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Intercellular Communication Through Septate Junction in the Drosophila Wing Disc Epithelium

Bachelor's Thesis (12 ECTS)

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Abstract:

Scribble (Scrib), a basolateral polarity determinant, plays a key role in the maintenance of epithelial cell polarity and homeostasis in epithelial tissues. The host lab recently revealed that Scrib is required for polarity maintenance not only autonomously but also non-autonomously through intercellular communications. However, molecular mechanisms behind non-autonomous pathway remain to be addressed. Considering Scrib localizes at the septate junction (homologous to vertebrate tight junction), it is hypothesized that the septate junction components may play a role in the intercellular mechanisms. To address this, in vivo RNAi screening of 18 septate junction components was performed. Coracle was identified as a candidate to suppress non-autonomous loss of Scrib in flanking wild type cells. While Macroglobulin, Fasciclin III, and Sinuous may play a role in later stages. Taken together, the findings indicate a possible genetic interaction between Scrib and the septate junction components to support intercellular communication for sustaining tissue homeostasis.

Keywords:

Septate junctions, apicobasal polarity, cell competition, wing imaginal discs

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

Rakkudevaheline kommunikatsioon läbi äädikakärbse (Drosophila melanogaster) tiivadiski epiteeli tiheliiduste

Lühikokkuvõte:

Scribble valk on raku polarisatsiooni basolateraalne determinant, mis mängib olulist rolli epiteelirakkude polarisatsiooni ja homöostaasi säilitamisel. Hiljuti näidati, et Scribble on vajalik polarisatsiooni säilitamiseks mitte ainult autonoomselt vaid ka mitteautonoomselt läbi rakkudevahelise kommunikatsiooni. Molekulaarseid mehhanismid, mis on olulised mitte-autonoomset regulatsiooni juures on käsitlemata. Eeldades, et Scribble lokaliseerub tihe liidustes (ingl septate junctions; imetajate tiheliiduste homoloogid) püstitati antud bakalaureusetöös hüpotees, et vastavate liiduste komponendid mängivad olulist rolli rakkudevahelistes mehhanismides. Sellest johtuvalt uuriti antud töös 18 erinevat liiduste komponenti kasutades in vivo RNAi sõeluuringut. Tulemusena leiti, et Coracle oli võimeline supresseerima mitte-autonoomset Scribble kadu ümbritsevates metsik-tüüpi rakkudes. Samas Macroglobulin, Fasciclin III ja Sinous mängivad rolli hilisemates staadiumites. Kokkuvõtvalt antud tulemused näitavad võimalikku interaktsiooni Scribble ja liiduste komponentide vahel, mis aitab toetada rakkudevahelist kommunikatsiooni säilitamaks koe homöostaasi.

Võtmesõnad: Tiheliidused, apikobasaalne polaarsus, rakkude vaheline konkurents, tiiva imaginaal-disk

CERCS: B350 Arengubioloogia, loomade kasv, ontogenees, embrüoloogia

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TERMS

Dorsal closure - process involving onset of signalling pathways and cell shape changes to close the dorsal epidermal gap during late embryogenesis.

Balancers - Balancers are chromosomes with multiple inversions which prevent homologous recombination from taking place in order to avoid the desired gene from being lost from the fly stock e.g., Tm6BTb is a third chromosome balancer, *Curly*-O (*CyO*) is a second chromosome balancer. Flies containing the former look short and big whereas the latter gives the flies curly wings. These balancers in a homozygous condition(e.g., CyO/CyO) are lethal.

Tracheal morphogenesis – a stage in late embryogenesis where there is an increase in tracheal diameter.

ABBREVIATIONS

ABP - Apical-basal polarity

AJ - Adherens junction

AP - Anterior-posterior

ATS - After temperature shift

BBB - Blood-brain barrier

Dlg - Discs-large

Dpp - Decapentaplegic

ECM - Extracellular matrix

Lgl - Lethal-giant larvae

PCP - Planar cell polarity

pSJ - Pleated septate junction

Ptc - Patched

RNAi - RNA interference

Scrib - Scribble

SJ - Septate junction

sSJ - Smooth septate junction

TJ - tight junction

LRR - leucine rich repeats

PDZ - postsynaptic density-95/Disc-large/ZO-1

MAGUK - membrane-associated guanylate kinases

$GAL80^{ts}$ - GAL80 temperature-sensitive

TCJ - tricellular junction

tSJ - tricellular septate junction

Septate Junction Component Abbreviations

Kune - Kune-Kune

NrxIV - Neurexin IV

Nrg - Neuroglian

Crim - Crimpled

Mcr - Macroglobulin complement-related

FasIII - Fasciclin III

Lac - Lachesin

Sinu - Sinuous

Pasi1- Pasiflora 1

Wrm - Wurmchen1

Cora - Coracle

Pasi2 - Pasiflora 2

Vari - Varicose

Cont - Contactin

ATPα - α subunit of Na+/K+ATPase

INTRODUCTION

Apical-basal polarity is a key feature of epithelial cells because its maintenance is vital for diverse cellular processes such as differentiation, cell division, and morphogenesis. *Drosophila melanogaster* has served as a valuable model organism to understand epithelial cell polarity mechanisms since most polarity determinant genes are conserved between humans and flies(Dow et al., 2003)(Mathew et al., 2002).

Three main protein complexes have been identified to be responsible for the regulation of polarity in the apical-basal axis – Crumbs, PAR and Scrib. Compartmentalization of each domain is through antagonistic interactions between the complexes (Bilder & Perrimon, 2000;Khoury & Bilder, 2020). The basolateral polarity is mainly maintained by the Scrib complex consisting of Scrib, Dlg (Woods et al., 1996), and Lgl. Loss-of-function mutations in these genes show loss of apical-basal polarity (ABP), overproliferation, and loss of epithelial tissue architecture. Previous studies have explored that loss-of-function of *scrib* in the anterior-posterior (AP) boundary of wing imaginal disc epithelia render the cells in this region 'suboptimal' triggering progressive loss of ABP in the abutting wild-type cells. However, it remains to be addressed how cellular communication takes place between optimal and suboptimal cells to regulate ABP in a non-autonomous manner.

Considering Scrib is enriched at septate junctions (SJ), invertebrate homologs of tight junctions (TJ), it is possible that SJ components may play a role in promoting or impeding loss of polarity to neighbouring cells. Loss-of-function studies using *Drosophila melanogaster* embryonic epidermis have enabled the identification of genes encoding proteins involved in the occluding function of SJ. Proteins dispensable for the formation and organization of SJ show aberrations in the ladder-like morphology of pleated SJ (pSJ). Furthermore, knockdown of some septate genes affect dorsal closure and tracheal morphogenesis (Laprise et al., 2006; K. S. Nelson et al., 2010). However, the role of SJ components in ABP is not well explored in postembryonic epithelia.

In this study, by employing the UAS/GAL4/GAL80^{ts} system, 18 septate junction components were conditionally knocked down alongside Scrib in the AP boundary of wing imaginal discs. One candidate was found to suppress the loss of polarity, 3 candidates were found to be important in later stages of polarity loss while the remaining components caused regressive loss of polarity.

1 LITERATURE REVIEW

1.1 *Drosophila melanogaster* as a model organism

The fruit fly is an organism belonging to the Kingdom, Animalia; Phylum, Arthropoda; Class, Insecta; Order, Diptera; Family, Drosophilidae; Genus, *Drosophila*; Species, *Drosophila melanogaster*.

