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Analysis of intercellular network that regulates apicobasal polarity of epithelial cells

Bachelor's thesis
12 EAP
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INFOLEHT

Epiteelirakkude apikobasaalset polaarisatsiooni reguleerivate rakkudevaheliste võrgustiku analüüs

Antud bakalaureusetöös uuritakse kuidas on rakkude polaarsus reguleeritud läbi rakk-rakk kommunikatsiooni. Kasutades meetodit, kus rakkude apikobasaalse polarisatsiooni eest vastutav võtmekomponent Scribble on konditsionaalselt alla surutud, seati antud töö eesmärgiks leida uusi potentsiaalseid geene, mis koostöös Scribble valguga reguleerivad raku polaarsust ja homeostaasi ning olulised neoplaasia formeerumisel. Eksperimentaalosa on jaotatud kahte ossa. Esiteks selgitati välja katsetingimused, kasutades konditsionaalset RNAi meetodit, järgnevaks sõeluuringuks. Teiseks püüti leida uusi polarisatsiooni eest vastutavaid geeni kandidaate läbi süstemaatilise sõeluuringu, kombineerides konditsionaalset RNAi ja Dfs äädikakärbse tüvesid, kus teatud geenid kustutatud.

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

CERCS (B350): Biomeditsiin

Analysis of intercellular network that regulates apicobasal polarity of epithelial cells

This study examines the apicobasal polarity regulated, by cell-to-cell communication. By employing conditional knockdown of Scribble, a key apicobasal polarity determinant, in *Drosophila* wing imaginal disc, this study aims to identify novel genes that cooperate with Scribble to regulate cell polarity and tissue homeostasis. Experimental plan is divided into two parts. First, experimental protocols are tested for establishing screening. Conditional RNAi method is used. Second, to find out novel genes through systematic screening, small scale screening is attempted. A combination of conditional RNAi and Dfs stocks in which genes have been deleted are used. The main objective of the experiment is to identify a strong synergistic phenotype of neoplasia.

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

CERCS (B350): Biomeditsiin

1. TABLE OF CONTENT

2. ABBREVIATIONS

ABP – Apicobasal Polarity

AJ – Apical junction

AJC – Apical junction complex

AMOT – Angiomotin

AMOTL1, AMOTL2 – Angiomotin like 1, angiomotin like 2

AP – Anterior-posterior

Ap – Apterous

aPKC - Atypical protein kinase C

ATS – After temperature shift

CCNE1 – Cyclin E1

CRB3-Pals1-PATJ - Crumbs Cell Polarity Complex Component 3- Protein Associated With LIN7 1- Pals1-associated tight junction

DIAP1 - Death-associated inhibitor of apoptosis 1

dKD – Double knockdown

Dlg – Disc large

Dpp morphogen – Decapentaplegic morphogen

DV – Dorsal-ventral

EGF – Epidermal growth factor

EGFR signaling - Epidermal growth factor receptor signaling

EMT - Epithelial-mesenchymal transition

ERGF-Ras - Epidermal growth factor receptor-Rat sarcoma virus

Ex – Expanded

FERM domain – 4.1 protein erzin radixin moesin

FMB – FERM binding motif

G1/S transition – Transition from G1 phase to S-phase (cell cycle)

GFP – Green fluorescent protein

GPCR – G-protein-coupled receptor

GTPase – Enzyme that bind to the nucleotide guanosine triphosphate (GTP)

GUK - Guanylate kinases

Iro-C - Iroquois complex

KD – Knockdown

L1 – First instar larval

L2 – Second instar larval

L3 – Third instar larval

LAP family – Leucyl aminopeptidase family

Lgl – Lethal giant larvae

LRR domain – Leucine-rich repeat domain

MAGUK – Membrane-associated guanylate kinases

MAPK - Extracellular signal regulated kinase

MEK - Mitogen-activated protein kinase

Mer – Merlin

NDR - nuclear Dbf-2-related

PAR-1, PAR-2… – Protease-activated receptor-1, 2…

PCR – Protein-coupled receptors

PDZ domain - Post synaptic density protein, Dlg1, *zonula occludens*-1

PKC - Protein kinase C

PTPN14 - Protein Tyrosine Phosphatase Non-Receptor

RING finger domain - Really Interesting New Gene finger domain

Scrib – Scribble

$Scrib^{FL}$ – Full-length Scrib

SH3 domain - SRC Homology 3

SJ – Septate Junction

TAZ - Transcription Adaptor putative Zinc finger

TJ – Tight junction

UAS – Upstream activating sequence

WD40 repeat - Tryptophan-aspartic acid (W-D) dipeptide, 40 amino acids.

Wg expression – Wingless expression

WW domain – Tryptophan (W) domain

YAP - Yes-associated *protein*

Yki – Yorkie

ZA – *zonula adherens*

ZO complex - *zonula occludens*

3. INTRODUCTION

Drosophila melanogaster or the fruit fly has been one of the best model organisms for many years to study various physiological, biological, molecular and intracellular processes. Research uses the various developmental stages of *Drosophila* to better understand and understand the developmental processes of an organism. To study the structural features of tissues, the imaginal disc of the larval wing is often used, one of the imaginal discs.

The use of imaginal discs in research has been served as an excellent model for understanding developmental processes, tissue growth and regeneration, intercellular signaling pathways that regulate growth, cell polarities and morphogenesis (Tripathi & Irvine, 2022).

Through the study of the structure of imaginal discs and epithelial cells, it was found that the regulation of epithelial cells is a complex process. Epithelial cells exhibit apical-basal polarity (ABP), planar polarity. Among them, the apical-basal polarity and its components are responsible for the correct shape of the cells and tissues, support the correct development of the organ, its function and homeostasis (Buckley & Johnson). The ABP is maintained by apical domain and a basolateral domain. Important regulatory components of ABP are the Par and Crumbs protein complexes in the apical domain and the Scribble protein complex in the basal domain. This study focuses on the Scribble complex, as loss of this complex, leads to tissue disorganization, disruption of intercellular contact, impaired control of tissue growth, and also tissue neoplasia (Bilder, 2004; Stephens et al., 2018; Zeitler et al., 2004).

This study aims to identify novel genes that are involved in regulating ABP through intercellular communication by combining conditional *scribble* RNAi with Deficiency lines of 3R chromosome in *Drosophila*. The goal is to establish a candidate for further gene research by finding a strong tumor phenotype. The absence of the gene will help to understand that without this gene, the preservation of ABP and, accordingly, the tissue of the imaginal disc is impossible. Identification of a strong tumor phenotype will help to make further screening of genes and determine their area of influence, which will help to better understand the intercellular interaction and the effect of this on ABP.

4. LITERATURE REVIEW

4. 1. *Drosophila melanogaster* as a model

Drosophila melanogaster, commonly called fruit fly, is an arthropod, a dipteran (member of an order of insects containing the two-winged or so-called true flies) insect, belonging to the family *Drosophilidae* (Krimbas, C.B. & Loukas, M. 1980). It is 3 mm long and is valuable organism in experimental biology; a cheap model for student projects. Additionally, they are easy to obtain and maintain in laboratories.

The life cycle of *Drosophila melanogaster* is short, and it is easy to grow a large number of individuals for genetic, biochemical, and molecular analyses. Fruit flies are usually cultured at 25˚C or 18˚C. The developmental period from fertilized egg to adult fly is usually 10 days, and the maximum lifetime is 60 to 80 days (it is dependent on the culture conditions). *Drosophila melanogaster* have four development stages: egg/embryo, larval, pupal and adult stage (Ashburner & Thompson, 1978).

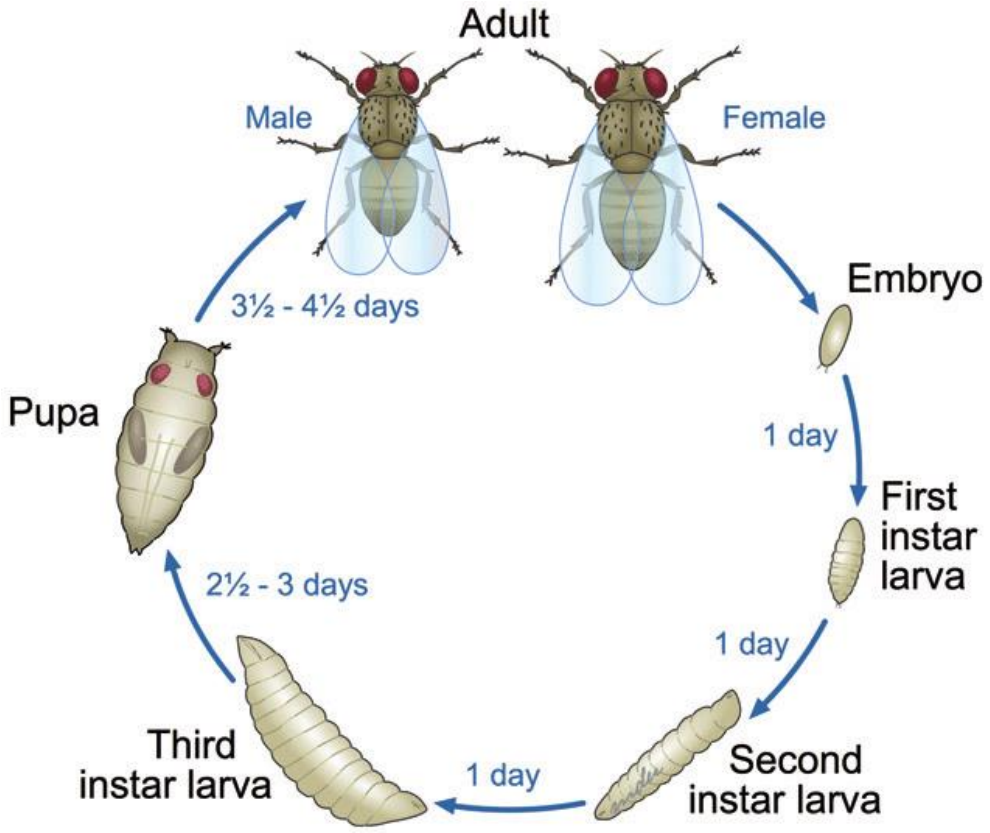

Figure 1. *Drosophila* melanogaster lifecycle. The whole life cycle of the fruit fly is relatively rapid and takes approximately 10-12 days at 25 ℃. The development is divided into stages: embryonic, larval (first instar, second instar and third instar), pupal and adult stage. (Ong et al., 2014).

4. 2. Larval stage

The next developmental stage after embryogenesis is larval stage. The larval stage splits into three instar stages: the first (L1), second (L2) and third (L3) instar larvals respectively (Figure 1; Tennessen & Thummel, 2011). The first and second instar larval stages last one day each, whereas the L3 stage lasts two days (Tennessen & Thummel, 2011). The late third larval instar stage is easy to recognize, because the larval climbs away from the food and gets ready to pupate (Figure 1; Tennessen & Thummel, 2011). It is characteristic that the larvae, unlike the adult, instead of organs, have the rudiments of organs, or in other words, imaginal discs. The larval of *Drosophila* has 19 imaginal discs (Marren & Mabey, 2010).

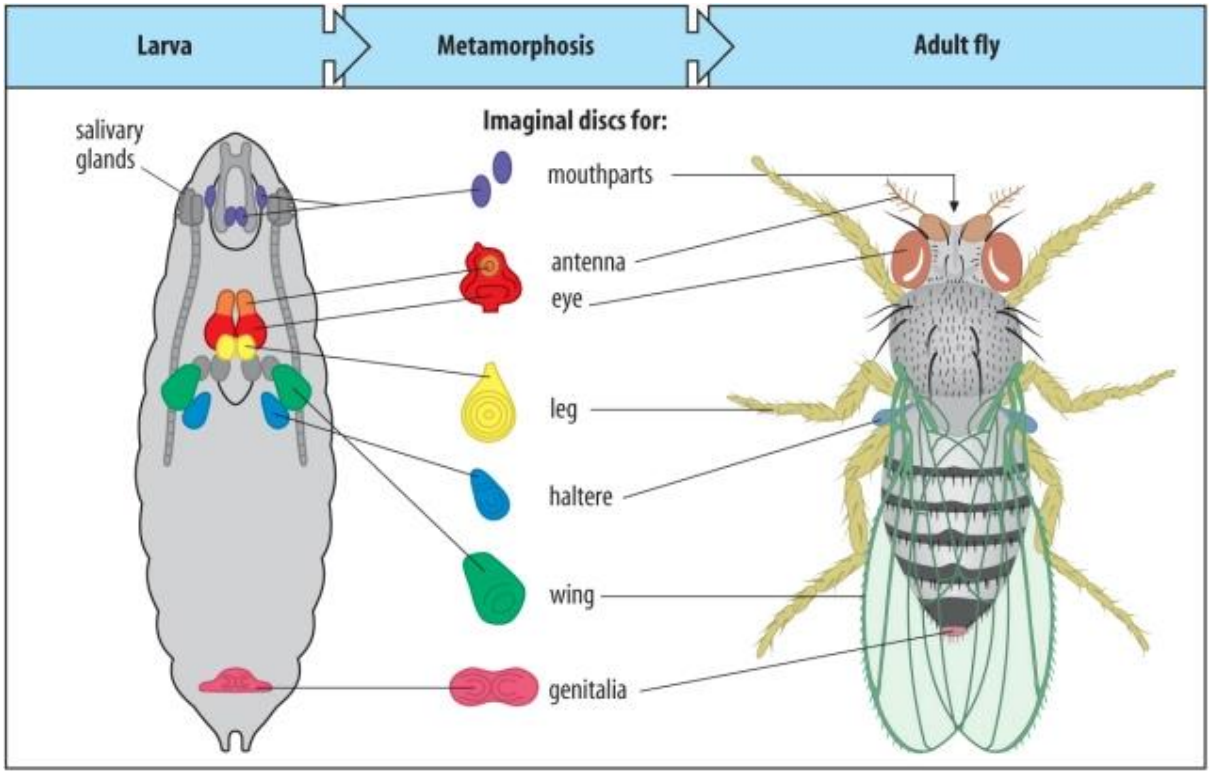

Figure 2. Anatomical structures of the larval compared to the structures with the adult fruit fly. Epithelial cells of the imaginal discs give rise to the adult external structures during metamorphosis (Adapted by https://sociogenomics.wordpress.com/2012/06/10/symmetry-and-evolution-a-genomic-antagonism-approach/).

