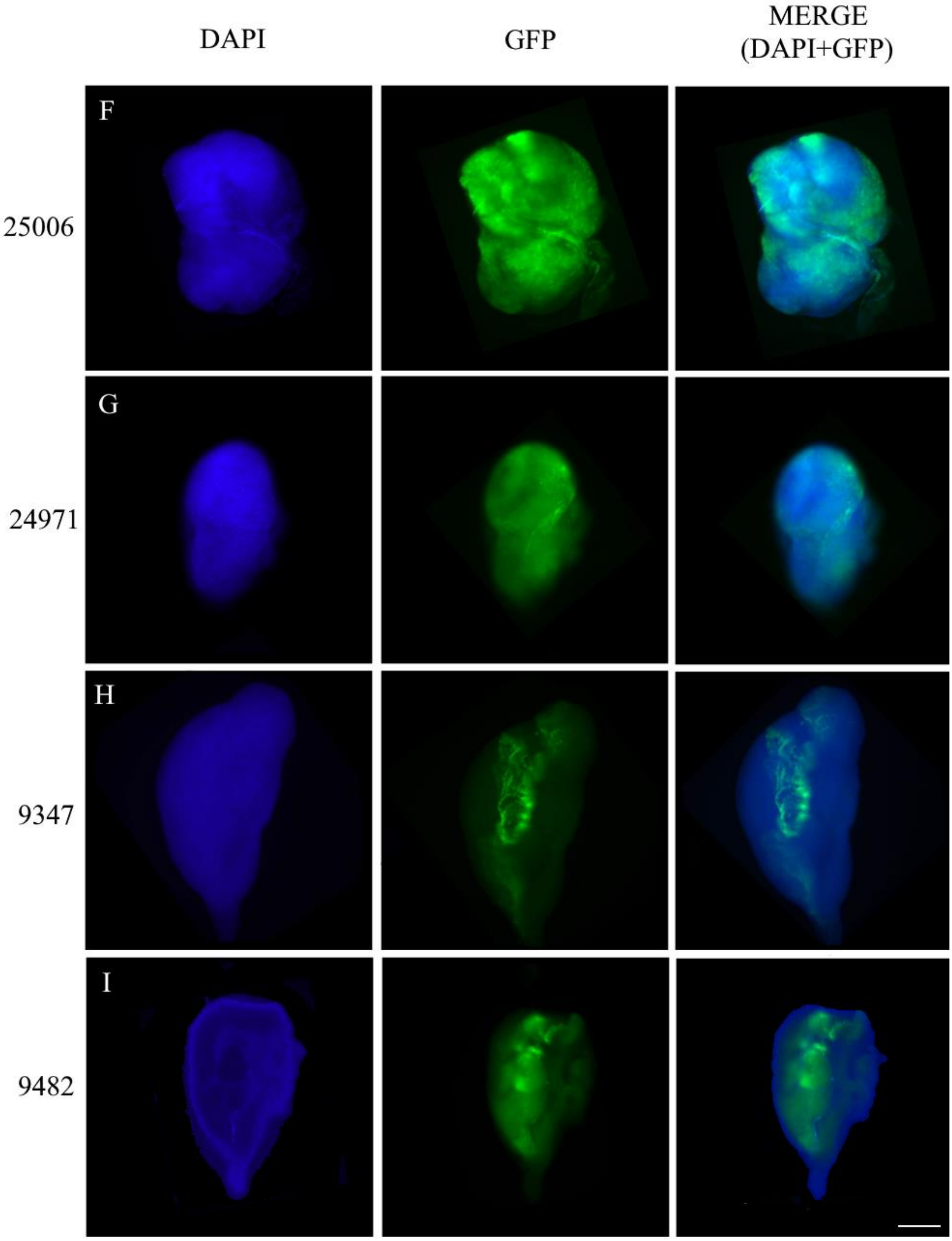


Figure 10. Primary Df screening results for 3D/3D protocol. +/+ and +/- controls are visible in the first two rows. All lines showed strong overgrowth, except lines Df 8103 and 8482 that showed mild overgrowth. Each column shows DAPI, GFP, and merged channels (DAPI+GFP) from left to right. Scale bar is 100 um.