Drosophila melanogaster is a holometabolous (*holo* for 'total' and *metabolous* for 'change') insect that develop from an egg to a worm-like larva which then undergoes moulting to become an adult entirely different from the initial stage of development. Its life cycle takes about ten days to complete at 25°C and may vary depending on the temperature (Figure 1A)(Hales et al., 2015). Growth of the adult body is limited due to the rigid exoskeleton, therefore, development occurs as imaginal discs which differentiate into adult organs during metamorphosis (Fig 1B)(Shingleton, 2010).

The fly has four pairs of chromosomes; *X*, *2L*, *2R*, *3L*, and *3R* (*L*-left arm, *R*-right arm) . The fourth chromosome is smaller, therefore, it is not significant in genetic experimentations. The Y chromosome in males has few genes required for the development of the male fly and motility of the sperm.

Drosophila has been utilized in the field of genetics for over a century. Initially inbred for entomological studies (*Thomas H. Morgan – Biographical - NobelPrize.Org*), the organism has been a valuable contribution in genetics from establishing the chromosome hereditary theory (Bridges, 1916; Morgan, 1910) to the identification of important body segmentation genes in fly embryo in developmental biology (Nüsslein-volhard & Wieschaus, 1980). In recent years, its utilization has expanded to other fields of research to identify tumour suppressor genes (Xu et al., 1995) and to elucidate human mechanisms in human diseases like Polycystic Kidney Disease (PKD) (Gamberi et al., 2017) and neurodegenerative Alzheimer's disease(Carmine-Simmen et al., 2009).

The fruit fly provides enormous advantages in comparison to other model organisms. First, the short life cycle enables results to be obtained rapidly. Second, its maintenance is cost-effective. Third, the fly genome is completely sequenced (Adams et al., 2000; Misra et al., 2002), so genes, their alleles and their functions are known, and are constantly updated into a database (such as FlyBase) when new genetic information emerges. Moreover, human and *Drosophila* genomes share a 75% similarity (Reiter et al., 2001). This conservation of genes means that any genetic manipulation carried out within *Drosophila* using reverse genetic

screens can enable the identification of the function of the same desired gene in human. Additionally, there are numerous genetic tools to edit *Drosophila* genome. One such tool is the GAL4/UAS system (see section Tools).

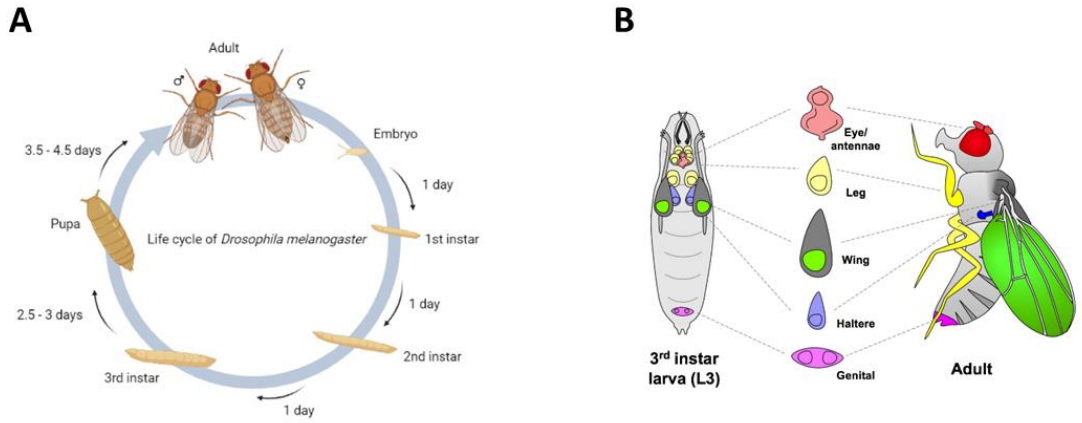

Figure 1: *Drosophila melanogaster*. (A) Different stages of *Drosophila* life cycle. (B) 3rd instar larvae containing imaginal discs destined to form adult organs. Image (B) source: The Harris Lab https://web.asu.edu/harris/research

1.2 Epithelial Tissue

Epithelial tissues are found in the lining of body surfaces, body cavities, organs, and glands. They play a role in absorption (epithelial lining of the intestine absorb nutrients from the digested food), secretion (epithelium in the glands release hormones, enzymes,etc.), protecting the surfaces from mechanical stress and providing a barrier against the entry of pathogens (epithelial lining of the skin).

Epithelial tissues are made up of epithelial cells which take up different morphological forms depending on their location and performed functions (Figure 2A). Simple squamous epithelia are flattened cells arranged in a single layer and are found in the lining of alveoli of lungs and inner lining of blood vessels. Simple cuboidal epithelial cells have a more cube-like appearance and are mostly found in glandular (secretory) tissues and lining of kidney tubules and ducts. Simple columnar epithelial cells are taller and are present in the lining of the stomach. In addition, these cells can be arranged into several layers to form stratified epithelial cells. In other cases, the lateral arrangement of simple columnar cells displays a stratified illusion, thus termed as “pseudostratified columnar epithelia”. These cells are found in respiratory airways. In *Drosophila*, simple squamous epithelia are prevalent in the

blood-brain barrier (BBB) and simple cuboidal epithelia exist in trachea, hindgut and imaginal discs.

1.3 Wing Imaginal Disc

Wing imaginal discs, like all other imaginal discs, are derived from the ectoderm. They are sacs of undifferentiated cells which give rise to the adult wing during metamorphosis. The wing disc contains a notum, hinge and pouch regions. Anterior and posterior compartments divide the wing disc into two parts,and dorsal and ventral regions divide the wing pouch area (Figure 2B).

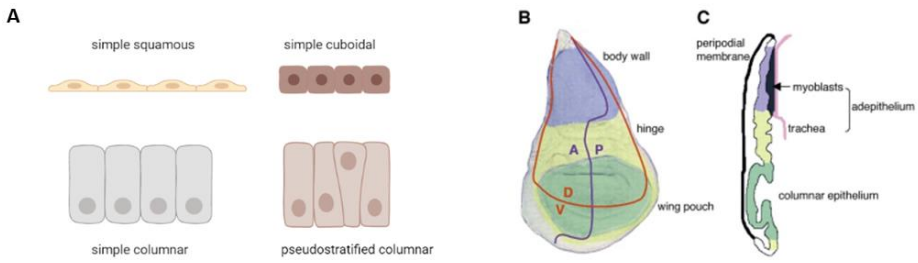

Figure 2: Different types of epithelia. (A) Morphological forms of epithelia (B)wing imaginal disc regions,A-anterior,P-posterior,D-dorsal and V-ventral (C) cross-section of the wing disc displaying epithelia types.Image source:(Schluck, 2013.)

1.4 Epithelial Cell

Each epithelial cell contains an apical side facing the outer environment such as the gut lumen, a basal side anchoring the cell to the extracellular matrix (ECM) via the basement membrane, and lateral sides attaching the neighbouring cells. This attachment is mediated by junctions.