4.3. Imaginal wing disc

The name imaginal disc comes from the Latin word *imago*, which means image and is used to refer to the mature stage of insects in entomology, a branch of insect zoology (From https://www.oxfordlearnersdictionaries.com/definition/english/imago?q=imago). While on their own, imaginal discs have a disc-like structure, from which the outer cuticular structures later develops (Marren & Mabey, 2010). The imaginal disc can be characterized as a sac-like epithelial structure within the insect larva that undergoes metamorphosis as it develops. Imaginal discs give rise to head, thoracic, limb, and genital structures (Figure 2).

During the first instar (L1) and second instar (L2) larval stages, it is important for the discs to take the correct shape, produce a sufficient number of cells, and establish compartment boundaries (Kumar, 2010; Kumar, 2011). In the third instar larval stage (L3), the cells of the imaginal discs continue to divide, and as the cells accept their terminal fate, the discs continue to form (Kumar, 2011). Most larval tissues are polyploid, that is, they have more than one pair of homologous chromosomes, while the cells of the imaginal disc are diploid, which promotes growth by increasing the number of cells (Tripathi & Irvine, 2022).

Drosophila has two imaginal wing discs, from which structures such as the wing hinge and the wing itself are formed, as well as the dorsal half of the body wall (T2, mesothorax), which is the main component of the thorax (Aldaz et al., 2010). The second segment is the posterior thorax, notum, part of the sides, and pleura (Aldaz et al., 2010).

At an early stage, the imaginal wing disc appears as a flat sac composed of cuboidal epithelial cells and whose apical sides face the lumen (Tripathi & Irvine, 2022). As the cell disc grows, differences occur between cells in morphogenesis, with cells on one side of the wing flattening out to form thin squamous epithelium, or in other words, peripodial epithelium (PE) (Auerbach 1936; McClure & Schubiger 2005, Tripathi & Irvine, 2022).

The physical connection of the epithelial cells of the imaginal wing disc is through intercellular junctions near their apical side (Tripathi & Irvine, 2022). Mechanical adhesion between cells and regulation of the apical shape of cells is provided by adherens junctions connected to the actin cytoskeleton (Farhadifar et al., 2007). Apical cells differ from each other in shape and size in the wing disc, which means that cells have different mechanics and behavior depending on location (Aegerter-Wilmsen et al., 2012; Legoff et al., 2013; Mao et al., 2013 ; Pan et al., 2018; Dye et al., 2021). Septate junctions are junctions basal to adherent junctions (Tripathi & Irvine, 2022). Their function is to form a paracellular diffusion barrier between the apical and basal surfaces (Tepass et al., 2001). The marginal zone, which contains transmembrane proteins

necessary for the regulation of polarity and intercellular signaling, is located apically with respect to adhesive junctions (Tepass 2012; Thompson 2013).

Disc development proceeds according to its subdivision into AP and DV regions (compartments) (Tripathi & Irvine, 2022). The formation of compartment boundaries occurs when the expression of genes that determine positional identity is hereditarily controlled.

As the dorsal expression of the transcription factor Apterous (Ap) (Cohen et al., 1992; Diaz-Benjumea & Cohen 1993; Blair et al., 1994) occurs in the second larval stage, separate dorsal and ventral compartments appear at the wing disc (Garcia-Bellido et al. 1976). Dorsal-specific expression of Ap is enhanced by EGFR signaling during L2 (Wang et al., 2000; Zecca & Struhl 2002b). Prior to L2, Ap expression is inhibited by Iro-C expression, but due to the fact that during L2 Ap expression occurs in a wider domain, whose borders cover the dorsal half as well as the notal region, the influence of Iro-C ceases (Wang et al., 2000 ; Zecca and Struhl 2002b). This is hypothesized to be because the dependence of α-transcription on EGFR is only transient and further dependent on autoregulation and maintenance of chromatin state (Zecca & Struhl 2002b; Oktaba et al., 2008; Bieli et al., 2015), while Iro-C continues to require EGFR signaling as the wing disc grows (Zecca & Struhl 2002a; Rafel & Milan 2008).

The above compartments are regulated by Wg and Dpp morphogens. A morphogen is a signaling molecule that affects cells by inducing specific cellular responses depending on its local concentration. As a result of the action of morphogens, the nature of tissue development in the process of morphogenesis is determined.

Dpp is a classic and key morphogen involved in the development of *Drosophila melanogaster* and is essential for proper patterning, early development of the embryo, as well as 15 imaginal discs, including wing discs. The function of Dpp is to regulate the expression of genes that are active in broad domains that surround the Dpp transcription band (Tripathi & Irvine, 2022).

Wg expression can be observed in the imaginal disc of the third larval stage, when the notum, hinge, blade, and margin can already be identified using molecular markers (Swarup & Verheyen, 2012). Localization of Wg usually occurs in the domains of the annular hinge region along the border of the DV compartment that separates the wing lobes, as well as in a wide strip in the dorsal part of the disc. During L2, *wg* is expressed in the ventral region of the wing imaginal disc and the wing field is determined (Simcox et al., 1996; Wang et al., 2000).is expressed in the dorsal part of the imaginal disc of the wing *vein* , which contributes to the determination of the notum (Simcox et al., 1996; Wang et al., 2000). Expression and correct expression of *vein* in the wing field is very important, as loss of expression leads to loss of notal structures, and incorrect expression in the wing field stops further wing formation (Baonza et al., 2000; Wang et al., 2000). Wg and EGFR are antagonistic to each other as Wg represses

vein expression in the dorsal region of the disc (Baonza et al. 2000) and vice versa, EGFR represses Wg expression in the dorsal region to limit Wg expression only in the ventral region (Baonza et al., 2000 ; Wang et al., 2000). As a result of this confrontation between Wg and EGFR, the wing disc is divided into wing regions and notum, thus explaining why the wing transforms into notum, although the function of Wg is absent (Swarup & Verheyen, 2012).

4.4. Apicobasal polarity

Cellular polarity is a fundamental feature of many cell types, describing the asymmetric distribution of its components within the cell (2).

Epithelial tissues that have arisen during the evolution of animals are capable of forming various forms, dividing the body into various physiological sections. Epithelial tissues are composed of epithelial cells whose plasma membrane is divided into domains. Domains, in turn, play an important role in the organization and physiology of the cell. They are subdivided into the apical - facing the external environment and the basolateral domain, which is in contact with the intercellular space of the body, these two domains also differ in the composition of proteins and lipids (Hutterer et al., 2004). The domains themselves are separated by a peripheral junction complex (CJC) that binds adjacent epithelial cells together and thereby forms a semi-permeable barrier to diffusion of solutes through the extracellular space (Farquhar & Palade, 1963).

The main difference between epithelial cells and other polarized cells is that epithelial cells form a series of specialized cellular connections with neighbouring cells that organize the epithelium and perform its functions as a paracellular barrier. These intercellular junctions are located along the lateral sides of the epithelial cells, thus complicating the formation of the apical-basal cell pattern, which includes four different cortical domains: apical, tight junction, commissural junction, and basolateral domain (St Johnston & Ahringer, 2010).

The importance of cell polarity in epithelial tissues lays in the fact that losing it leads to tissue disorganization, which subsequently causes cancer in humans (Royer & Lu, 2011). However, it remains unknown whether the loss of cell polarity is a cause or a consequence of cancer, despite the well-studied tumor function of the complexes in *D.melanogaster*. There is a theory that epithelial cell polarity may suppress tumors in mammals by participating in the installation and maintenance of the three-dimensional organization of epithelial cells. This theory is supported by two facts: first, polarity proteins are cellular targets for oncogenes, and second, tumor suppressors regulate polarity pathways (Royer & Lu, 2011).

The apico-basal polarity has two functionally important roles, one of which is to regulate asymmetric cell division and the other to support the apical junction complex (AJC) (Royer & Lu, 2011).

The polarity mechanisms of the main epithelial cells are capable of both preventing tumor initiation and blocking its metastasis and malignancy due to its association with AJC. AJC contains complexes of tight and adhesive junctions, and its structure depends on the integrity of the complexes of apical and basolateral polarity. In the later stages of oncogenesis, a key

component of adhesions, E-cadherin, is lost, which may contribute to the epithelial-mesenchymal transition (EMT), which is a critical step in metastasis.

The process of embryogenesis in Drosophila is well understood and helps to represent the processes of epithelial polarity establishment (Knust & Bossinger, 2002; Müller, 2000; Tepass et al., 2001. In the embryonic ectoderm of fruit fly, cells are formed during cellularization. Cellularization is the process by which an individual cell membrane is created for each nucleus (Knust & Bossinger, 2002). During this process, dotted adhesive junctions are formed along the lateral cell cortex and are marked by the beta-catenin homolog Armadillo. Once the cellularization process is completed, the adhesive junctions fuse and form the *zonula adherens* (ZA), a narrow adhesive band that surrounds the apical part of the cell. Further, a second junction, the so-called septal junction, is formed just basal to ZA, which then forms a barrier that will control diffusion through the intercellular space (Hutterer et al., 2004).

The embryonic epidermis secretes the larval cuticle, which is secreted exclusively from the apical surface, so characteristic morphological defects are observed in the mutants. By classical mutation screening (Nüsslein-Volhard et al., 1984) , the following genes were identified: *bazooka*, *crushes* and *stardusts*. These are the three main genes that play an important role in establishing epithelial polarity. (Hutterer et al., 2004). *Stardust* encodes a guanylate kinase that is membrane bound and conserved in the short intracellular Crumbs domain (Bachmann et al., 2001, Hong et al., 2001.).

4.5 Regulation of apico-basal polarity

The regulation of cellular polarity also plays an important role in ensuring the normal functioning of the cell, while cells, at the same time, use various mechanisms to ensure the abundance and activity of the polarity determinants. Basically, kinases and phosphatases regulate the localization and activity of polarity proteins (Hong, 2018; Schuhmacher et al., 2019; Wu & Griffin, 2017). Although there have been studies that have been able to characterize the molecular pathways that mediate proliferation and survival after oncogenic signaling (Hanahan & Weinberg, 2000), the mechanisms that oncogenes use to deregulate tissue organization during transformation, as well as the contribution of cellular organization and polarity (Aranda et al., 2018).

Polarity regulation is an extensive network of various signals, but the role of these signals in oncogenesis remains unknown (Bilder et al. 2003; Goldstein & Macara, 2007; Macara, 2004; Martin-Belmonte & Mostov, 2008a). The reason why the role of signals remains unknown is the problem of finding a suitable model, or sometimes lack thereof, for recreating and subsequently studying the structures *in vitro* (Aranda et al., 2018). The methods that are used traditionally, unfortunately, cannot recreate the complex interactions that can be observed in the three-dimensional space of a complex organ, moreover, cancer cell lines are not able to maintain the structural and functional properties of the organ from which they originated (Aranda et al., 2018). However, methods already exist that can recreate and show oncogenic signaling modulated by epithelial organization, and in due course oncogenes can show disorganization (Debnath et al., 2003; Hebner et al., 2008; Muthuswamy et al., 2001; Underwood et al., 2006; Weaver et al., 1996; Xiang & Muthuswamy, 2006). These studies provide a conceptual framework and experimental tools that could be used to explore molecular pathways that deregulate cellular organization (Aranda et al., 2018).

There is a single molecular mechanism, common to all the different types of polarization and well conserved among species, that creates and maintains polarity. In the case of apicobasal polarity, due to the three protein complexes, which act in concert, interact with each other, as well as with structural components of the cytoskeleton and intercellular junctions, thereby providing polarity (Assémat et al., 2008; Dow & Humber, 2007; Etienne- Manneville & Hall, 2003b). For example, the Scribble, Par, and Crumbs protein complexes play an important role in defining the basolateral and apical domains, as well as the apicolateral border, however, some proteins in these complexes have shown themselves in oncogenesis, thereby identifying themselves as tumor suppressors. (Aranda et al., 2018; Bilder, 2004; Lee & Vasioukhin, 2008).

4.6. The regulation complexes of apico-basal polarity

Three main protein complexes Par, Crumbs and Scribble regulate the apicobasal polarity of epithelial cells. Par complex and Crumbs complex locate in apical domain and Scribble complex is based in basal polarity. The regulation is based on antagonistic influence of these three protein complexes to each other.