Overall, the 3D/3D protocol consistently showed overgrowth across all of the lines examined. However, the reliability of these findings was called into question due to the unexpected presence of overgrowth and even tumor phenotypes in the +/- control, intended for visualizing wings devoid of overgrowth.. Given the prevalence of overgrowth across multiple lines, numerous candidates emerged for secondary screening. Consequently, none of the lines utilized in the 3D/3D protocol can be dismissed as possible tumor suppressors. It is imperative to revisit these lines under an alternative protocol to attain more accurate and precise results.

3.3.3 Df screening using 4D/3D protocol

In the 4D/3D protocol, a total of 6 Dfs were investigated. Fly lines examined in this protocol include Df 94971, 25075, 27580, 8957, 9210, and 9482. Each line was assessed for the percentage distribution across different phenotypic categories, as depicted in Figure 11.

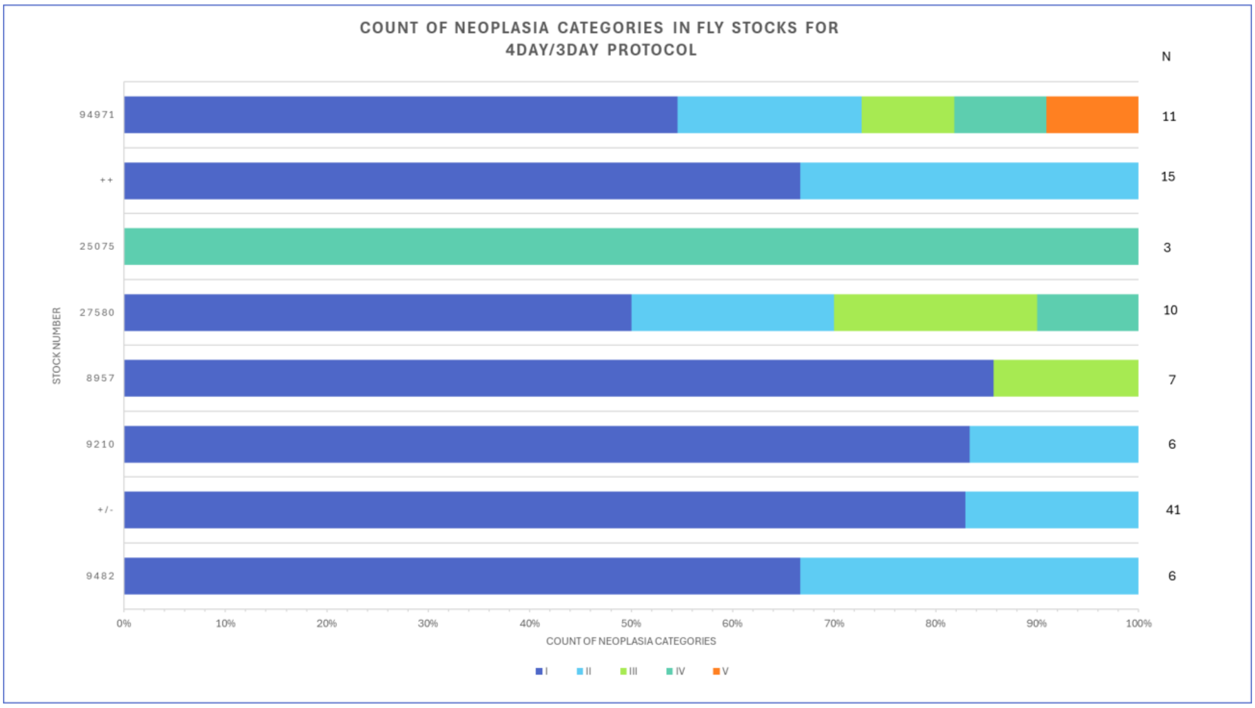


Figure 11. Count of neoplasia categories observed in fly stocks for 4D/ protocol. The graph shows +/- control, cross with Df line 26525 as +/+ control, and other crosses utilized in the experiment. The graph categorizes the observations into 5 distinct stages of neoplasia, dark blue - no overgrowth (I), light blue - mild overgrowth (II), green - medium overgrowth (III), turquoise - strong overgrowth (IV), and orange - tumor (V).