Cellular junctions carry out different functions depending on their multi-protein complex composition. Cadherin superfamily proteins are involved in the cell-to-cell attachment, whereas integrin superfamily proteins are involved in the attachment of the cell to the ECM. The lateral attachment of cells is mediated by the adherens junctions (AJ) consisting of transmembrane protein E-cadherin (DE-Cadherin in *Drosophila*), p120-catenin, α-catenin and β-catenin (intracellular adaptor proteins) which link the plasma membrane to the actin filaments. In vertebrates, desmosomes connect intermediate filaments from one cell with its neighbouring cell. Desmosomes are composed of cadherin-related proteins (desmoglein and

desmocollin) and similar AJ adaptor proteins (plakoglobins and plakophilins). Furthermore, tight junctions in vertebrates and septate junctions in invertebrates provide a barrier against paracellular diffusion of unnecessary substances. Gap junctions are channels composed of connexins in vertebrates and innexins in invertebrates. They are involved in the transfer of small solutes, metabolites and ions across the plasma membrane.

On the basal side of the epithelial cells, cell-matrix junctions and hemidesmosomes (composed of integrin-family proteins) anchor the actin filaments and intermediate filaments, respectively, to the ECM. Desmosomes and hemidesmosomes are not present in *Drosophila* due to the absence of intermediate filaments

1.5 Occluding Junctions

Occluding functions of tight junctions and septate junctions are vital to maintain homeostasis in living organisms. TJs that are present in the BBB of the central nervous system prevent unwanted substances, pathogens, or toxic molecules from diffusing into the brain tissues from the surrounding capillaries. Likewise, it is important that neurotransmitters and neuropeptides do not enter the blood. In *Drosophila* BBB, SJs restrict paracellular diffusion in glial cells (Baumgartner et al., 1996).Furthermore, blood-testis barrier (BTB) in Sertoli cells of the seminiferous tubules are required to avoid any leak of spermatocyte into the bloodstream which would trigger immune response and interfere spermatogenesis (Gow et al., 1999).

Tight junctions are composed of Occludin (Furuse et al., 1993), Claudin(Furuse et al., 1998) and cytoplasmic proteins ZO-1(Stevenson et al., 1986), ZO-2 (Gumbiner et al., 1991), and ZO-3(Haskins et al., 1998). In addition, member of the immunoglobulin superfamily called Junction Adhesion molecule (JAM) is present at the TJs in both endothelial and epithelial cells (Martìn-Padura et al., 1998).

In vertebrates, TJs are positioned apical to AJs (Figure 3A and Figure 4). Ultrastructural images show TJs between cells as points of contact (Figure 3A and B). In *Drosophila*, there are two different types of septate junctions depending on the germ layer from which the epithelial cells were derived from. Epithelia derived from the ectoderm (*Drosophila* imaginal discs) contain SJ with a more ladder-like appearance and is termed as pleated SJ (pSJ) (Figure 3C). These SJs are localised basal to the AJ (Figure 4). Endodermal epithelia (*Drosophila* midgut) contain SJ arranged parallelly (ultrastructure shows fingerprint-like

appearance) (Figure 3D). These SJ are located apicolaterally and the epithelia contain basal spot AJs instead of the apical adhesion belt (Figure 4). sSJ and pSJ also vary in protein composition; sSJ contains Snakeskin, Mesh and Tetraspanin-family protein, the proteins in pSJ are discussed below. The proteins in common are Dlg (Discs-Large), Lgl (Lethal-giant larve), FasIII (Fasciclin III) and Coracle (Cora). Herein, "septate junction/SJ'' refers to pSJ.

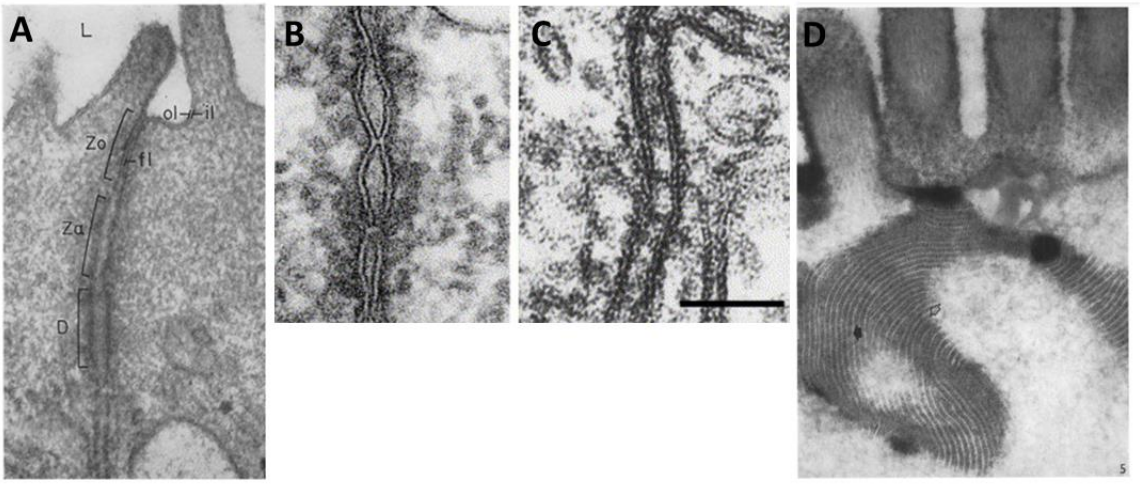

Figure 3: Ultrastructure Images of the Types of Occluding Junctions in Vertebrates and Invertebrates

(A) Section form the lining of the gall bladder from guinae pig shows two epithelial cells containing junctional complexes ZO (zonula occluden), ZA(zonula adheren), D (desmosome). Other labels are ol (outerleaflet), il (inner leaflet) and fl (fusion line). The intercellular space is entirely sealed near the tight junction/ZO but is seen to widen more basally. Image source:(Farquhar & Palade, 1963).

(B) Ultra-thin section of mouse epididymis showing the tight junction. Tight junctions contain intercellular focal contacts. Image source:(Furuse & Tsukita, 2006)

(C) Ultra-thin section of silkworm trachea showing pleated septate junction(pSJ) with a ladder-like cleft. Image source:(Furuse & Tsukita, 2006)

(D) Smooth sepatate junction (sSJ) from the midgut of moth (*Ephestia kuehniella*) contain more parallel organization. Image source:(Flower, 1975)

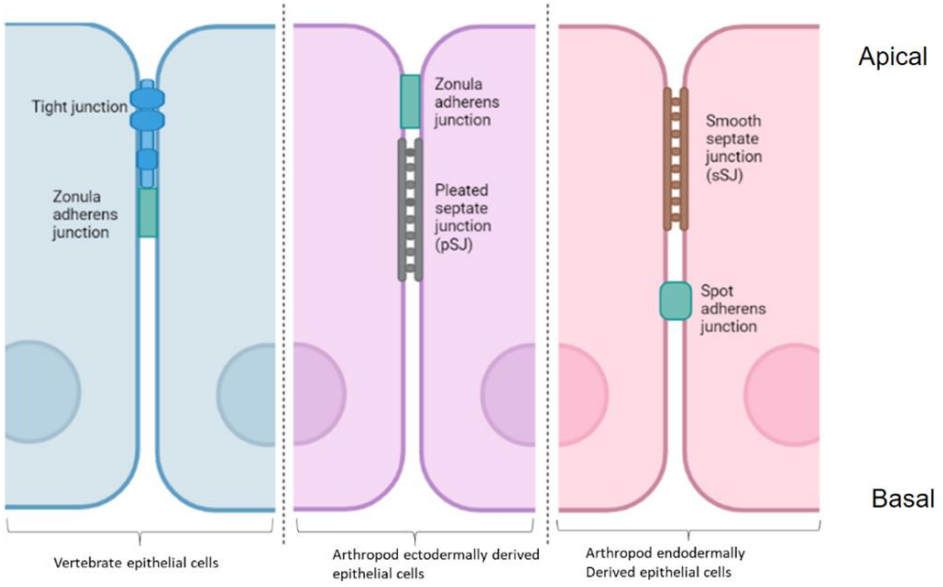

Figure 4: Schematic Representation of the Junction Complex Types and Organization. Tight junctions localize above AJ in vertebrates(blue). Invertebrate epithelia are of two types; ectodermal epithelia(purple) and endodermal epithelia(pink). Ectodermal epithelia contain pleated SJ(pSJ) which form basal to AJ, whereas endodermal epithelia contain smooth SJ(sSJ) in the apical region. Endodermal epithelia contain AJ as spots instead of the circumferential adhesion belt. Desmosomes have been omitted from vertebrate epithelia for clarity. Image is adapted from a figure in (Izumi & Furuse, 2014).