4.6.1. Par complex

Par proteins - from the English “partitioning defective”, were first identified in nematode *Chaenorhabits elegans* as mutant proteins, because they were involved in a defect in the anterior-posterior (anterior-posterior) separation of proteins in the early embryo (Kemphues et al., 1988) . Analysis of the par genes showed that proteins differ structurally and functionally: scaffold/adapter proteins PAR-3 and PAR-6 with several domains of protein-protein interaction, serine/threonine kinases PAR-1, PAR-4, PAR-2 protein containing the RING finger domain,typical of ubiquitin ligases and a member of the 14-3-3 PAR-5 signaling protein family (Suzuki & Ohno, 2006; Goldstein & Macara, 2007). Using the generation of antibodies to each PAR protein, the localization of each protein in the *C. elegans* embryo at the unicellular stage was revealed. PAR-3 and PAR-6 are located in the anterior cortex, PAR-1 and PAR-2 are located in the posterior cortex, and PAR-4 and PAR-5 are evenly distributed in the cytoplasm (Rose & Kemphues, 1998). Also, PAR proteins have their own subcellular hierarchy of localization - a polarity signal is given, i.e. entry of the sperm into the egg, in response to which the PAR-3 and PAR-6 proteins begin to localize at the anterior (anterior) pole, and the localization of PAR-1 and PAR-2 is directed to the posterior pole, but at the same time, reverse signals from PAR -1 and PAR-2 make localization of PAR-3 and PAR-6 limited (Ebnet et al., 2008). At the moment, it is known that due to the relationship between the anterior and posterior PAR proteins, asymmetric membrane domains are formed along the anterior-posterior axis. In addition to the PAR-2 protein, all other PAR proteins are also present in *Drosophila*, which makes this mechanism conserved and provides insight into the formation of distinct membrane domains in *Drosophila* epithelial cells (Guo and Kemphues, 1996).

The PAR-aPKC system is a common polarity mechanism since it has been found that PKC-3 found in *C. elegans* and responsible for establishing asymmetry in the single cell embryonic stage by binding and interacting PAR-3 PAR-6 has an aPKC orthologue that is found in *D. melanogaster.*

As mentioned earlier, epithelial cells have two domains - a basal one that contacts other cells and an apical domain that does not contact cells and faces the lumen of the organ. Posterior

pole proteins are locked in the basal domain (PAR-1), while anterior pole proteins such as aPKC, PAR-3, and PAR-6 are located in the apical domain.

Tight junctions (TJs) contain a variety of peripheral proteins that are divided into scaffold/adapter proteins, regulatory proteins (GTPases, G-proteins, kinases and phosphatases), and transcription factors that regulate RNA processing (Ebnet et al., 2008). Scaffold proteins interacting with transmembrane proteins as a result form multiprotein complexes. Examples of scaffold proteins are the ZO complex (ZO-1 - ZO-2 - ZO-3), the CRB3-Pals1-PATJ complex, and the PAR-3-aPKC-PAR-6 complex (Ebnet et al., 2008). The ZO complex binds transmembrane proteins to the cytoskeleton, ZO-1, ZO-2, ZO-3 all proteins of the ZO complex interact with F-actin, thereby forming the main link of the actin cytoskeleton in TJs (Ebnet et al., 2008). CRB3 is a *Drosophila* homologue of Crumbs (a transmembrane protein), while Pals1 and PATJ are cytoplasmic scaffold proteins. In vertebrates, CRB3 in epithelial cells is based on the apical membrane, but is more concentrated in TJs. where it interacts directly with Pals, which in turn is connected to PATJ (Shin et al., 2006). The Crumbs homologue in *Drosophila* is a determinant (in genetics, a hypothetical germplasm unit that controls the development of a well-defined tissue or organ) in the apical membrane and its overexpression leads to an increase in the sphere of influence of the apical membrane domain (Wodarz et al., 1995).

4.6.2. Crumbs complex

Another complex that is responsible for the apico-basal polarity is the Crumbs complex. Along with the Par complex, the Crumbs complex is located in the apical domain. The complex in Drosophila is formed from four main proteins: Crumbs, Stardust (Pals1 homologue), Patj (tight junction protein associated with PALS1), and Lin-7 (Bulgakova & Knust, 2009; Margolis, 2018). The Crumbs protein is a transmembrane protein that consists of two domains, a large extracellular and a small cytoplasmic domain (Bulgakova & Knust, 2009). The large extracellular domain contains 29 repeats like EGF (epidermal growth factor) and four repeats like the laminin-1 globular domain (Bulgakova & Knust, 2009). The small cytoplasmic domain consists of 37 amino acids (Bulgakova & Knust, 2009). The Patj protein consists of four PDZ domains and contains one L27 domain at the N-terminus (Pielage et al., 2003). Lin-7 is a short 195 amino acid protein that contains a PDZ domain at the C-terminus and L27 at the N-terminus (Bachmann et al., 2004). Crumbs and Stardust proteins are required during the embryonic stage to provide support for epithelial cell polarity (Bachmann et al., 2001; Hong et al., 2001; Tepass & Knust, 1990; Tepass & Knust, 1993).

4.6.3. Scribble complex

The next and also important protein complex that is involved in the regulation of apico-basal polarity is the Scribble complex. Along with Par and Crumbs, the Scribble complex was first identified in *Drosophila melanogaster*. The Scribble complex itself consists of Scrib (Scribble), Dlg (disc large), and Lgl (lethal giant larvae) proteins (Barreda et al., 2020). Each protein has its own name origin - Scrib got its name because the observed phenotype of the fruit fly mutant epithelium was disorganized, Dlg - the larvae showed excessive growth of imaginal discs and the last protein, Lgl got its name from overgrown larvae that were observed to be unable to stop proliferation and differentiation of larval tissues (Elsum et al., 2012). Subsequently, homologues of these proteins were also discovered in other organisms, from worms to humans. Scrib is a protein from the LAP family, which typically contain one to four PDZ domains, as well as 16 LRRs (leuchine-rich repeats) (Bryant & Huwe, 2000). Proteins from the LAP family provide control of cell shape, size, and subcellular localization of the protein (Bryant & Huwe, 2000). The Scrib protein has four PDZ domains, which in turn consist of 80-90 amino acids, which interact with each other through protein-protein interactions (Elsum et al., 2012). The PDZ and LRR domains ensure the efficient and correct operation of Scrib, which consists in the correct localization of the protein and its targeting to the membrane (Elsum et al., 2012). The LRR domain is still the most important, since it's loss leads to the complete loss of Scrib protein functions, and vice versa, being overexpressed in *Drosophila* Scrib mutant lacking PDZ domains, it can provide normal functioning similar to the wild type gene *in vivo* (Zeitler et al., 2004).

The Dlg protein originates from the MAGUKthat provide binding of membrane structures, adhesion, and signal transduction (Pan et al., 2004).family MAGUK characterized by the presence of PDZ, SH3 (Scr3 homolog), and GUK domains (Woods & Bryant, 1993). Although SH3 is not a catalytic domain, it binds substrates to enzymes, thereby controlling their enzymatic activity (Gonzalez-Marisca et al, 2000). GUK domains do not have an ATP binding site and its functioning ensures the relationship with the SH3 domain and proteins, which in turn are associated with microtubules or with the actin cytoskeleton (Elsum et al., 2012; Hanada et al., 2000; Bauer et al., 2010).

The Lgl protein consists of several WD-40 repeats and conserved phosphorylation sites (Elsum et al.,2012). At one time, WD-40s are structural motifs that consist of about 40 amino acids and usually end with a tryptophan–aspartic acid (WD) dipeptide (Neer et al., 1994). The functions of WD-40 include signaling, vesicle transport, cytoskeletal assembly, and also cell division (Smith et al., 1999).

The importance of scrib, dlg, and lgl is confirmed by the fact that mutations in Drosophila of these genes contribute to the development of tumors in epithelial tissues (Humbert et al., 2008).

This happens due to the loss of apicobasal polarity in cells, differentiation and proliferation (Humbert et al., 2008). From which it can be concluded that these three proteins act as tumor suppressors and provide regulation of tissue architecture (Humbert et al., 2008).

On *Drosophila scrib*, *lgl* and *dlg* mutants, it was shown that the formation of tumors in epithelial cells is caused by incorrect basolateral localization of apical proteins, the absence of a dense band (which is formed by adhesive junctions) necessary for the formation of adhesive zones, which in turn establish tight connections between epithelial cells and epithelial tissue architecture (Bilder & Perrimon, 2000; Bilder et al., 2000). Scrib, Dlg, and Lgl are also involved in Drosophila neuroblast differentiation and influence cell migration of Drosophila ovarian border cells (Zhao et al., 2008; Szafranski & Goode, 2007; Szafranski & Goode, 2004).

Scrib, Dlg, and Lgl are localized in the Drosophila epithelial cell cortex, at the basolateral junctions, basal to the adherent junctions (Bilder and Perrimon, 2000). The Scrib LRR domain plays an important role in plasma membrane localization, and the PDZ domain recruits Scrib to the junctional complex in Drosophila epithelial cells and neuroblasts (Yamanaka & Ohno, 2008; Zeitler et al., 2004; Albertson et al., 2004).

The regulation of the apicobasal polarity of cells is carried out mainly due to antagonistic mutual influences between Lgl and aPKC (Elsum et al.,2012). aPKC phosphorylates Lgl, thereby preventing the latter from localizing to the cortex and being active (Yamanaka & Ohno, 2008; Koppen et al., 2001; Wirtz-Peitz & Knoblich et al., 2006). Lgl binds to the Par complex and inhibits aPKC activity; knockdown of aPKC can eliminate defects in Drosophila scrib, lgl, and dlg mutants (Rolls et al., 2003; Grzeschik et al., 2010; Leong et al., 2009; Bilder et al., 2003).

In a study of Drosophila that had mutant sites in the developing eye, it was found that cyclin E (G1-S phase cell cycle regulator) and E2F1 (cell cycle transcription factor) are expressed thereby disrupting eye development in the area where cells exit cell cycle (Elsum et al.,2012). In addition, Diap1, an inhibitor of Drosophila apoptosis, is activated, blocking cell death during development (Grzeschik et al., 2010; Grzeschik et al., 2007). This increased activity of cyclin E, E2F1, and Diap1 is due to the fact that the Hippo pathway of negative control of tissue growth is inhibited (Elsum et al.,2012). The Hippo signaling pathway includes a kinase cascade that consists of the Hippo and Warts protein kinases, whose function is to phosphorylate and inactivate the transcriptional coactivator Yorkie (Halder & Johnson, 2011). Inactivation of Hippo signaling pathways occurs due to the loss of Lgl activity, which leads to increased expression of Yorkie targets such as CCNE1 (cyclin E), E2F1 and Diap1 - tissue growth regulator genes (Grzeschik et al., 2010; Grzeschik et al., 2010; Parsons et al., 2010). It should be noted that in case of loss or depletion of the remaining proteins of the Scribble complex -

Scrib and Dlg, Hippo signaling is not disturbed, but with the condition that the apicobasal cell polarity is not disturbed (Grzeschik et al., 2010; Doggett et al., 2011). However, Scrib depletion in Drosophila increases F-actin accumulation, which in turn negatively affects Hippo signaling by reducing it, as well as increasing tissue growth (Fernandez et al., 2011; Sansores-Garcia et al., 2011). If, nevertheless, the apical-basal polarity of the cells is disturbed and depletion of Scrib and Dlg is present, then this also inactivates the Hippo pathway, since the localization zones of the apical domain expand and the level of aPKC and Crumbs increases (Grzeschik et al., 2010;Grzeschik et al., 2010).

Scrib involvement has also been noted in the EGRF-Ras GTPase signaling pathway that controls cell proliferation and survival in Drosophila cells (Dow et al., 2008; Nagasaka et al., 2010). A kinase cascade consisting of Raf, MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular signal regulated kinase)] and MAPK mediates the EGRF-Ras signaling pathway (Kern et al., 2011).

4.7. Hippo pathway

All cells have fundamental biological processes such as cell differentiation, proliferation and apoptosis, i.e. cell death. Naturally, these processes need to be controlled and coordinated. During the development of the organism and when the organs and the body grow, the cells must produce the right number of cells for this, and differentiation must ensure the function of the developed organs (Yu & Guan, 2013) . Further, at an older age, tissues need to be renewed, due to the death of old cells and the division of new ones (Tomasetti et al., 2019). Apoptosis is necessary for cells so that cells with mutations, old cells, and damaged cells self-destruct and do not lead to the development of oncology or other processes that are detrimental to the body, and stem cells will replace them (Yu & Guan, 2013).

Recent studies have shown that the Hippo pathway is responsible for the regulation of many cellular processes. For example, the Hippo pathway promotes cell death and differentiation, and it can also inhibit cell proliferation (Di Cara et al., 2015). The Hippo pathway was first identified in Drosophila during a genetic mosaic screening of tumor suppressor genes (Yu & Guan, 2013). *hpo, sav* and *wts* are negative regulators of tissue growth, and inactivation of any of these genes will result in increased cell proliferation and suppressed cell death. All these genes encode different families of proteins, *hpo* encodes kinase proteins from the Ste20 family, *sav* encodes proteins that contain WW and coiled-coil domains and *wts* encodes kinase proteins from the NDR (nuclear Dbf-2-related) family (Huang et al., 2005).

Yorkie (Yki) is a transcriptional co-activator protein in the Hippo pathway. This is a downstream component through which three interconnected branches pass through which the upstream regulation of the path occurs. (Oh & Irvine, 2010). Yki is regulated by the previously mentioned suppressor proteins, since in some cases Yki can act as an oncogene and provoke uncontrolled tissue growth (Oh & Irvine, 2010).

The regulation of the Hippo pathway depends on intracellular processes. The Hippo pathway is regulated by apicobasal polarity, PCR mechanical cues and GPCR signaling and actin cytoskeleton.