In the +/+ control, a cross with host stock and Df 26525 was utilized. Surprisingly, this stock predominantly exhibited no overgrowth (Figure 12A), with 67% falling into the I category and 33% into the II category. This outcome contradicts expectations, as the +/+ control, possessing one copy of *scrib* gene, should ideally display tumor phenotypes.

Conversely, the +/- control exhibited no overgrowth (Figure 12B), with 83% categorized as I and 17% as II category. This aligns with expectations, +/- , harboring two copies of the *scrib* gene, should not manifest tumors.

Line 94971 yielded mixed results, primarily displaying no overgrowth (Figure 12C), with 55% in I category, 18% in II, 9% in III, 9% in IV, and 9% in V. Given the variability, repeating the experiment with this line is advisable.

Line 25075 demonstrated uniform strong overgrowth across all wings (Figure 12D), placing them in category IV. This line appears as a promising candidate line for secondary screening.

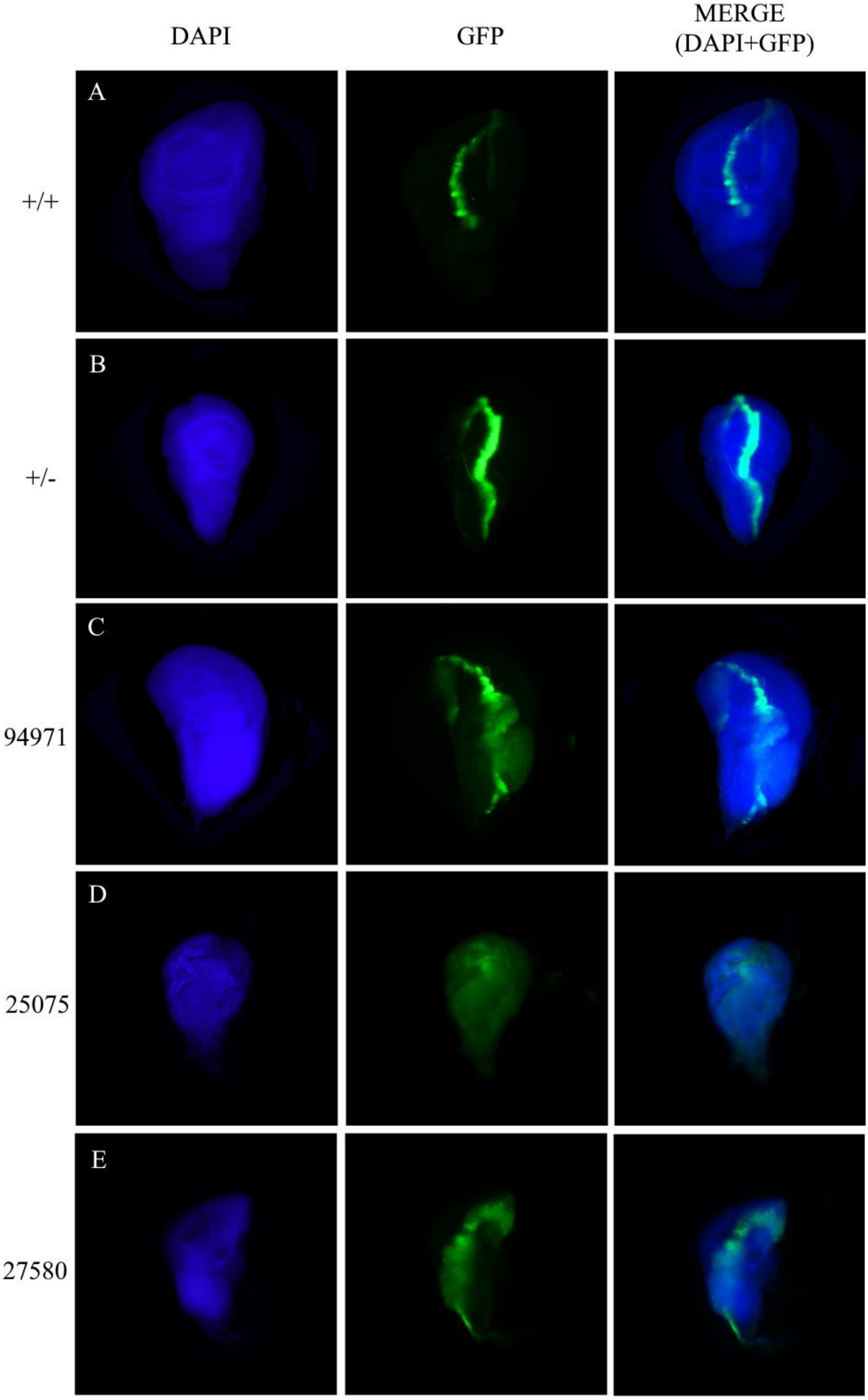
Line 27580 also exhibited mixed results, predominantly indicating no overgrowth (Figure 12E), with 50% in category I, 20% in II, 20% in III, and 10% in IV category. Repeating the experiment with this line could provide clearer insights.