Despite there being some molecular and structural differences between TJ and SJ, similarities have also been found. For instance, *Drosophila* proteins Neurexin IV (Nrx IV), Contactin (Cont) and Neuroglian (Nrg) have vertebrate homologs NCP1, Contactin and NF155 which interact to maintain the paracellular barrier in the axo-glial cell junction (Bhat et al., 2001; Charles et al., 2002). The resemblance of paranodal SJ to invertebrate SJ suggests an evolutionary origin for SJ in the earliest metazoan.

Furthermore, TJ claudin-family proteins are found in *Drosophila* SJ (Behr et al., 2003). This provides an additional reason to use *Drosophila* as a model organism to understand Claudin protein counterparts in humans.

1.6 Cell Polarity

1.6.1 Overview

The asymmetric distribution of cellular contents into their distinctive compartments which moderate dynamic behaviours can be defined as cell polarity. In vertebrates and invertebrates, this is fundamental to regulate cell differentiation, cell migration(Etienne-Manneville & Hall, 2001), and processes of development.

Cell polarity is not only unique to multicellular organisms. Single-celled organisms such as *S. cerevisiae* (Baker's yeast) require polarity for budding (yeast cell division). The budding of the daughter cell from the mother cell is mediated by small GTPase Cdc42(cell division control protein 42) which signals arrangement of actin filaments and subsequent vesicular transport to the plasma membrane (W. J. Nelson, 2003).

In a typical monolayered epithelium, there is apicobasal polarity in the apical-to-basal axis and planar cell polarity (PCP) in the same plane of the epithelium(Cortijo et al., 2012). PCP is regulated by the Frizzled pathway which, in *Drosophila*, determines the direction of bristle growth (Vinson & Adler, 1987). A more recent study found a link between the two planes(Banerjee et al., 2017).

1.6.2 Apicobasal Polarity Determinants (ABP)

Maintenance of epithelial architecture depends on the interplay between polarity complexes present in the apical and basolateral domains of the epithelial cells. There are three main polarity complexes which regulate the apicobasal polarity – Crumbs, Partitioning-defective (Par) and Scribble localized in the apical, apical-lateral and basolateral domains, respectively (Figure 5).

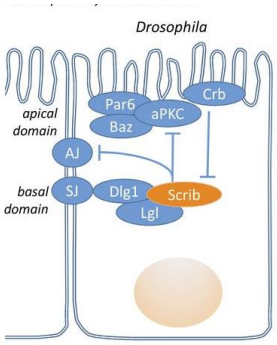

Figure 5: *Drosophila* ABP determinants. Crumbs complex is the most apical followed by Par and then Scribble.The Scribble complex localizes at the SJ. The three polarity modules negatively regulate one another to avoid the mislocalization into the wrong compartment. Image source:(Bonello & Peifer, 2019)

Crumbs consist of Crumbs (Crb), Stardust, Patj and Lin7. Par complex contains Bazooka (Baz), atypical protein kinase C(aPKC) and Par6. Scribble complex is composed of Dlg , Lgl,and Scrib. Research over the years has revealed the fundamentality of these complexes in regulating ABP (Tepaß & Knust, 1990; Hutterer et al., 2004; Khoury & Bilder, 2020). Proper compartmentalization requires the antagonistic interactions between the complexes(Figure 5). Scrib inhibits apical proteins such as Crumbs from localizing basolaterally(Bilder & Perrimon, 2000) while phosphorylation of Lgl by aPKC inhibits its apical localization.

In addition to the conventional polarity determinant complexes, Yurt/Cora group (core septate junction components, section 1.8) is involved in the basolateral polarity maintenance by antagonizing Crumbs. This occurs during organogenesis before septate junction is established.

Epithelial cells acquire migratory properties when ABP is lost. This epithelial-mesenchymal transition (EMT) process is a characteristic in metastatic cancers and is initiated by a transcription factor (SNAI1). The member of the Par complex,aPKC,was found to phosphorylate SNAI1 leading to its degradation allowing maintenance of ABP and preventing EMT (Jung et al., 2019).

1.7 Scribble Complex

1.7.1 Overview

In *Drosophila*, overproliferated epithelial tissue that has lost its monolayered organization, epithelial architecture, and ability to differentiate, can be termed as neoplasm. These characteristics are observed in imaginal disc epithelia when *lgl*, *dlg* (Figure 6B), and *scrib* (Figure 6C) are knocked down (Gateff & Schneiderman, 1974; Woods et al., 1996). These genes are thus termed as neoplastic tumour suppressors.

There was ectopic distribution of each protein in embryos mutant for the other two genes (Bilder et al., 2000). This revealed a functional interdependency between the proteins in the Scribble module. Furthermore, it is plausible to state that polarity and proliferation are tightly connected.

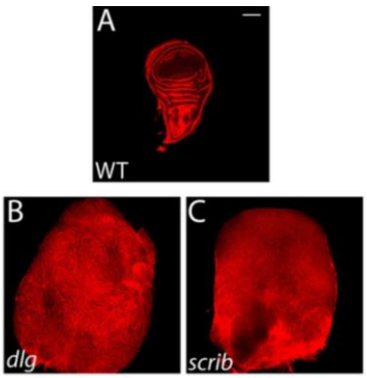

Figure 6: Third instar wing imaginal disc with *dlg* and *scrib* knockdown. Discs have been stained with F-actin staining to reveal dramatic overgrowth and architecture defects of *dlg* (B) and *scrib* (C) knockdown relative to wild-type (A). Image source:(Bunker et al., 2015)

Despite the genetic link between the three proteins, there is no direct interaction. However, it has been shown that Lgl can interact with the LRR domain of Scrib (Figure 7)(Kallay et al., 2006). In *Drosophila* neuromuscular junction, GUK-holder binds to the PDZ2 domain of Scrib and GUK domain of Dlg forming a tripartite complex (Mathew et al., 2002). Phosphorylated Lgl has also been found to interact with Dlg (Zhu et al., 2014).

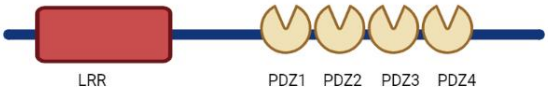

Figure 7: Protein Domains of Scrib. LRR domain is at the N-terminal (left end) and four PDZ domains are at the C-terminal (right end).

1.7.2 Scrib

Scrib protein contains 16 LRR and 4 PDZ domains(Figure 7). By carrying out a series of allelic mutations, the domains responsible for the different functions of Scrib were identified. PDZ domain is required for cell proliferation and LRR is required for the attachment of Scrib to the plasma membrane(Zeitler et al., 2004).