There are two proteins that work together to regulate cell differentiation and proliferation, and also have tumor suppression functions, these are Merlin (Mer) and Expanded (Ex) (McCartney et al., 2000; Maitra et al., 2006). Both proteins originate from the FERM (4.1, Ezrin, Radxin, Moesin) domain-containing family (Yu & Guan, 2013). More recent studies have identified the Kibra protein (the protein contains the WW and C2 domains) that interacts with Mer and Ex and the three of them activate Wts (Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010). Mer, Ex and Kirba are localized in the apical domain of polarized epithelial cells

(Boedigheimer and Laughon 1993; Boedigheimer et al., 1997; Yu et al., 2010). The Mer and Ex proteins have the role of linkers for binding the apical plasma membrane and the actin cytoskeleton (Bretscher et al., 2002). The C2 domain of the Kirba protein binds to phospholipids and directs the Mer and Ex proteins to the cell surface (Kremerskothen et al., 2003).

The Drosophila Crumbs protein is localized in the apical domain, more precisely in a separate zone adjacent to ZA - zonula adherens (Bulgakova & Knust, 2009). Crumbs is a transmembrane protein whose short intracellular domain contains FMB (FERM binding motif). The interaction of FBM with Ex models the localization and stability of Ex, thereby regulating the activity of Hippo pathway kinases and Yki (Chen et al., 2010; Ling et al., 2010; Robinson et al. 2010).

The Par complex in the apical domain is also involved in the regulation of the Hippo pathway along with the Crumbs complex. If the Par component of the aPKC complex is overexpressed, this can trigger Yki activity and tissue overgrowth (Grzeschik et al., 2010; Sun & Irvine 2011) . That is, Yki will begin to behave like an oncogene. The Par complex can be counteracted by the basal Scribble complex, but if the Scrib or Lgl proteins are depleted, then Yki will be active (Grzeschik et al., 2010; Menendez et al., 2010; Sun & Irvine 2011).

The AMOT protein family found in mammalian cells and including the proteins AMOT, AMOLT1 and AMOLT2 interact with many TJ components, thereby maintaining TJ integrity and cell polarity (Wells et al., 2006). AMOT proteins and the Yki YAP/TAZ homologue in mammals interact regardless of the YAP/TAZ phosphorylation status and are therefore determined by PPxY motifs in AMOT and WW domains in YAP/TAZ (Chan et al., 2011; Wang et al., 2011; Zhao et al., 2011). AMOT proteins can inhibit YAP/TAZ activity either by insertion into the actin skeleton or TJ resulting in decreased nuclear localization and activity, or proteins can induce phosphorylation at Lats target sites (Zhao et al., 2011; Yu & Guan, 2013). So far, no orthologue of the AMOT protein family has been found in Drosophila, which suggests that the regulation of the Hippo pathway in mammals and Drosophila may be different (Yu & Guan, 2013).

AJ has two components that can regulate the Hippo pathway α-Catenin and PTPN14 (protein tyrosine phosphatase 14). Their difference lies in the fact that α-Catenin will interact with YAP if it is phosphorylated, while PTPN14 can interact with YAP directly using PPxY motifs on its part and WW domains on the YAP side (Huang et al., 2012; Liu et al., 2012; Wang et al., 2012; Schlegelmilch et al., 2011). In mammals and *Drosophila*, the organization of the N-terminal FERM domain is similar. The PTPN14 orthologue in *Drosophila* Pez has been shown to inactivate Yki through interaction with Kibra (Poernbacher et al., 2012).

Ajuba is another protein that has been found to modulate the Hippo pathway. It interacts with the Sav and Lats kinases, both in mammalian and Drosophila cells, thereby inhibiting the action of YAP/Yki (Das Thakur et al., 2010).

The hippo pathway is regulated by apico-basal polarity through the recruitment of pathway kinases to the apical domain, thereby either activating or inactivating Yki/YAP/TAZ at cellular junctions, which should eventually lead to Yki/YAP/TAZ inactivation to prevent oncogenesis (Yu & Guan, 2013).

4.8. The last findings of Scrib

The study of scrib alleles showed that the *scrib*^{5} provokes excessive proliferation in the wing imaginal disc, but the allele is able to maintain ABP, which means that the N-terminal domain of the LRR is enough for ABP, but only for maintenance, this is not enough to control growth (Khoury and Bilder, 2020; Zeitler et al., 2004). This study showed that if *scrib*^{5} are in contact with wild-type (WT) cells, then the *Drosophila* presence of Scrib5 protein (Gui et al., 2021). Conversely, if *scrib*^{5} are surrounded by the same cells, then excessive proliferation is observed in the imaginal wing discs, which is accompanied by Yki activation. To sum it up, *scrib*^{5} cannot independently maintain ABP in the cells (Gui et al., 2021), for this they need to be in contact with wild-type cells, which suggests that the regulation of ABP may still depend on intercellular signals. that influence the behavior of the cell in the tissue.

In addition, studies have shown that cells with full length Scrib (ScribFL) can regenerate ABP in *scrib*^{2}*/scrib*^{5} (Gui et al., 2021) and continue normal tissue growth (Gui et al., 2021).

The study also looked at what happens to ABP after scrib knockdown and before Gui epithelial cell overproliferation (Gui et al., 2021). It has now been proven that the initial loss of Scrib in flanking cells occurs independently of Yki activity (Gui et al., 2021). This was indicated by the fact that Wts co-expression in scrib KD cells restrained the overexpression of ex-lacZ, which led to the suppression of hyperplastic phenotypes (Gui et al., 2021).

Next, the molecular mechanisms responsible for intercellular regressive alignment of ABP were studied (Gui et al., 2021). It is known that epithelial cells communicate with each other through AJ (Pinheiro and Bellaïche, 2018) whose constituent components are DE-Cad and β-cat, which are associated with α-cat.

The interaction of α-cat with Scrib was also considered, as α-cat expression was significantly reduced in scrib KD and DE-Cad KD (Gui et al., 2021). The interaction is explained by the fact that α-cat is biochemically associated with the LRR and PDZ3/4 Scrib domains (Gui et al., 2021). It was also found that there is a genetic relationship between α-cat, SJ components and Scrib (Gui et al., 2021) to maintain regulation even in the absence of Scrib should be due to the fact that α-cat must be located in SJ. Answering the questions whether the decrease in Scrib mediated by the decrease in Scrib expression in dKD cells is autonomous or not, and which alignment of ABP (intercellular progressive from wild-type cells, or intercellular regressive from KD cells) is more dominant. It is believed that the intercellular progressive alignment of ABP is still dominant, since the preservation of ABP was most likely associated with the directive signal that WT cells give, just through intercellular progressive alignment (Gui et al., 2021).

5. EXPERIMENTAL PART

5.1 AIMS OF THE THESIS

The overall goal of the project is to identify and establish novel functions for genes located on the 3R chromosome of the fruit fly that could be related to ABP regulation and tissue homeostasis. Based on the previous findings (Gui et al., 2021). It is hypothesized that there are genes that cooperate with Scribble for ABP. The objectives of the thesis are as follow:

1. To carry out the screening of novel genes, experimental protocols are tested.
2. To find out novel genes through systematic screening, small scale screening is attempted.

For the experiment, the RNAi *in vivo* method was used to knock down genes (especially *scrib*) at the third instar larva stage in order to identify candidates with the neoplasis phenotype for further gene screening. Additional experiments performed by immunohistochemistry were needed in order to better understand the underlying molecular mechanisms associated with Scrib loss and ABP disruption.

5.2 MATERIALS AND METHODS

5.2.1 Establishing a protocol for screening

To establish a protocol for screening, this study first tested different conditions of conditional knockdown of scrib as previously described (Gui et al., 2021, PREPRINT). For information about deleted or partially deleted genes, see Table 1.

This includes to confirm whether loss of Scrib mediated tissue overproliferation occurs in a time dependent manner and a Scrib dosage dependent manner. The following crosses were used:

For the first part of the experiment to test experimental protocols:

- Scrib RNAi x ptc-Gal4>GFP, ex-lacZ ; Gal 80ts

For the second part to test one copy scrib background and two copies scrib

- One copy scrib background
    - 7633 Df (3R) x ptc-Gal4>GFP, ex-lacZ ; scribRNAi-Gal80ts
    - 7638 Df (3R) x ptc-Gal4>GFP, ex-lacZ ; scribRNAi-Gal80ts
    - 7675 Df (3R) x ptc-Gal4>GFP, ex-lacZ ; scribRNAi-Gal80ts
- Two copies scrib (wild type)
    - Oregon-R x ptc-Gal4>GFP, ex-lacZ ; scribRNAi-Gal80ts

All experimental parts had the same conditions for the procedure:

24 hours egg laying, 4 days room temperature, 2 days / 5 days 29°C, dissection

Screening experiment flow

Flies were reared on standard medium and provided with dry yeast to promote egg laying. After crosses were set, the eggs were collected for 24 hours, the tubes were kept at room temperature for 4 days and shifted to 29°C for 2 days. Thereafter late third instar larval were collected. Then wing imaginal discs were dissected, fixed, stained, and prepared for imaging. Samples were dissected in batches of 3 deficiency lines x host stock crosses, together with a negative control each time to monitor any variations among the samples. The deficiency inclduing scrib allele (Df 8105) was not used as positive control (one copy of scrib), because of the stock lost while

stock expressing RNAi against Oregon-R was used as a control (two copies of scrib) to exclude the possibility of observed effects being from the RNAi itself.

Dissection

For each sample, around 10 large third instar larvae crawling on the sides of the tube were collected, controlled for a GFP potivity and transferred to a 2x2 well plate with PBS. The larvae were dissected one at a time under the stereo microscope, on a silicone disc covered with PBS. The posterior end of the larva was removed using forceps, removing all fat bodies. Using forceps, the larval is turned inside out (inverting method), the brains and other unnecessary structures are removed, thereby leaving discs attached to the head. The samples were transferred to an Eppendorf tube filled with cold PBS and kept on ice until fixation.

Fixation

3.7% paraformaldehyde (PFA) (Sigma-Aldrich) in PBS with 0.1 % triton (PBT) was used for fixation. The PBS in each tube containing the samples was removed and replaced with 900 µl PBT. In a fume hood, 100 µl of PFA (37% concentration) was then added to the tube. The samples were left 20 min at room temperature for fixation, then the formaldehyde solution was removed and the samples were washed 3 times with 1 ml PBT.

Staining

Primary screening samples were stained with DAPI. The PBT in the tube containing the samples was removed until 100 µl was left, and 2 µl of DAPI was added for a concentration of 1:50. Samples were incubated either at room temperature 30 minutes before removing DAPI and washing with PBT three times with 10 min incubation. This stage was used after secondary antibody staining for candidate samples where lacZ, Dlg and DE-Cad staining were used.

For candidate samples where lacZ, Dlg and DE-Cad staining were used, all the PBT was removed and replaced with a 400 µl blocking buffer. Blocking buffer was prepared with 5% goat serum (GS) (Sigma-Adrich) in PBT. The samples were left at room temperature for 2 hours, then the blocking buffer was removed. After 200 µl of blocking buffer was removed.

Primary anti-β-galactosidase mouse antibody was diluted in a blocking buffer at a concentration of 1:500, added to the samples and incubated at 4 °C overnight. In case primary anti-disc large-s mouse antibody and anti-DCAD2-s rat antibody were diluted in a blocking buffer at a

concentration 1:50, added to the samples and incubated at 4 °C overnight. The samples were washed 3 times with PBT, letting stand 10 min each time.

The secondary antibody, Alexa Fluor® 568 Goat anti-mouse IgG (Thermo Fisher Scientific) and Alexa Fluor® 568 Goat Anti-Rat IgG H&L (Thermo Fisher Scientific) , were diluted in PBT at a concentration of 1:200 for anti-β-galactosidase and 1:500 for other primary antibodies and added to the samples. The samples were kept away from light and left at room temperature for 2 hours, then the samples were washed 3 times with PBT, letting stand 10 min each time. After that was the DAPI stage with 30 minutes of incubation, washed three times. Samples were put on PBT and stored at 4°C away from light until mounting.

Mounting and Imaging

Microscope slides were prepared by adding two narrow stripes of tape 0.5-0.8 cm apart to make the sample area and support the cover slip to prevent it from crushing the samples. Between narrow stripes of tape was added 10 µl of 70% glycerol The head parts with wing discs were transferred using a brush on a silicon disc covered PBT for a removing wing disc from the head part. After removing, imaginal disc was transferred in 70% glycerol. The cover slip was added and sealed on all sides using transparent nail polish. Samples were stored at 4°C away from light until imaging. Samples were imaged using Olympus BX51 brightfield microscope with 20X dry objective.

Tabel 1. Fly stocks used for crossing with *scrib* RNAi and determination of strong neoplasia phenotype.

Completely deleted and partially deleted genes	Stock number	Inserted chromosome
CG8526, CG8534, CG9458, CG9459, CG9467, CG16904, CG12418_ eloF, FBXO11, Glut4EF, Teh1,	7633	3R
Ak3, art1, Cad86C, H2- CG4073, TTL15, CG4511, CG4565, CG4570, CG4596, Leash, CG6465, CG6567, CG6574, CG14683, CG14684, CG14687, Phyhd1, CG14689, CG14691, CG14692, CG14693, CG14694, CG14695, CG14696, CG31272, CG31278, CG31373, CG31391, CG31467, CG42394, CG42633, desi, fau, fdh, hth, Mcm5, MED7, mRpL37, pug, Rbp1, SelR, Skeletor, Sodh-2, TkR86C, TfIIFbeta, tomboy20, tpc1, Tsp86D, CG45076, CG45078, asRNA:CR44018, lncRNA:CR44230, asRNA:CR45015, asRNA:CR45016, asRNA:CR45195	7638	3R
GluProRS, AP-1sigma, CG5854, Ndc1, CG5902, CG12268, CG13599, CG13601, CG43998, CG13603, Epp, CG31140, CG31141, CG31142, CG33108, lncRNA:CR31451, gdh, KrT95D, LSm3, Rpt2, Rab7, RpS19b, sba, CG43999, mir-9381, asRNA:CR46092	7675	3R

This table lists those genes and gene regions that are deleted or partially deleted. In the future, after establishing a strong tumor phenotype, knowing which genes are deleted, it will be possible to screen for their mutations. All information about gene and stocks is possible to find https://bdsc.indiana.edu/index.html and https://flybase.org/.