Line 8957 predominantly displayed no overgrowth (Figure 12F), with 86% categorized as I and 14% as III category. However, four wings lacking GFP were excluded from the analysis. Notably, the I category wings originated from one experiment, while the III category and GFP-lacking wings stemmed from another, warranting caution in interpretation.

Line 9210 mostly exhibited no overgrowth (Figure 12G), with 83% classified as I and 17% as II category.

Similarly, line 9482 primarily displayed no overgrowth (Figure 12H), with 67% falling into I category and 33% into II category.

Lines 25011 and 26529 omitted from the results as their wings did not express during imaging.



DAPI
GFP
MERGE
(DAPI+GFP)
A
+/+
B
+/-
C
94971
D
25075
E
27580

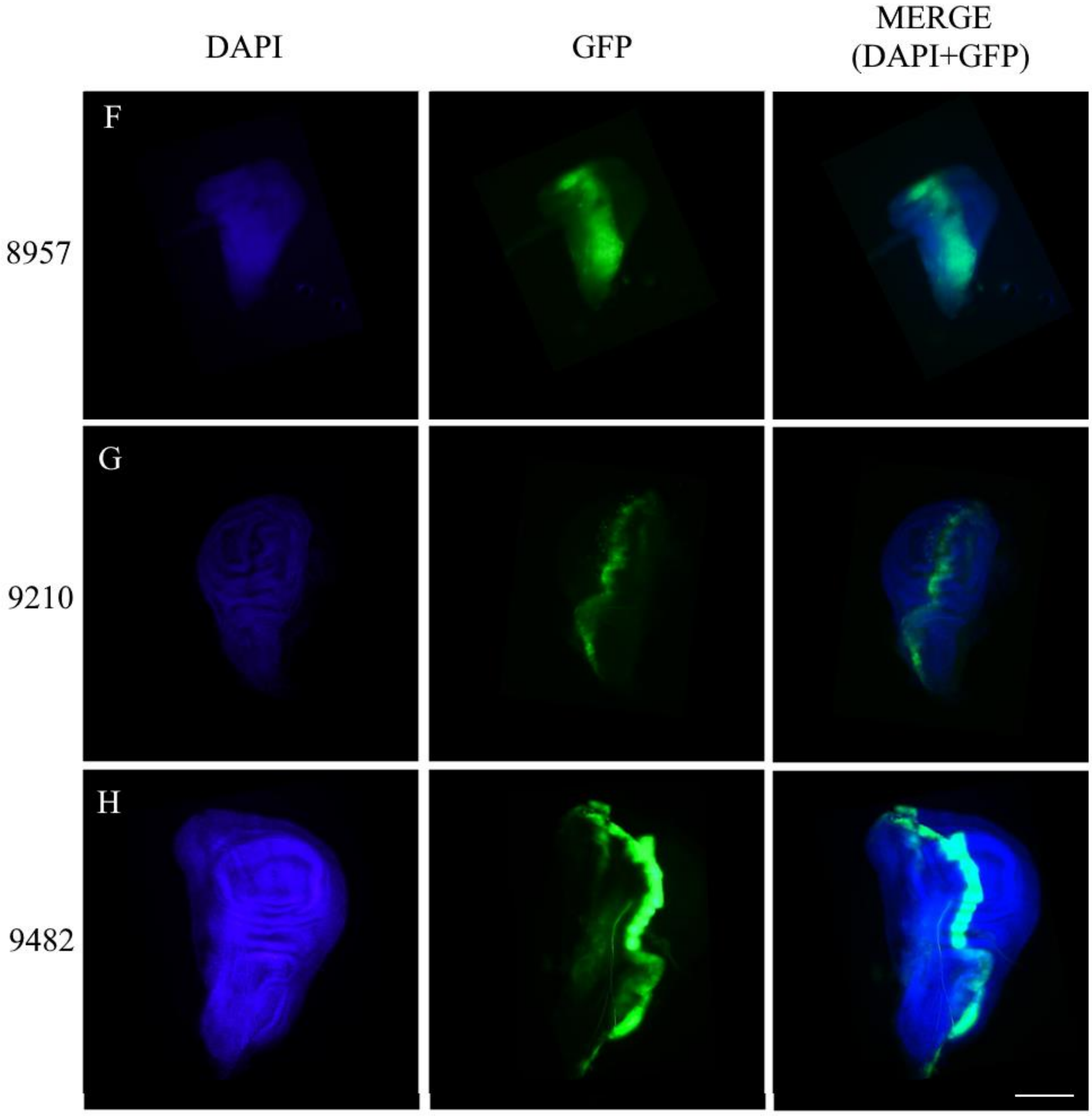


Figure 12. Primary screening results for 4D/3D protocol. +/+ (A) and +/- (B) are used as controls. Dfs 94971 (C), 27580 (E), 9210 (G), 9482 (H), and 8957 (F) exhibit no overgrowth phenotype. Dfs 25075 (D) and display neoplasia phenotype with strong overgrowth. Each column shows DAPI, GFP, and merged channels (DAPI+GFP) from left to right. Scale bar is 100 um.

Overall, the 4D/3D protocol revealed minimal overgrowth, with even the +/+ control exhibiting no tumor (Figure 11). Consequently, the results were deemed unreliable, as there may be other Dfs expected to display overgrowth but remain undetected due to the inaccuracies in the positive control. Nonetheless, one line (Df 25075) demonstrated sufficient overgrowth to warrant consideration for secondary screening. Additional lines, such as Dfs

94971, 27580, and 8957, should undergo retesting with an improved protocol, given their presentation of wings with some overgrowth phenotype.

3.4 DISCUSSION

Lines that are deficient of genes of interest were screened to understand which of them are involved in maintenance of ABP and epithelial cell homeostasis when interacting with Scribble (Fischbach, 2022). This method was required to better understand which genes are involved in mechanisms responsible for cell communication and epithelial morphogenesis (Huang et al., 2023). These approaches are based on the hypothesis that there is a synergy between loss of Scribble and other components for progression of neoplasia. Two protocols were looked at during this thesis. The key difference was in the amount of days the fly offspring were left at the 18°C incubator after the egg laying. In one protocol it was 3 days, in the other it was 4 days. In both protocols issues with controls occurred.