Next, studies proceeded to explain how loss of polarity lead to overgrowth. Yorkie (Yki) , part of the Hippo pathway, is normally phosphorylated by Warts (Wts) to be retained in the cytoplasm. When unphosphorylated, it moves to the nucleus to activate genes involved in

proliferation. Discs with the *scrib* mutation have high Yki activity resulting in overproliferation.

Research in the host lab recently revealed that Scrib is required to maintain polarity not only cell-autonomously but also in a non-cell-autonomous manner. Loss of Scrib expands from the site of knockdown to neighbouring cells over time. This phenomenon when loss of polarity occurs from suboptimal cells to wildtype cells is described as intercellular regressive aligment of ABP (Figure 8). It was found that AJ component, α-catenin, can interact with core SJ component to regulate localization of Scrib (Gui et al., 2021).

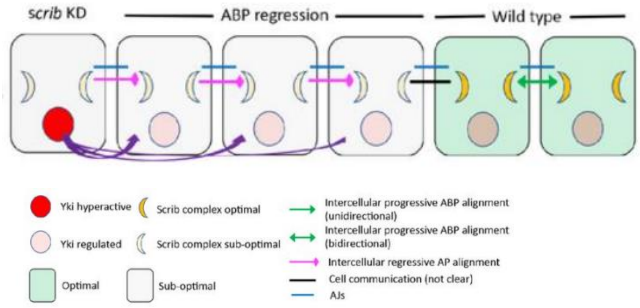

Figure 8: Intercellular Regressive Alignment of ABP. Yorkie signal activity after *scrib* knockdown facilitates loss of ABP. Image source:(Gui et al., 2021)

1.8 Septate Junctions

Septate junction is composed of multiple proteins with diverse protein domains. In addition to their role in epithelial barrier function, some proteins are required for dorsal closure and tracheal morphogenesis. Septate junction components can be characterized into core, resident, and accessory proteins.

1.8.1 Core Components

Core proteins are located in the SJ and are important for the formation and regulation of the junction (Oshima & Fehon, 2011). These proteins are highly interdependent such that removal of one component affects the whole composition.

Sinuous (Sinu) (Wu et al., 2004), Kune-kune (Kune) (K. S. Nelson et al., 2010), and Megatrachea (Mega)/ Pickel (Pck) (Behr et al., 2003) are proteins belonging to the claudin superfamily. Claudins contain four membrane domains, intracellular N-terminal and C-terminal, a large extracellular loop and two smaller loops. At the C terminal end is a binding site for

the PDZ domain. Mutant embryos show aberrant distribution of the proteins from their residence in the region basal to AJ ,without loss of apicobasal polarity. These proteins are required for barrier function. In contrast to Kune and Mega, Sinu is required for SJ organization but not formation. Loss-of-function of *sinu* results in SJ losing its ladder-like structure and the number of septa, but is not involved in the formation of SJ.

There are two septate junction components from the 4.1 superfamily of proteins: Cora and Yurt. Both contain N-terminal FERM domain which binds to intracellular cytoplasmic proteins. Loss of Cora result in several embryonic defects such as dorsal closure, tracheal morphogenesis, establishment of SJ and permeability function. Cora associates with Nrx-IV at the plasma membrane. Although the human 4.1 protein directly interacts with human Dlg, this interaction is not seen in *Drosophila.* Cora mutants show normal localization of Dlg and the pair does not coimmunoprecipitate (Ward IV et al., 1998).

Other core septate junction components include: Na,K-ATPase involved in the pumping of ions across the plasma membrane contains subunits α and β. These were found to involve in SJ formation providing a role apart from ATPase catalytic activity(Paul et al., 2007); Neuroglian , a member of the Immunoglobulin(Ig) superfamily of CAM(cell adhesion molecules) that forms a complex with Cor/NrxIV/Na+K+ATPase(Genova & Fehon, 2003); and Varicose(Vari) is a member of the MAGUK proteins that act as scaffold proteins(like Dlg). It has been shown to involve in paracellular barrier function as well as tracheal morphogenesis and adult hair patterning(Moyer & Jacobs, 2008). Finally, Pasi1 and Pasi2, are also required for in the permeability function in the BBB and have been added to core components list recently(Deligiannaki et al., 2015). Additional core components are Lachesin(lac) and Wurmchen(wrm).

1.8.2 Resident Components

Resident proteins are located in the junction but are not involved in the formation and regulation. Some resident components include Scrib, Dlg ,FasIII (Fasciclin III) and Yurt. Recent research shows *yurt*/*cora* double mutant results in loss of apicobasal polarity in all ectodermal epithelia(Laprise et al., 2009)

1.8.3 Accessory Components

These proteins are not located in the junction but are necessary to form and maintain it. Examples include Ly6 superfamily proteins Boudin(Hijazi et al., 2009), Coiled(Hijazi et al.,

2011) and Crooked(Nilton et al., 2010). Interestingly, Boudin was found to non-autonomously rescue SJ aberrant phenotype in wing disc epithelia.

Furthermore, tricellular junctions (TCJ) studies are becoming popular topic in the recent years(Bosveld et al., 2018). These junctions form at the point where three cells meet. Tricellular septate junction(tSJ) proteins are important to maintain bicellular septate junction integrity. Components of tSJ are Gliotactin, M6 and Bark(Esmangart de Bournonville & le Borgne, 2020).

1.9 Tools

1.9.1 RNA interference (RNAi)

Gene silencing by RNAi(Figure 9) has become a widely used technique in reverse genetics to study how loss of function of a gene may affect the phenotype of an organism. Initially discovered in *C. elegans*(Fire et al., 1998), this method is now part of the Genetic Toolkit in *Drosophila* and can be used in combination with the GAL4/UAS system (see below).

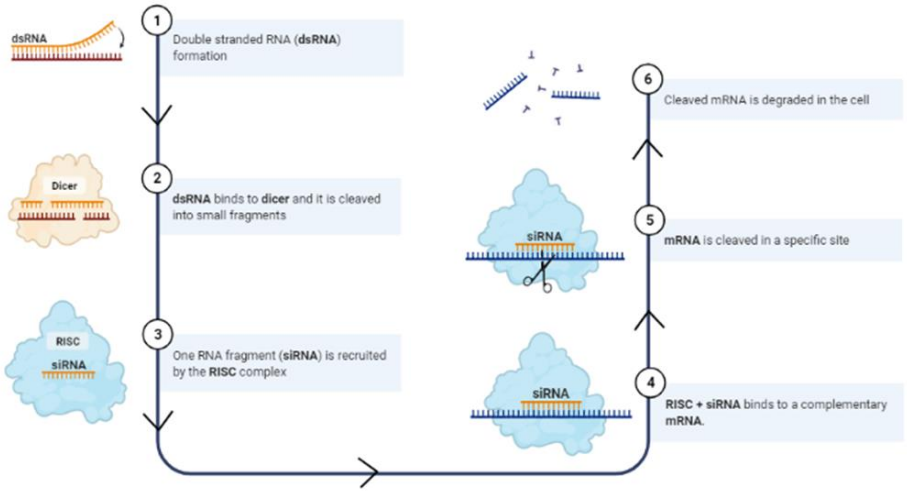

Figure 9: Mechanism of Gene Silencing. The double-stranded RNA (dsRNA) is cleaved into ~19bp single-stranded (siRNA) which integrate into the RISC complex. The siRNA is directed to its homologous mRNA to which it binds and is eventually degraded.