5.2.2 RNAi screening and immunohistochemestry

The GAL4/UAS system is often used to regulate fruit fly gene expression (Duffy, 2002). GAL4 is a regulatory protein that was first discovered in the yeast *S.cereviridae* and functions as a transcription factor that triggers the activation of the UAS (Upstream Activation System) enhancer. This system works in conjunction with the thermosensitive proteinGAL80^{ts}. This is a protein that is active at low temperatures, for example 18℃ or 20℃, binds to GAL4 and prevents GAL4 induced transcription (Brand and Perrimon, 1993; Mondal et al., 2007; Rodríguez et al., 2012; Diaz-Garcia et al., 2016). If the temperature is raised to 29℃, then the GAL80^{ts} becomes inactive, cannot retain GAL4, and the gene is expressed (Brand and Perrimon, 1993; Mondal et al., 2007; Rodríguez et al., 2012; Diaz-Garcia et al., 2016). Thus, the GAL80^{ts}/GAL4/UAS system is used to knock down *scrib* to ensure that the Scrib protein is not expressed and to study the impact of the lack of expression of the Scrib protein on ABP in a spatiotemporal manner.

For this part of experiment were used late third instar larvals, which were obtained after crossing Scrib RNAi x ptc-Gal4>GFP, ex-lacZ ; Gal80ts.

The procedure was consisted of:

1. Dissection
2. Fixation
3. Praimary antibody stainig
4. Secondary antibody stainig
5. Mounting and screening

The more detailed description of each process is described above. All concentrations and describtions of antibodies is possible to find in Table 2.

Table 2. Description of primary and secondary antibodies.

Candidate	Primary antibody	Host species	Link
ex-lacZ	Anti-β-galactosidase	Mouse	https://www.thermofisher.com/antibody/product/beta-Galactosidase-Antibody-Polyclonal/A-11132
DE-Cad	Anti-DCAD2	Rat	https://dshb.biology.uiowa.edu/DCAD2
Dlg	Anti-disc large-s	Mouse	https://dshb.biology.uiowa.edu/4F3-anti-discs-large
IgG	Goat-anti-rat 568	Rat	https://www.thermofisher.com/antibody/product/Goat-anti-Rat-IgG-H-L-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-11077
IgG	Goat Anti-Mouse IgG H&L (Alexa Fluor® 568)	Rabbit	https://www.abcam.com/goat-mouse-igg-hl-alexa-fluor-568-ab175473.html

Primary antibodies were added to a 5% blocking buffer (Normal Goat Serum + 1xPBT), so their concentration is calculated for a large proportion of the blocking buffer. Secondary antibodies are mixed with 1xPBT.

5.2.3 The second experimental part

During second experimental part were maken a crosses to test one copy scrib background and two copies scrib

- One copy scrib background
    - 7633 Df (3R) x ptc-Gal4>GFP, ex-lacZ ; scribRNAi-Gal80ts
    - 7638 Df (3R) x ptc-Gal4>GFP, ex-lacZ ; scribRNAi-Gal80ts
    - 7675 Df (3R) x ptc-Gal4>GFP, ex-lacZ ; scribRNAi-Gal80ts
- Two copies scrib (wild type)
    - Oregon-R x ptc-Gal4>GFP, ex-lacZ ; scribRNAi-Gal80ts

For this part of experiments were used late third instar larvals and practical part was consist of:

1. Dissection

2. Fixation
3. DAPI
4. Mounting and screening

The more detailed description of each process is described above. The description of deleted genes in Dfs is possible to find in Table 1. All concentrations and describtions of antibodies is possible to find in Table 2.

5.3 RESULTS

5.3.1 Immunohistochemistry

To compare the changes of the phenotype of the wing disc after two (2D) and five (5D) days after temperature shift (at 29℃) the Yki activity were analysed. Yki activity can be measured by an increase in the level of ex-lacZ, which detected by the primary antibody anti-β-galactosidase. In Figure 3, one can see the difference of ex-lacZ signal between two days and after five days after temperature shift at 29℃. Previous studies have found that Yki activity is up-regulated by conditional *scrib* KD (Gui et al., 2021). If, after two days of incubation (Figure 3) at 29℃, the imaginal disc retains its shape, ABP is maintained, and only a GFP band can be seen, this indicates that WT cells still inhibit the development hyperproliferated cells, while after five days of incubation at 29℃, the disc is severely deformed, ABP is lost, and neoplasia phenotypes are observed throughout the imaginal disc. This supports the fact that long-term incubation at 29℃ results in a loss of Scrib, leading to ABP dysregulation and hyperactivation of Yki activity.

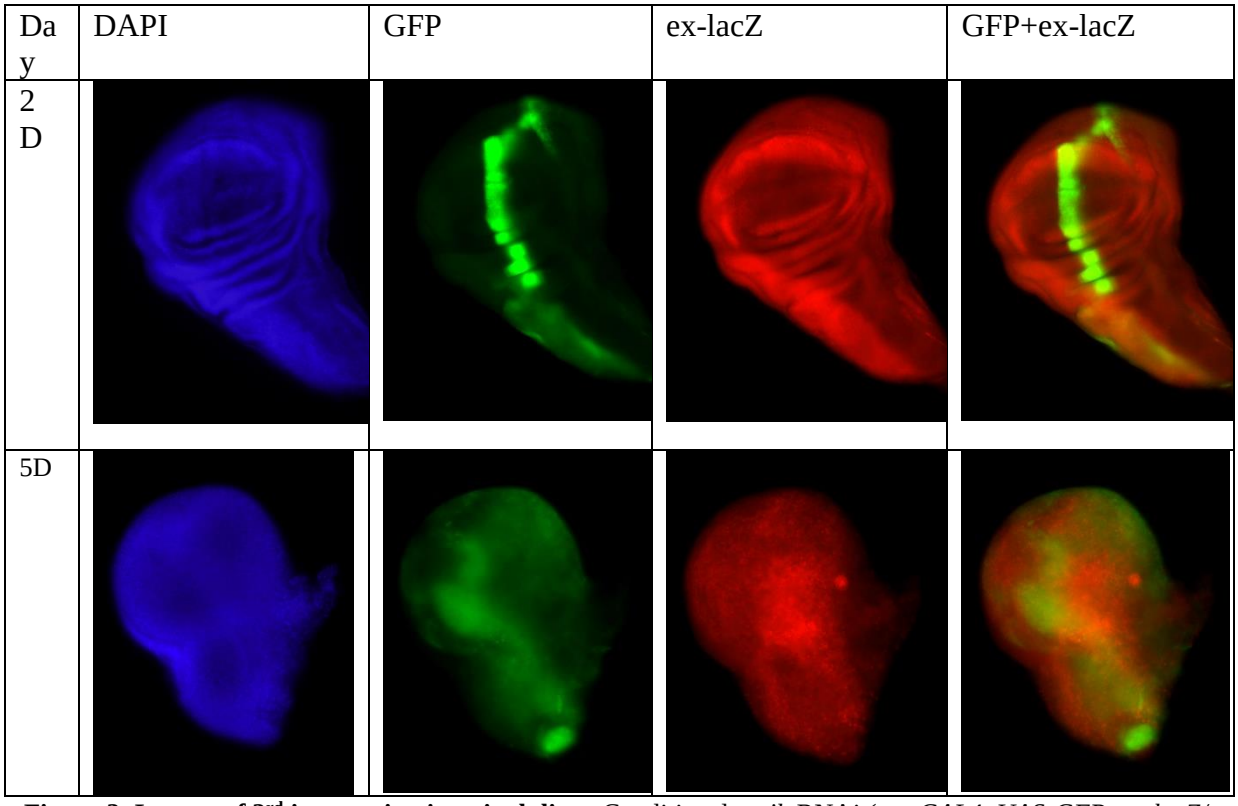

Figure 3. Images of 3rd instar wing imaginal discs. Conditional *scrib* RNAi (*ptc-GAL4, UAS-GFP, ex-lacZ/+; GAL80^{ts}, UAS-scrib RNAi*) two days 2D (upper panel) or five days (5D) (lower panel) induction of *scrib* knockdown. DAPI (blue) were used to visualize DNA, GFP (indicates Scrib KD green), ex-lacZ (red), and merged image of GFP and ex-lacZ.

Next, the effect of the Dlg protein was investigated on the loss of ABP as Dlg is marker of basolateral polarity. Studies show (Figure 4) that after 2D of incubation, ABP and the shape of the disc are preserved, accordingly there are no significant changes in the spatial distribution of Dlg. For comparison, it is worth paying attention to the imaginal discs that were subjected to long-term (5 days) incubation, where *scrib* knockdown (GFP positive cells) are broadly observed and Dlg expression is significanly affected.

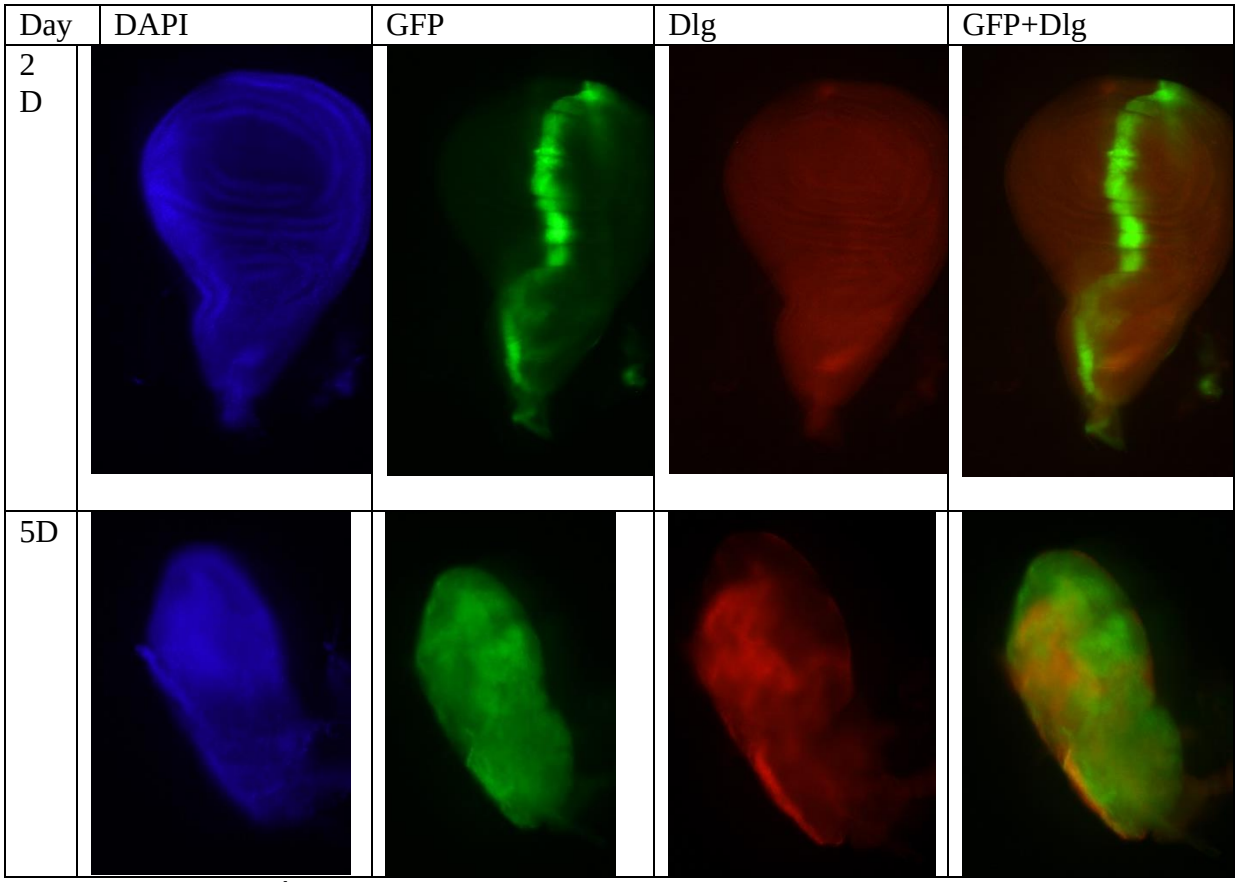

Figure 4. Images of 3rd instar wing imaginal discs. Conditional scrib RNAi (ptc-GAL4, UAS-GFP, ex-lacZ/+; GAL80^{ts}, UAS-scrib RNAi) two days (2D) (upper panel) or five days (5D) (lower panel) induction of scrib knockdown. DAPI were used to visualize DNA (blue), GFP (indicates Scrib KD, green), Dlg (red), and merged image of GFP and Dlg.

Previously, it was known that one of the functions of DE-Cad (one of the key components of the AJ through which cell communication is carried out) is to maintain the integrity of the epithelium. The results confirm that conditional knockdown of *scrib* (caused by ptc-GAL4) After 5 days of incubation resulted in loss of epithelial integrity (Figure 5).