When looking at the 3D/3D protocol it was clear that all results show severe neoplasia. The problem with these results was the fact that also +/- control shows overgrowth. This should not be so as the stock contains two copies of the *scrib* gene. Since the overgrowth in +/- control was present, the results were false and there was no way to determine if existing results of Dfs are viable. These lines should be looked over again, when a working protocol has been established. In 3D/3D protocol two lines were promising candidates since their phenotype was more severe than the control line's. Line 25006 has many deleted genes including Tpi that is involved in ATP production. The other candidate for further screening is line 24971. This line has a deleted gene tRNA:Lys-TTT-2-4 that is predicted to be involved in translation, among many other deleted genes.

When looking at the 4D/3D protocol the opposite problem occurred. It was clear that most of the results showed no overgrowth. This includes +/+ control. This control only consists of one copy of *scrib* gene and should show overgrowth. Because of this reason the rest of the results may be false as well. In this protocol Dfs 25011 and 26529 showed no GFP at all therefore they could not be included in the results graph. However, even with this protocol showing a tendency to have no overgrowth, one Df 25075 showed overgrowth. This line can be considered as a good candidate for further screening. One of the genes deleted in this stock is Bub3 that encodes a protein that functions in the spindle assembly checkpoint (SAC) pathway. This gene is involved in cell mitosis. Another gene deleted from this region is CAP-D2, which plays a crucial role in shaping mitotic chromosomes. It could be a contributing factor for neoplasia, since well executed mitosis is important for tissue homeostasis. It is also important to note that lines such as 94971, 27580 and 8957 showed some type of overgrowth (Figure 12 C,E,F) and are worth trying to look into after a working protocol has been established. Another interesting deficiency line is 9482. This line has been used in both protocols. The results enhance the problem of both protocols showing overwhelming tendencies to either have no overgrowth or have a lot of it. In 3D/3D protocol this line has very diverse results (Figure 10I).

In further research a 3,5D/3D protocol should be constructed. First the control lines should be used to see if they show desirable results. After the controls have given viable results the candidate lines should be checked. It would also be useful to use all of the lines again with a working protocol to not miss any possible candidates for genes responsible for tissue homeostasis.

It would have been best to use the same controls for both protocols to minimize the different components of protocols that may contribute to the outcome. Another option would be to use both controls for both protocols. This would be more time consuming and take more resources but it would give a clearer understanding if the problem of false results lies in the days left after egg laying or in the control cross itself.

Further changes in the amount of days the larvae are left in the 29°C incubator can be made to review if it has any impact to the neoplasias to the wing imaginal disc. And changes in temperature for the first days could be changed to either lower at 16°C or higher at 19°C to further research the possible outcomes of different factors contributing to the experiment.

Further research includes looking into the candidates using secondary screening which looks at individual genes within the region of deleted genes in the specific Df. If the wing imaginal disc displays neoplasia in secondary screening then this gene could work with Scribble and influence the homeostasis of epithelial cells. Looking at the gene’s function within the cell hypothesis could be made about this function's influence on the intracellular mechanisms.

SUMMARY

Overall, this work showed that neither the 3D/3D or the 4D/3D protocol are optimal for screening. The 3D/3D protocol showed suspiciously too much of overgrowth and the 4D/3D too little. New protocol should be constructed with changed time or temperature conditions to later establish a working protocol.

A strong candidate for further screening is Df 25075. It was screened using 4D/3D protocol and showed IV category overgrowth. It has a deleted gene that is used in spindle assembly checkpoint during mitosis that would be a good candidate gene to research.

Other possible candidates from 4D/3D protocol include Dfs 94971, 27580 and 8957. The lines show some wings with different stages of overgrowth. But since the protocol is not completely viable and there are a lot of wings in each line with no overgrowth, these can not be considered as strong candidates.

All lines from 3D/3D protocol showed overgrowth, but two lines - 25006 and 24971 – were considered as promising candidates for further screening since their phenotype was more severe compared to the control.

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