1.9.2 UAS/GAL4/GAL80^{ts}

GAL4 is a yeast transcriptional activator which can bind to Upstream Activating Sequence (UAS) to express downstream target genes. GAL4 expression can be regulated by a tissue-specific promoter to induce the transcription of the target gene in a tissue-specific manner(Figure 10A). Here, GAL4 expression is driven by the *patched* promoter and the target RNAi is expressed specifically in the *ptc* region (between AP boundary)(Figure 10B).

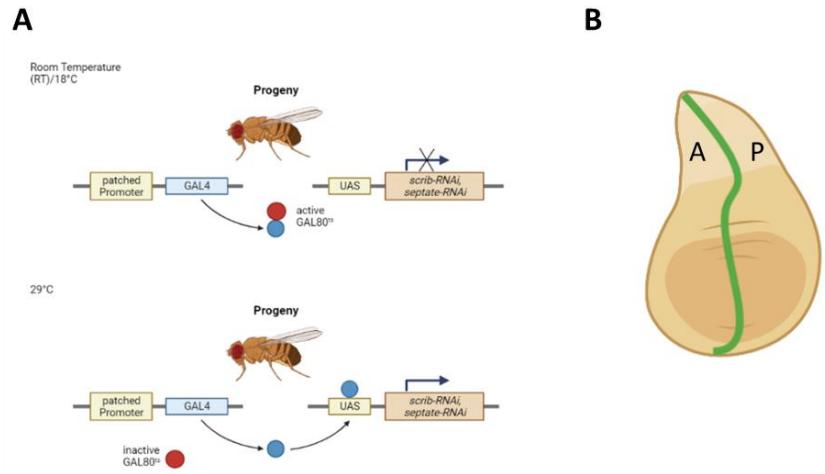

Figure 10: Conditional knockdown of RNAi using UAS/GAL4/GAL80^{ts} system. *Patched (ptc)* can be coupled with GAL4 to drive the expression of UAS-*scrib-RNAi and* UAS-*septate-RNAi* (A), in the anterior-posterior(AP) boundary of the wing imaginal disc (B).

Temperature-sensitive GAL80 protein (GAL80^{ts}), on the other hand, can bind to the GAL4 transcriptional activation domain preventing the RNA polymerase from initiating transcription. At 18°C, GAL80^{ts} is active and inhibits GAL4 activity. At 29°C, GAL80^{ts} is inactive and GAL4 drives the expression of the target gene (McGuire et al., 2003)(Figure 10A). Thus, GAL4/UAS along with GAL80^{ts} proves valuable to assess epithelial tissue growth over time (spatio-temporal manner).

2 THE AIM OF THE THESIS

- Hypothesizing that septate junction components are involved in intercellular communications regulating apicobasal polarity and tissue homeostasis, this study aims to carry out *in vivo* RNAi screening and identify candidate genes that have genetic interactions with Scrib.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Fly Strains

The following fly strains were used:

RNAi in the third chromosome: *atpα* (#33646) , *cora* (#35003) and *cont* (#34867) (Bloomington *Drosophila* Stock Center, BDSC, Indiana,USA).

RNAi in the second chromosome: *nrg*(#37496), *sinu*(#38258), *lac*(#38536), *nrx-IV*(#39071), *cora*(#51845), *M6*(#54032), *wrm1*(#63596), *mcr*(#65896), *fas3*(#77396), *kune*(#38295), *bark*(#67014), *crim*(#65362) (Bloomington *Drosophila* Stock Center, BDSC, Indiana,USA), *pasi1*(v102223), *pasi2*(#105806) and *vari*(v104548) (Vienna Drosophila Resource Center,VDRC, Austria).

The control lines: #36303 and #36304 (Bloomington *Drosophila* Stock Center,BDSC, Indiana,USA) are used as the control lines of the third chromosome and second chromosome, respectively.

Fly stocks *ex-lacZ*, *ptc-GAL4*, UAS-*EGFP* /CyO; *scrib* RNAi, Gal80ts/Tm6BTb genotype (herein referred to as *scrib*-RNAi) were generated through the recombination of the following stocks: *ex-lacZ*, *ptc-GAL4*, UAS-*EGFP* (Gui et al., 2021) , UAS-*scrib* RNAi (#35748),and *tub*P-*GAL80*^{ts}(#7017) (Bloomington *Drosophila* Stock Center, BDSC, Indiana,USA).

3.1.2 Fly Crosses

Female flies containing septate junction component RNAi were crossed with male flies containing the *scrib*-RNAi. Female flies with the control RNAi lines were crossed with male flies containing *scrib*-RNAi. All collected female flies were virgins to ensure that there were no stored sperm from previous matings. While the total number of flies varied in each cross, the ratio 3:1 (females:males) was maintained.

The progeny viability was qualitatively assessed by observing the abundance of thirdinstar larvae in each cross.

3.1.3 Temperature Shift and Conditional Knockdown

The UAS/GAL4/Gal80 temperature-sensitive system is used for the conditional knockdown of the target gene in a spatiotemporal manner (see literature review section, Tools). Therefore, the knockdown of *scrib* and septate genes only occur at 29°C.

Two conditions were implemented to investigate the extent of the proliferation of the wing imaginal disc (Figure 11):

i) 2 days (2D) after temperature shift (ATS)(Figure 11A)

The day the fly cross is considered as day 0. After 24 hours the flies are tipped into a new vial. By day 1, the laid eggs are visible on the surface of the food. The vial is kept at room temperature (18°C) for four days (from day 1 to day 5). On day 5 (the day of the temperature shift) the vial is transferred to the 29°C incubator for two days. On day 7, the third instar larvae are dissected under the microscope to obtain the wing imaginal discs. The larvae appear on the walls of the vial as they move away from moist conditions in the food to drier surfaces.

ii) 5 days (5D) after temperature shift (ATS)(Figure 11B)

Similar to (i), on day 0 the flies are crossed and after a 24-hour mating period the flies are transferred to a fresh vial (on day 1) while the vial containing eggs is placed at room temperature for another day. Here, the temperature shift takes place earlier (on day 2) to observe a progressed tissue proliferation in contrast to the condition above. The vial is kept at 29°C for five days and third-instar larvae are dissected on day 7.

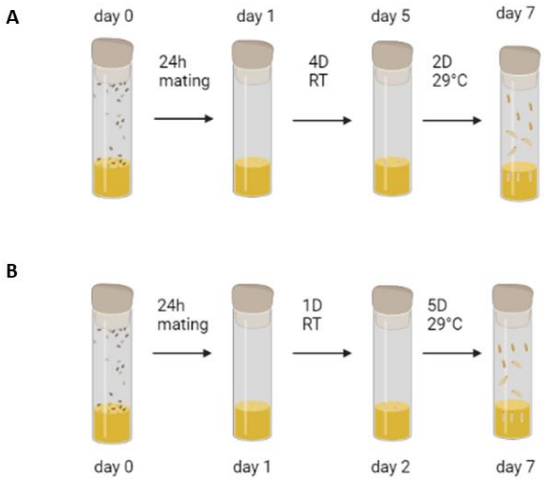

Figure 11: Schematic representation of the procedure. (A) two days (2D) and (B) five days (5D) after temperature shift(ATS)/29°C/after knockdown initiation. Room temperature (RT) is 18°C. Larvae begin to appear on the food already on the 5th day. On the 7th day, third instar larvae on the walls of the vial are selected for dissection.