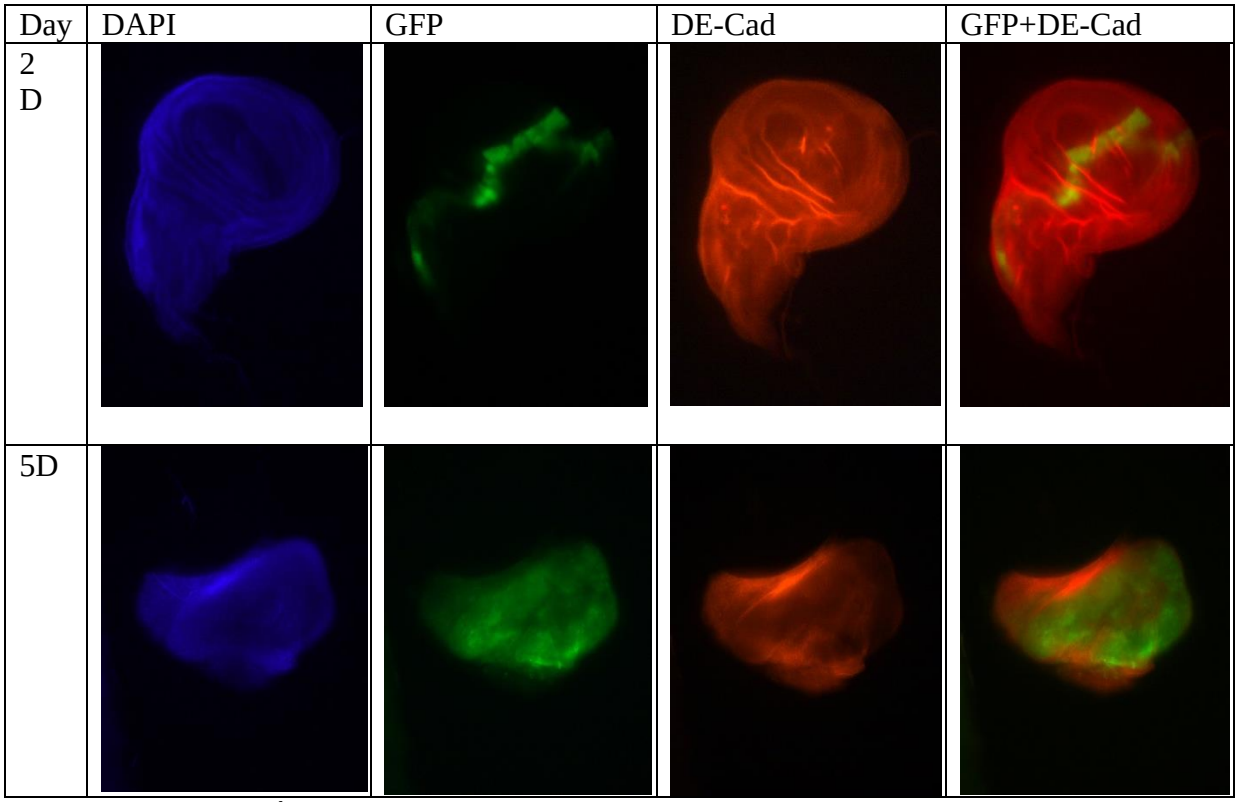

Figure 5. **Images of 3rd instar wing imaginal discs.** Conditional *scrib* RNAi (*ptc-GAL4, UAS-GFP, ex-lacZ/+; GAL80^{ts}, UAS-scrib RNAi*) two days (2D) (upper panel) or five days (5D) (lower panel) induction of *scrib* knockdown. DAPI were used to visualize DNA (blue), GFP (indicates Scrib green), DE-Cad (red), and merged image of GFP and DE-Cad.

5.3.2 The results of the second experimental part.

The next step is to explore in more detail the effect of Scrib on ABP loss for gene screening, For this purpose, cross-breeding was performed with Dfs males in which the gene is absent or partially absent on the 3R chromosome (for a description of Dfs, see chapter Materials and methods Tabel 1). Crossing was carried out with three different Dfs (the choice was made randomly).

For better understanding, control sample (conditional *scrib* RNAi alone) was prepared. For this, wild type Oregon-R genotype was used instead in conditional *scrib* RNAi.

Stocks 7633, 7638 and 7675 were used for the experiment, Tabel 1 describes which genes are missing in these stocks. Based on the control, at this stage of the study, none of the candidates is suitable for further screening. The main idea was that after two days of incubation at 29℃, a strong neoplasia phenotype was expected to have overproliferation throughout the imaginal

disc. The results obtained (Figure 7-12) show that after crossing with *scrib* RNAi, all selected candidates reveal similar phenotypes to the control (Figure 6). This suggests that the deleted genes may not have synergy with *scrib* knockdown, since the disc shapes are preserved after two days incubation. After five days of incubation (Figure 8, 10, 12), the results are as expected, since on the fifth day the disc loses its shape due to the loss of ABP.

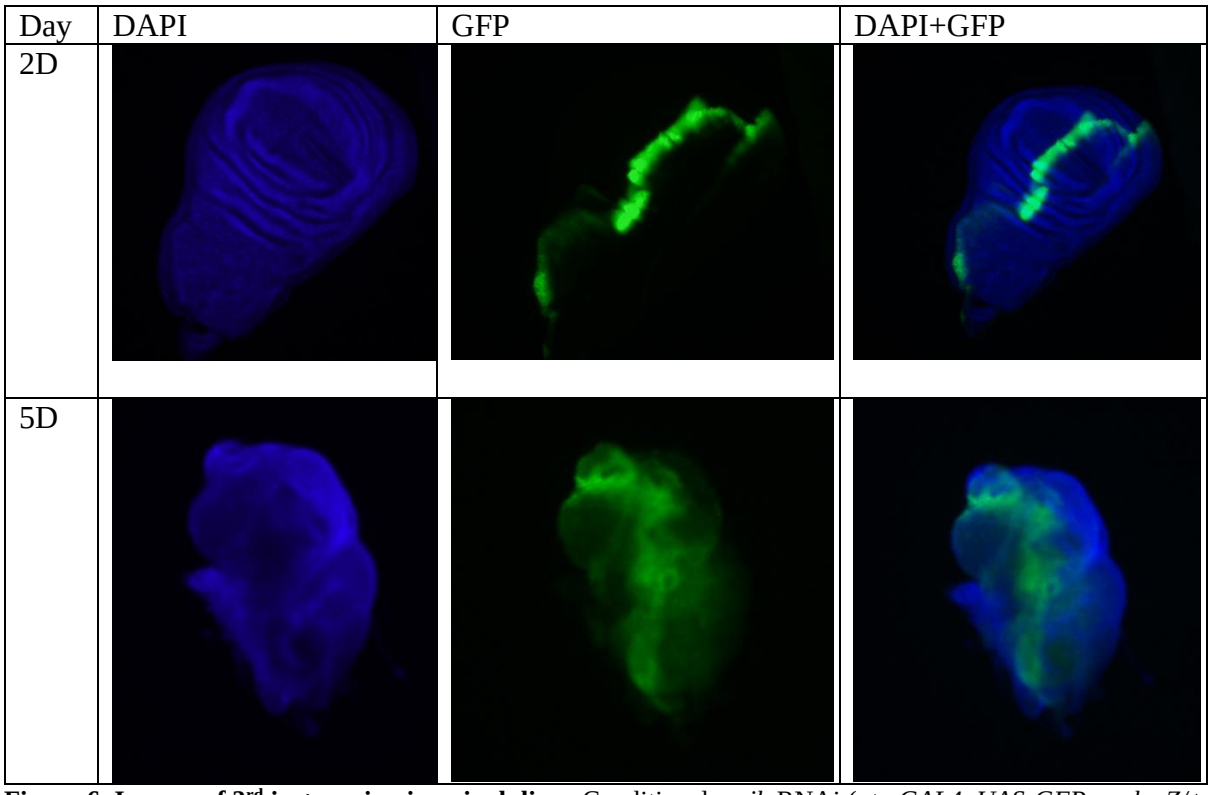

Figure 6. Images of 3rd instar wing imaginal discs. Conditional *scrib* RNAi (*ptc-GAL4, UAS-GFP, ex-lacZ/+; GAL80^{ts}, UAS-scrib RNAi*/ Oregon-R) two days (2D) (upper panel) or five days (5D) (lower panel) induction of *scrib* knockdown. DAPI were used to visualize DNA (blue), GFP (indicates Scrib KD, green) and merged image of GFP and DAPI

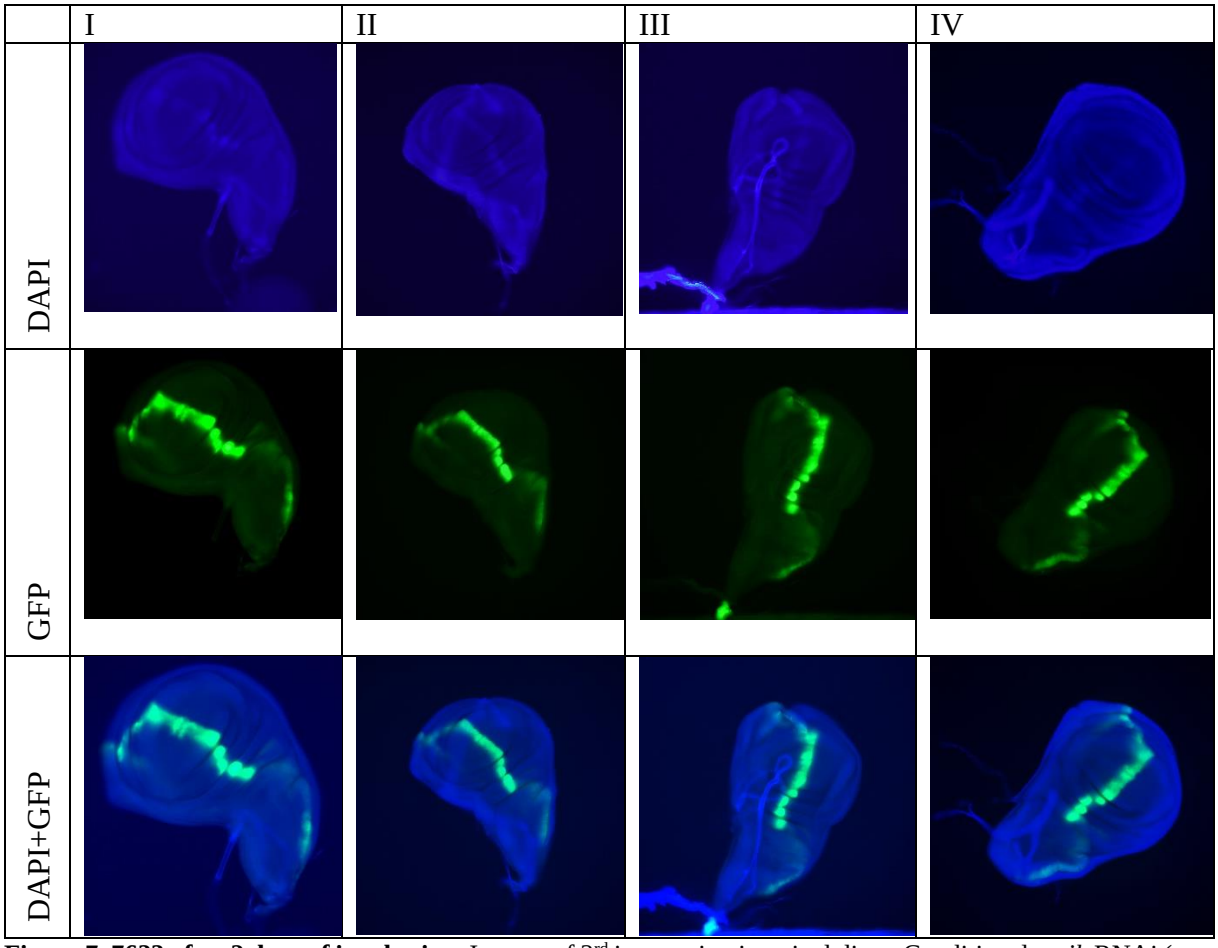

Figure 7. 7633 after 2 days of incubation. Images of 3rd instar wing imaginal discs. Conditional *scrib* RNAi (*ptc-GAL4, UAS-GFP, ex-lacZ/+; GAL80^{ts}, UAS-scrib RNAi*/ Df 7633) two days (2D) -induction of *scrib* knockdown with Df line #7633. DAPI were used to visualize DNA (blue), GFP (indicates Scrib KD, green) and merged image of GFP and DAPI. Four independent samples are presente.

It is an example, of negative results. Right know it is possible to say, that these candidates are not suitable for future research. After 2 days of incubation, it shows that imaginal wing disc have a good GFP stripe, what is a result, that ABP is regulated and supported by neighbour cells around GFP stripe.

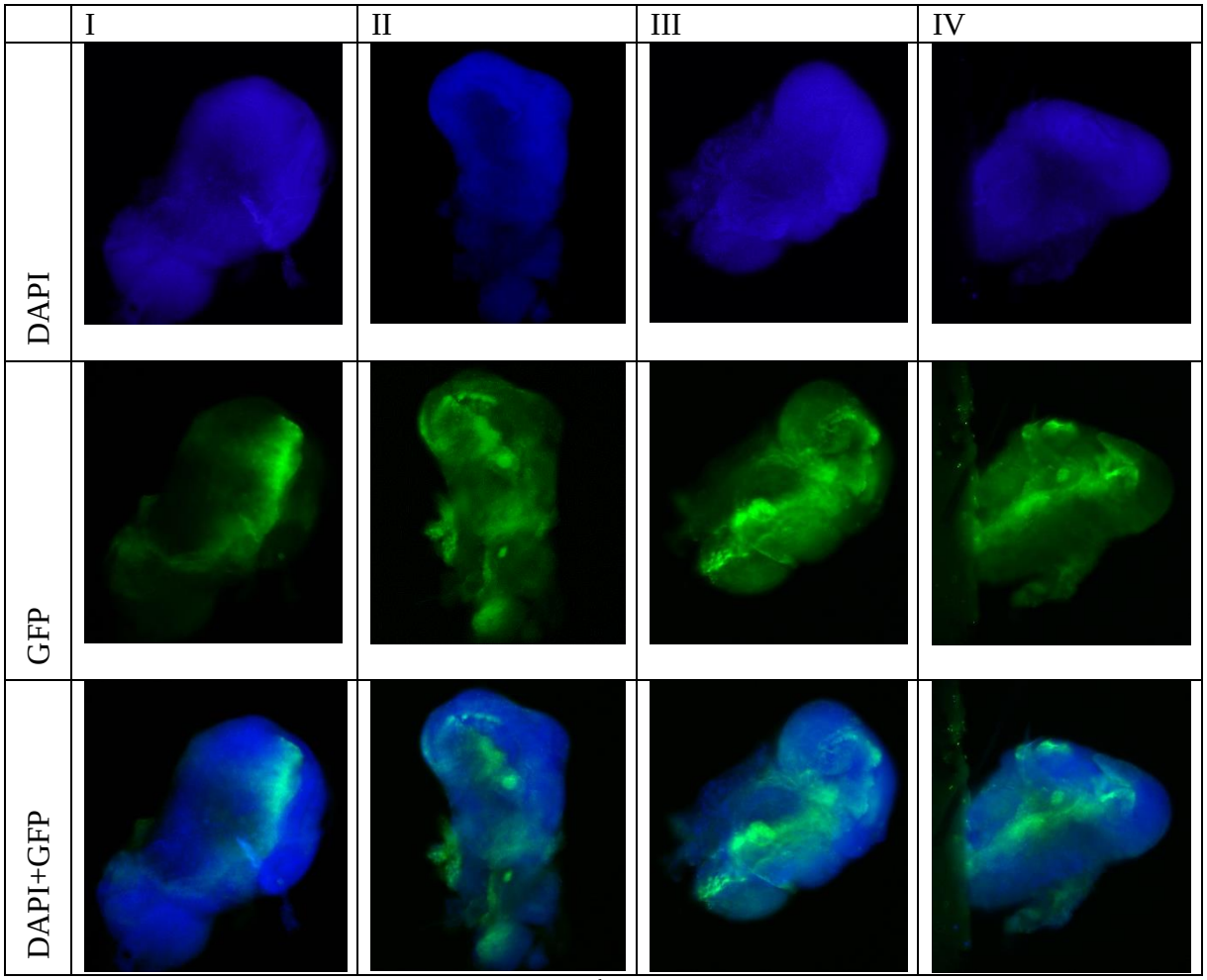

Figure 8. 7633 after 5 days of incubation. Images of 3rd instar wing imaginal discs. Conditional *scrib* RNAi (*ptc-GAL4, UAS-GFP, ex-lacZ/+; GAL80^{ts}, UAS-scrib RNAi*/ Df 7633) five days (5D) induction of *scrib* knockdown with Df line #7633. DAPI were used to visualize DNA (blue), GFP (indicates Scrib KD, green) and merged image of GFP and DAPI. Four independent samples are presente.

Here it is possible to see, that after 5D of incubation imaginal wing disc is typically deformed and tumor spreads globally.

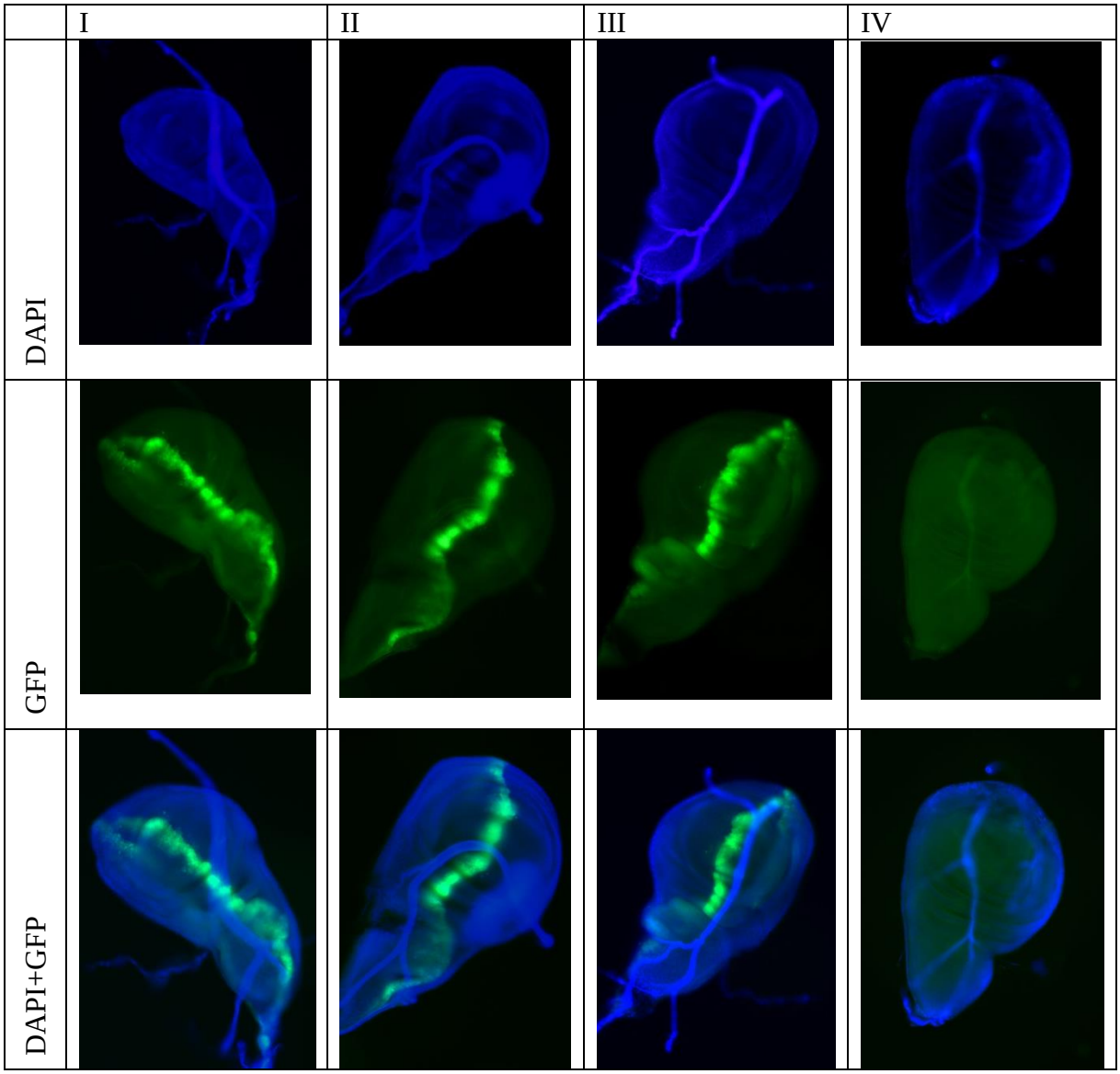

Figure 9. 7638 after 2 days of incubation. Images of 3rd instar wing imaginal discs. Conditional *scrib* RNAi (*ptc-GAL4, UAS-GFP, ex-lacZ/+; GAL80^{ts}, UAS-scrib RNAi*/ Df 7638) two days (2D) -induction of *scrib* knockdown with Df line #7638. DAPI were used to visualize DNA (blue), GFP (indicates Scrib KD, green) and merged image of GFP and DAPI. Four independent samples are presente.

There are the imaginal discs, which look like negative control and 7633 after same conditions. This stock is not suitable for next researches, because deleted genes did not to effect on the discs and tumor growth.

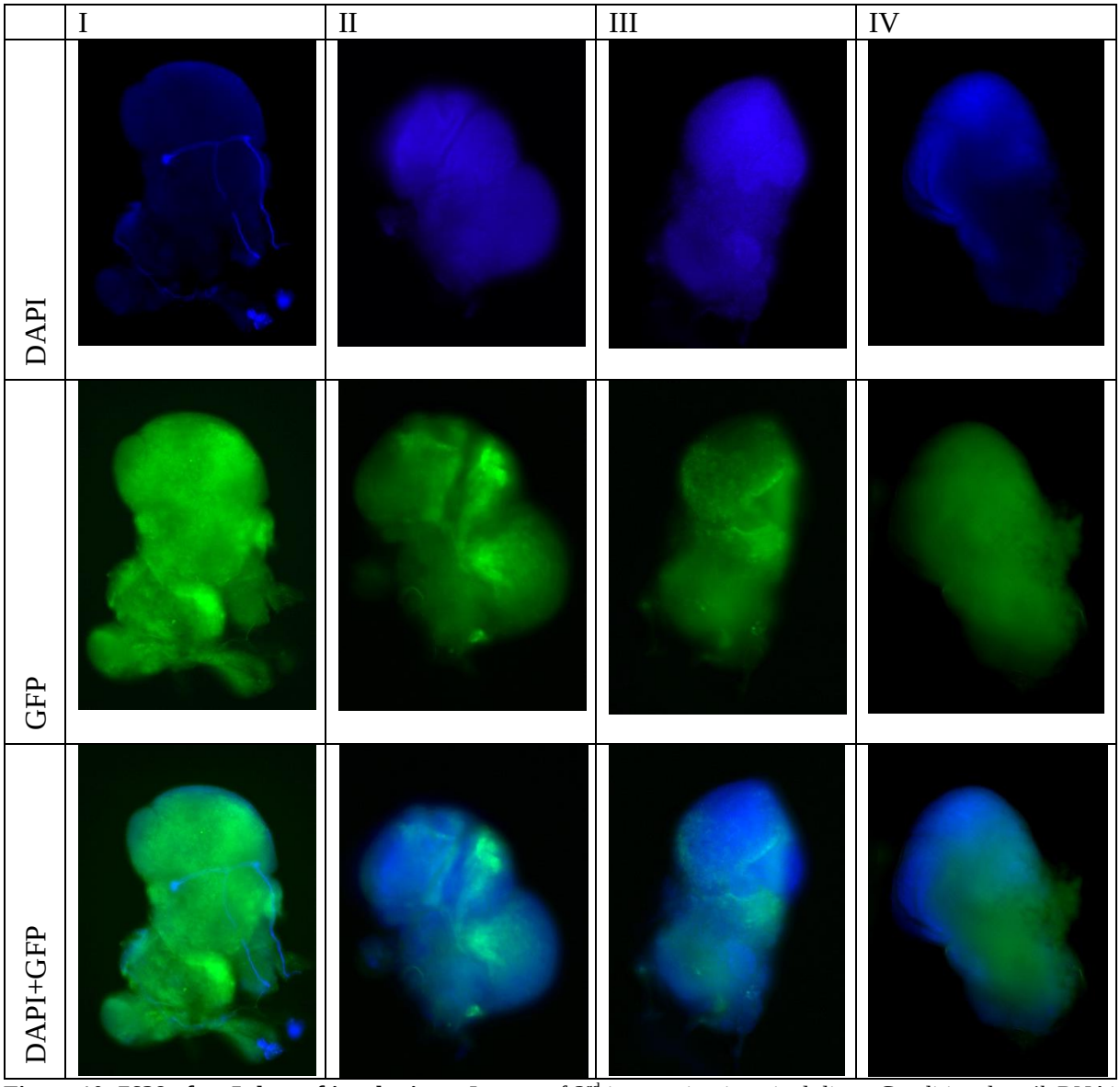

Figure 10. 7638 after 5 days of incubation. . Images of 3rd instar wing imaginal discs. Conditional *scrib* RNAi (*ptc-GAL4, UAS-GFP, ex-lacZ/+; GAL80^{ts}, UAS-scrib RNAi*/ Df 7638) five days(5D) induction of *scrib* knockdown with Df line #7638. DAPI were used to visualize DNA (blue), GFP (indicates Scrib KD, green) and merged image of GFP and DAPI. Four independent samples are presente.

There is the same result like was in negative control and 7633 in same conditions.