3.1.4 Immunohistochemistry

The third instar larvae were dissected in PBS(phosphate buffered saline). The dissected quarter of the upper body was fixed in 3.7% formaldehyde solution (37% formaldehyde diluted in PBT(PBS + 0.1% Tween 20) for 20 minutes. The washing step was performed twice using 500µl of PBT with an incubation time of 5 minutes between washes. DAPI (4′,6-diamidino-2-phenylindole)(1:1000, Thermo Fisher) was then added to 500µl of PBT and incubated for 30 minutes. The washing step was performed again to remove any residual DAPI. The larvae were further dissected in PBT to obtain the wing imaginal discs. The isolated discs were finally mounted in 70% glycerol (glycerol + PBS). In an exceptional case, to check for the distribution of FasIII, mouse anti-FasIII primary antibody (1:50, Developmental Studies Hybridoma Bank) was used. Mouse Alexa Fluor 568(1:500, Thermo Fisher) was added as the secondary antibody.

3.1.5 Imaging and Area Calculation Using ImageJ

All samples were viewed under the Olympus BX51 fluorescence microscope using 20X objective. The obtained images were opened in ImageJ and area was calculated as follows: set scale > convert image to 8bit > adjust threshold > measure. All calculations(GFP(%GFP area) = (area of GFP stripe/ area of wing disc) x 100) and graphs were generated in Excel.

3.2 RESULTS

3.2.1 Initial Elimination

Initially, 18 septate junction components have been listed for the screening and these were narrowed down to 11 based on the fecundity of the flies. Table 1 summarizes the results of the crosses carried out 2D and 5D ATS. Highlighted in grey are the fly crosses that failed to produce larvae for dissection.

Table 1: Percentage GFP area for septate junction candidates from 2D and 5D ATS.

Name of cross	Type of SJ	Wing disc area	GFP area	%GFP area	Wing disc area	GFP area	%GFP area
Second chromosome		2D ATS			5D ATS		
Kune	core						
Nrx-IV	core						
Nrg	core	0.328	0.037	11.3	0.251	0.099	39.4
Crim	core						
Bark	tSJ						
Mcr	core	0.176	0.011	6.25	0.209	0.108	51.7
Fas III	resident	0.181	0.012	6.63	0.201	0.104	51.9
Lac	core	0.389	0.028	7.20	0.433	0.112	25.9
M6	tSJ	0.183	0.01	5.46	0.372	0.155	41.7
Sinu	core	0.256	0.0245	9.57	0.235	0.152	64.7
Pasi1	core	0.174	0.011	6.32	0.248	0.065	26.2
Wrm	core						
Cora	core	0.165	0.009	5.45	0.397	0.116	29.2
Pasi2	core						
Vari	core	0.169	0.012	7.10	0.251	0.0963	38.4
control		0.211	0.0253	12.0	0.203	0.094	46.3
Third chromosome		2D ATS			5D ATS		
Cont	core						
ATPα	core	0.182	0.016	8.8	0.183	0.0750	41.0
Cora	core	0.134	0.018	13.4	0.207	0.107	51.7
control		0.14	0.0148	10.6	0.228	0.107	46.9

3.2.2 Control Lines

Two controls were used to compare with the septate lines expressed on the third chromosome and second chromosome (Figure 12) (Figure 13). The GFP-positive *patched* (*ptc*) stripe in the AP boundary represents *scrib* RNAi cells. The surrounding GFP-negative cells represent wildtype cells (Figure 12). 2D ATS, the discs appear to have their normal architecture and the GFP stripe is narrow with a %GFP area of about 12% (Table 1) . However, 5D ATS, the cells undergo proliferation and disc appears overgrown. The %GFP area after 5D increased to about 46% (Table 1).

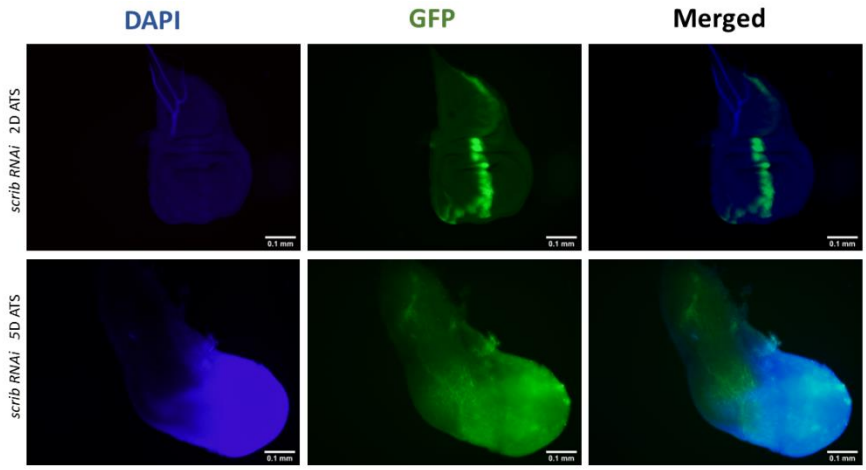

Figure 12: Images of 3rd instar wing imaginal discs. Conditional scrib RNAi (ptc-GAL4, UAS-GFP, ex-lacZ/+; GAL80ts, UAS-scrib RNAi/ +) two days 2D and 5D induction of scrib knockdown. DAPI were used to visualize DNA (blue), GFP (green), and merged image of GFP and DAPI. Scale bar: 0.1mm

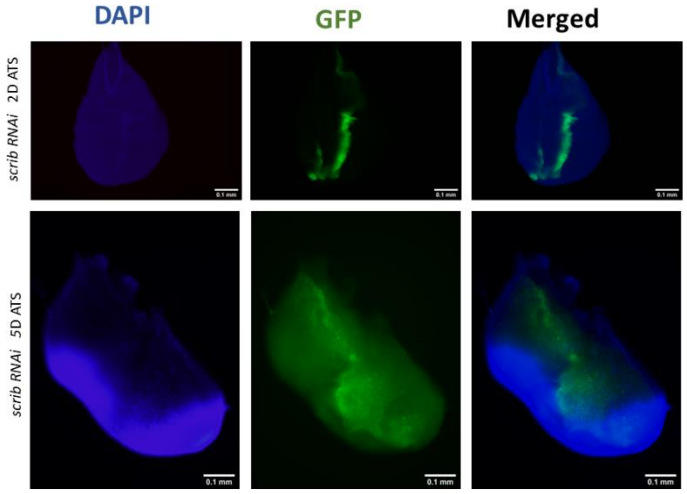

Figure 13: Images of 3rd instar wing imaginal discs. Conditional scrib RNAi (ptc-GAL4, UAS-GFP, ex-lacZ/+; GAL80ts, UAS-scrib RNAi/ +) two days 2D and 5D induction of scrib knockdown. DAPI were used to visualize DNA (blue), GFP (green), and merged image of GFP and DAPI. Scale bar: 0.1mm

3.2.3 Conditional Knockdown of Septate Junction Components in Third Chromosome

NB! Septate names mentioned mean septate and scrib RNAi for e.x:- ATPα means ATPα and *scrib* knockdown together.

All the discs looked similar to the control with only *scrib* knockdown but the GFP stripe appeared stronger in *cora* knockdown(Figure 14C). When the area of the GFP stripe was quantified, only Cora showed an increase in %GFP area (13.4%) while ATPα showed a decrease of 8.8% (Figure 14D).