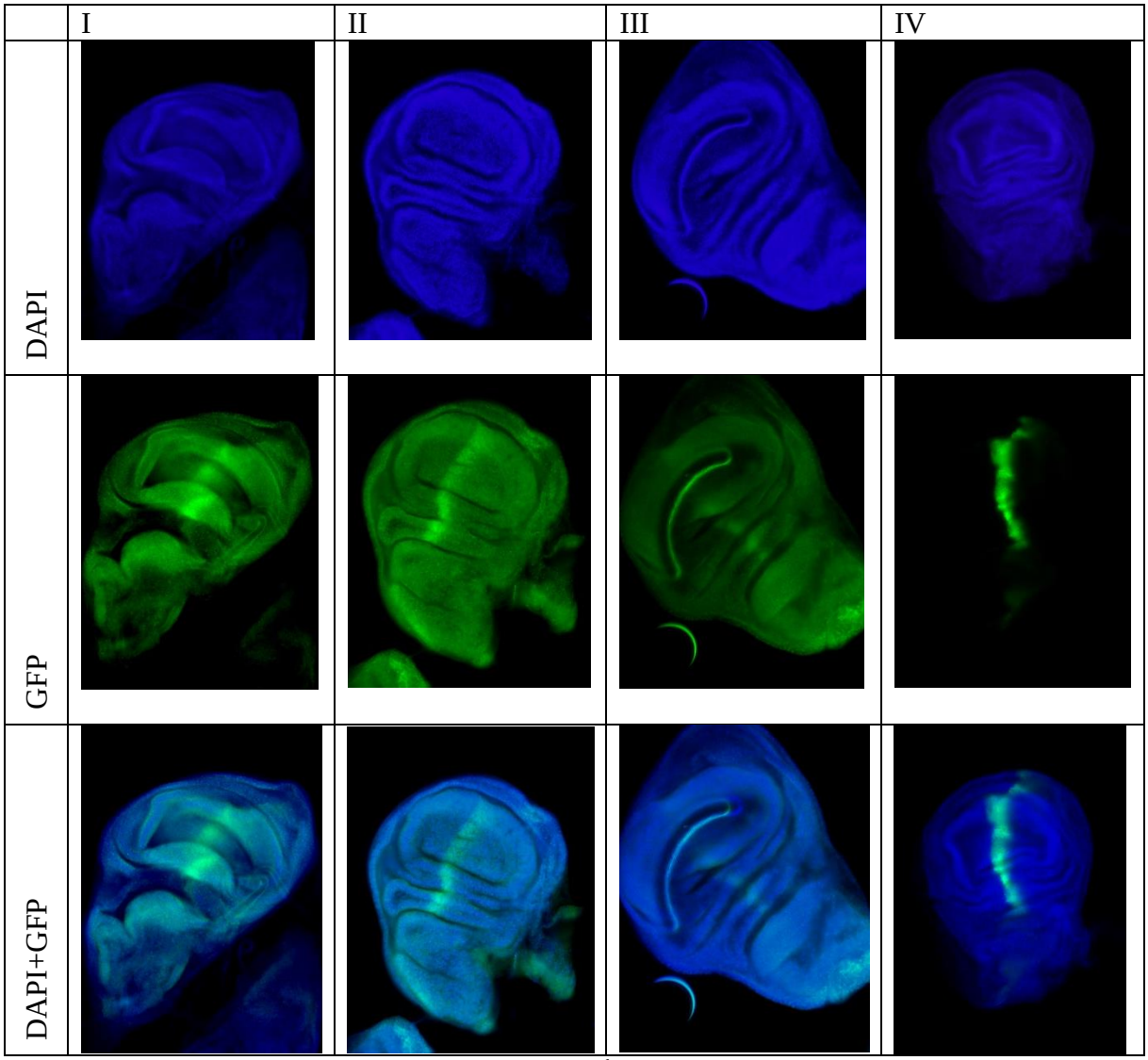

Figure 11. 7675 after 2 days of incubation. Images of 3rd instar wing imaginal discs. Conditional *scrib* RNAi (*ptc-GAL4, UAS-GFP, ex-lacZ/+; GAL80^{ts}, UAS-scrib RNAi*/ Df 7675) two days (2D) -induction of *scrib* knockdown with Df line #7675. DAPI were used to visualize DNA (blue), GFP (indicates Scrib KD, green) and merged image of GFP and DAPI. Four independent samples are presente.

There are only two discs which have a bright GFP strip and two another do not have it. It could be due to the fact that, two discs which do not have a bright GFP strip were in the glycerol (70%) for a long time, after that also GFP strip could be lost. But by the way, this candidate is also not suitable for a next step of research.

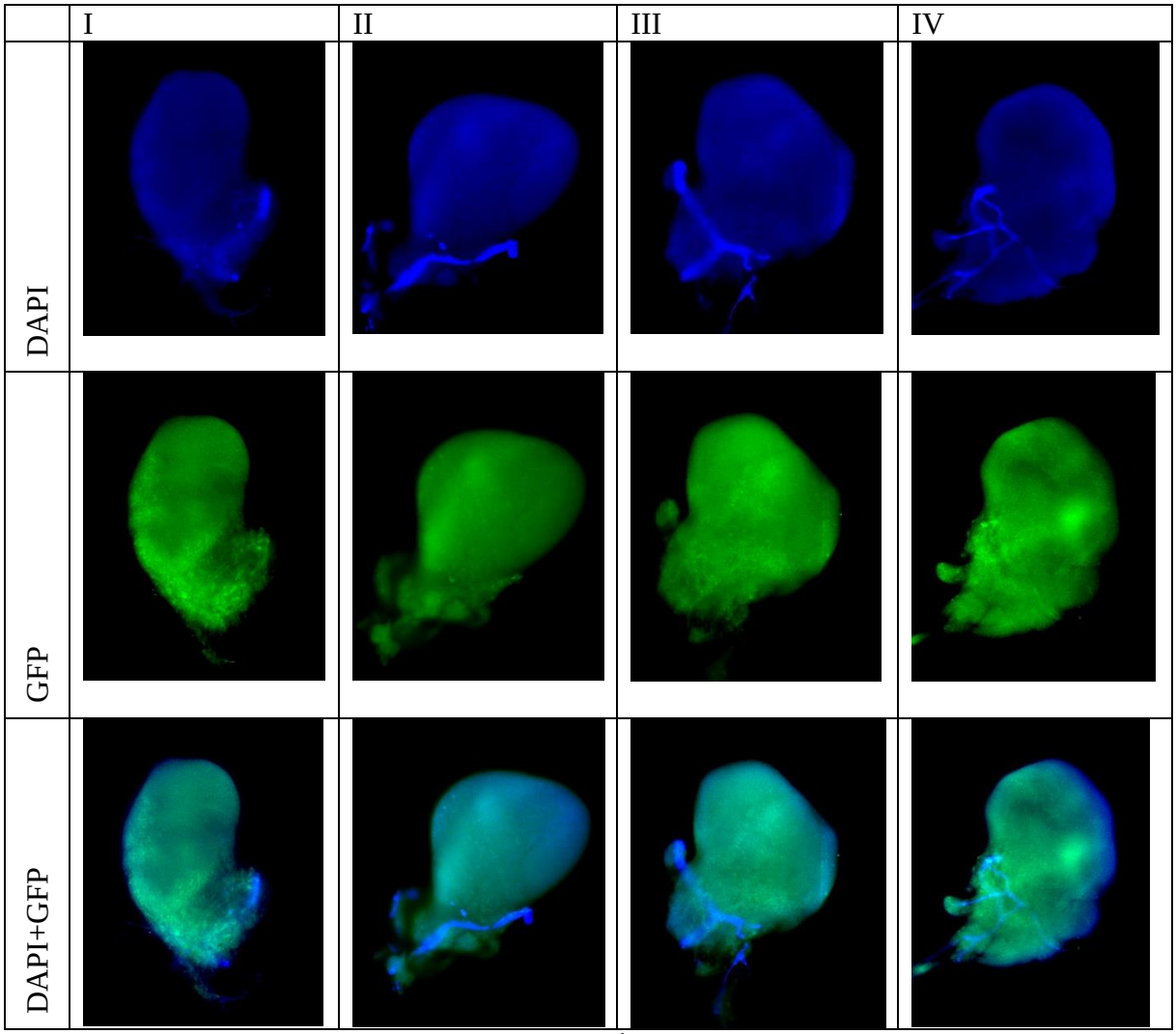

Figure 12. 7675 after 5 days of incubation. Images of 3rd instar wing imaginal discs. Conditional *scrib* RNAi (*ptc-GAL4, UAS-GFP, ex-lacZ/+; GAL80^{ts}, UAS-scrib RNAi*/ 7675) five days (5D) induction of *scrib* knockdown with Df line #7675. DAPI were used to visualize DNA (blue), GFP (indicates Scrib KD, green) and merged image of GFP and DAPI. Four indepndent samples are presente.

There are the same results like it was descripted in negative control, 7633 and 7638.

6. DISCUSSION

Having studied the concept of ABP, the Scribble complex, one of the key complexes on the implementation of ABP regulation, became attractive model to understand the gene functions, during *Drosophila melanogaster* development. Proper regulation of ABP is essential for continued tissue formation and organ function. If it is lost or dysregulated, then this can be one of the causes of neoplasia in humans.

Thanks to the model organism *Drosophila melanogaster* , in particular the L3 larvae, we are getting closer to undestand molecular mechanisms behind ABP dysregulation, caused by loss of Scribble complex. The imaginal wing disc perfectly modulates the epithelial tissue consisting of epithelial cells that contain the ABP of interest to us.

In this two-part experiment, it was important to first identify by immunohistochemistry the influence of Yki, Dlg and DE-Cad, which was described in Jinghua Gun et al., 2021 "*Intercellular alignment of apical-basal polarity coordinates tissue homeostasis and growth*" as the results described in this paper have indeed become a breakthrough in the topic of ABP regulation. And secondly, to identify candidate genes by crossing with Dfs lines of the 3R chromosome.

According to the first part of the experiment, it can be said that the influence of the three markers of ABP disturbance became clear also due to their staining with antibodies. Firstly, it gave a good experience in learning dissect L3 larvae in order to obtain imaginal wing discs, and secondly, it helped to better understand the work of some proteins and molecules during ABP disruption. For example, ex-lacZ signal is likely to be controlled by phosporylation or dephosphorylation of Yki, which indicates that the Hippo signaling pathway is dysregulated, therefore, Yki, a key effector of Hippo signaling pathway, is upregulated, leading to uncontrolled tissue growth.

During conditional knockdown of *scrib* Dlg disruption is obsereved non-autonomously. For example, Figure 4 shows loss of Dlg after five days of conditional *scrib* RNAi visible in both GFP positive and negative cells.

The second part of the experiment was an attempt to screen the genes involved in ABP regulation by employing Df lines with *scrib* RNAi . For this, we used Dfs stocks that lack or partially lack the chromosome. The aim was to identify a strong tumor phenotype after two days of incubation, which would be evidence that the missing gene has an effect on tumor development. To detect such a candidate, it was necessary to select 10 independent larvae and calculate how many of the 10 candidates had a tumor after two days of incubation.

In a comparative analysis, the template of which was a negative control (the result of a cross with an Oregon-R WT variant with all genes on the 3R chromosome), it was found that none of the randomly selected candidates (7633, 7638, 7675) had a strong tumor phenotype. As shown in Figures 7, 9, 11, most discs have a GFP stripe, indicating that ABP is supported by neighboring cells surrounding this stripe.

Of course, statistically these results can be called into question, for a number of reasons:

1. The results provided only 4 discs instead of the declared 10. This can be explained by the fact that it was not possible to collect 10 larvae at a time due to the fact that adults laid few eggs, did not all developed to L3 and not all tested for GFP positivity.
2. Secondly, the human factor played, during which ordinary larvals were chosen instead of those who have a balancer. The presence balancer is necessary to maintain a mutation in the chromosome and also to avoid recombinations.
3. To confirm the existing conclusions, or to refute them, it is worth repeating the experiment again.

Summarizing all the above arguments, at this stage there is no candidate with a strong neoplasia phenotype, and further development of screening will bring candidete genes.

7. SUMMARY

In order to better understand the regulation and intercellular communication affecting ABP, the L3 larval of *D.melanogaster* was used as a model. The main goal of the experiment was to identify the candidete genes that synergize with Scrib to suppress neoplasia. To establish screening protocol, preliminary studies were carried out using the method of immunohistochemistry. These approaches allow to investigate changes in the structure of the imaginal disc after the introduction of scrib RNAi and to understand how much the imaginal disc is deformed after the loss of Scrib and, consequently, loss of ABP regulation.

During the second part of the experiment, chromosome deficiency lines were combined with *scrib* RNAi in order to determine the genes cooperating with Scrib on tumor growth. It was expected that after two days of incubation at 29°C, the imaginal disc becomes a neoplasia, but no candidate with a strong tumor phenotype was identified in this thesis project. All studied Dfs stocks (7633, 7638 and 7675) were similar to the control. To confirm these results, more sample analysis such as more than 10 samples is required for statistical analysis. Extension of these approaches will bring novel candidate genes that are cruicial for tissue growth and homeostasis.

8. RESÜMEE

Epiteelirakkude apikobasaalset polarisatsiooni reguleeriva rakkudevahelise võrgustiku analüüs

Darja Tarassova resümee

Antud bakalaureusetöös kasutati mudelina äädikakärbse *Drosophila melanogaster* kolmanda kasvujärgu vastse tiiva imaginaaldiski, et paremini mõista, kuidas apiko-basaalne polaarsus (ABP) on reguleeritud ning kuidas rakkudevaheline kommunikatsioon seda mõjutab. Eksperimentide peamine eesmärk oli tuvastada kandidaatgeenid, mis omavad raku polarisatsiooni komponendi Scribble-ga sünergiat neoplaasia mahasurumiseks.

Sõeluuringu loomiseks viidi esmalt läbi eeluuringud kasutades immunohistokeemilist analüüsi. Kasutades *scrib* RNAi-d võimaldas see välja selgitada, millisel määral on arenev tiiva kude muutunud pärast Scrib-i allaregulatsiooni ning kuidas see mõjutab ABP regulatsiooni. Katse teises osas kombineeriti kromosoomi spetsiifilise regiooni deletsiooni (ingl *deficiency line*, Df) kärbseliinid *scrib* RNAi kärbseliinidega, et määrata geenid, mis sünergias Scrib-ga mõjutavad neoplaasia arengut.

Sõeluuringu loomiseks viidi esmalt läbi eeluuringud kasutades immunohistokeemilist analüüsi. Kasutades *scrib* RNAi-d võimaldas see välja selgitada, millisel määral on arenev tiiva kude muutunud pärast Scrib-i allaregulatsiooni ning kuidas see mõjutab ABP regulatsiooni. Katse teises osas kombineeriti kromosoomi spetsiifilise regiooni deletsiooni (ingl *deficiency line*, Df) kärbseliinid *scrib* RNAi kärbseliinidega, et määrata geenid, mis sünergias Scrib-ga mõjutavad neoplaasia arengut.

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