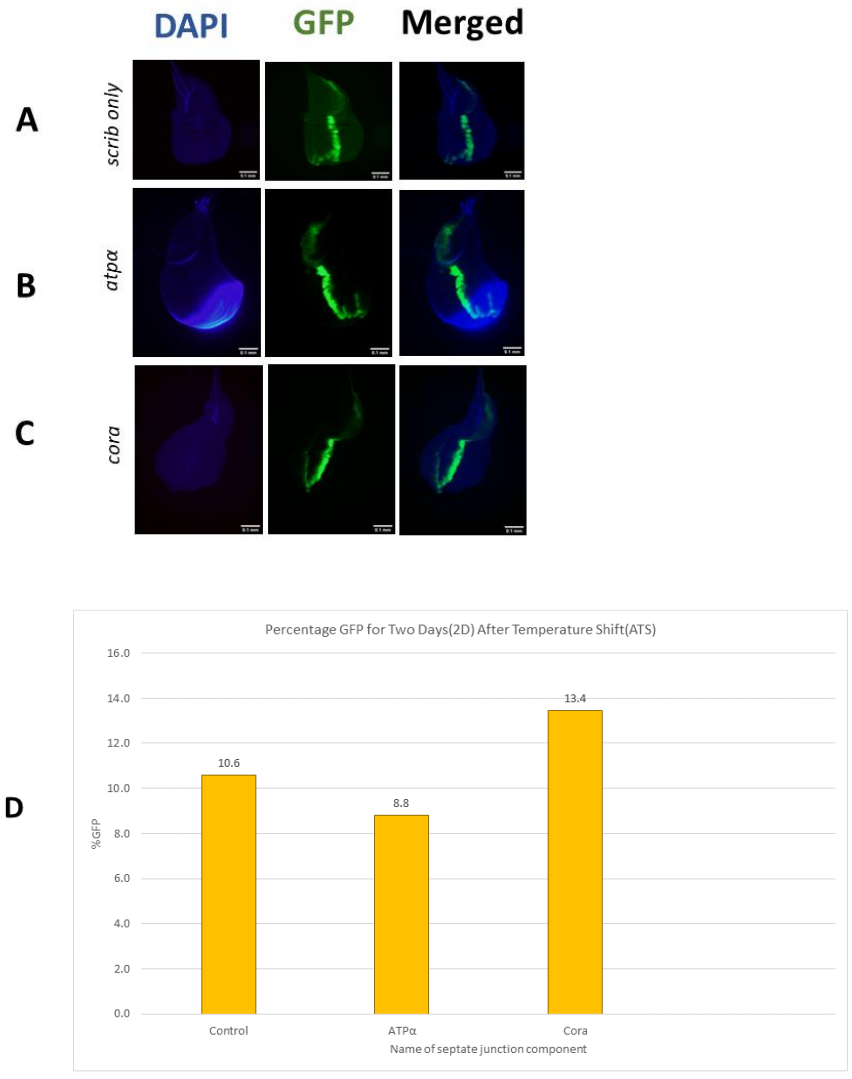

Figure 14: Images of 3rd instar wing imaginal discs. Conditional septate RNAi (ptc-GAL4, UAS-GFP, ex-lacZ/+; GAL80ts, UAS-scrib RNAi/ UAS-septate RNAi) two days of *scrib* knockdown. DAPI was used to visualize DNA (blue), GFP (green), and merged image of GFP and DAPI. (A) scrib-RNAi alone (B)*atpα* (C)*cora* (D)graphical representation of %GFP area. Scale bar: 0.1mm

5D ATS, all the discs increased in size similar to the control(Figure 15). ATPα showed strong GFP but was less distributed compared to control (Figure 15B). Cora had the highest increase in GFP area reaching a coverage of over 50%(Figure 15C and D).

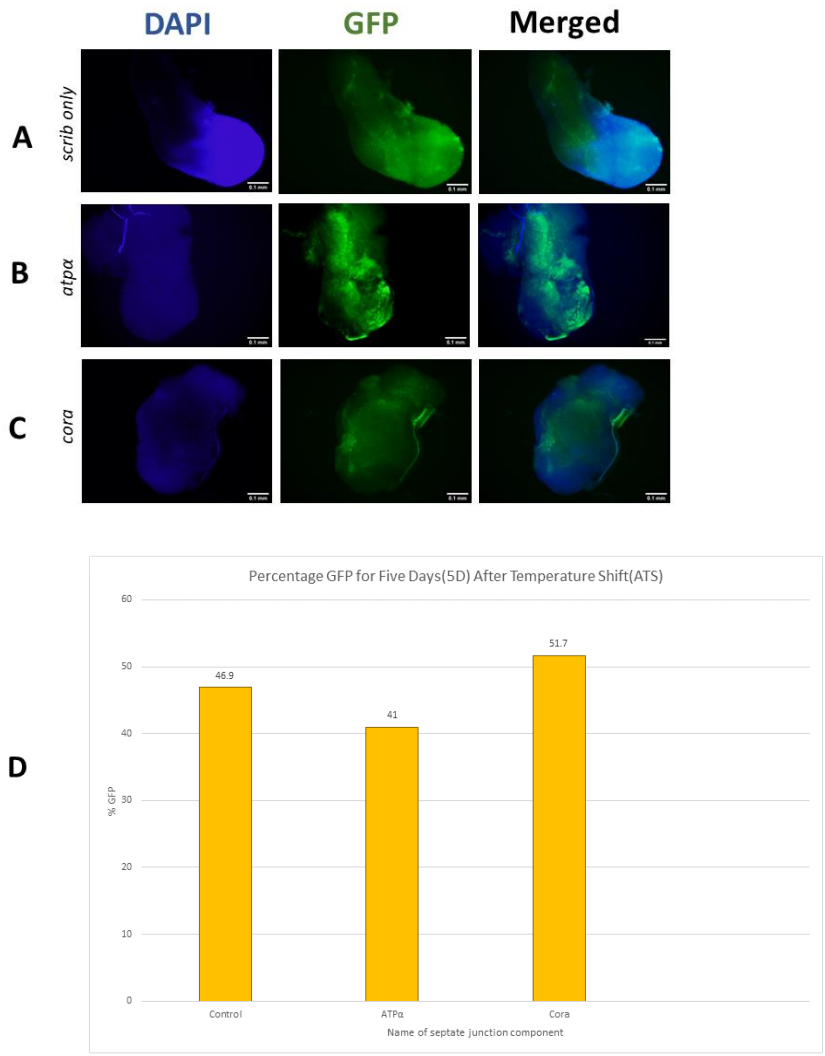

Figure 15: Images of 3rd instar wing imaginal discs. Conditional septate RNAi (ptc-GAL4, UAS-GFP, ex-lacZ/+; GAL80ts, UAS-scrib RNAi/ UAS-septate RNAi) five days of *scrib* knockdown. DAPI was used to visualize DNA (blue), GFP (green), and merged image of GFP and DAPI. (A) scrib-RNAi alone (B)*atpα* (C)*cora* (D) graphical representation of %GFP area. Scale bar:0.1mm

3.2.4 Conditional Knockdown of Septate Junction Components in Second Chromosome

2D ATS, GFP stripe appeared to be stronger in most discs and interestingly the stripes looked narrower than the control (Figure 16). In line with the observations, the GFP area appeared to decrease in all the discs when quantified (Figure 16K).

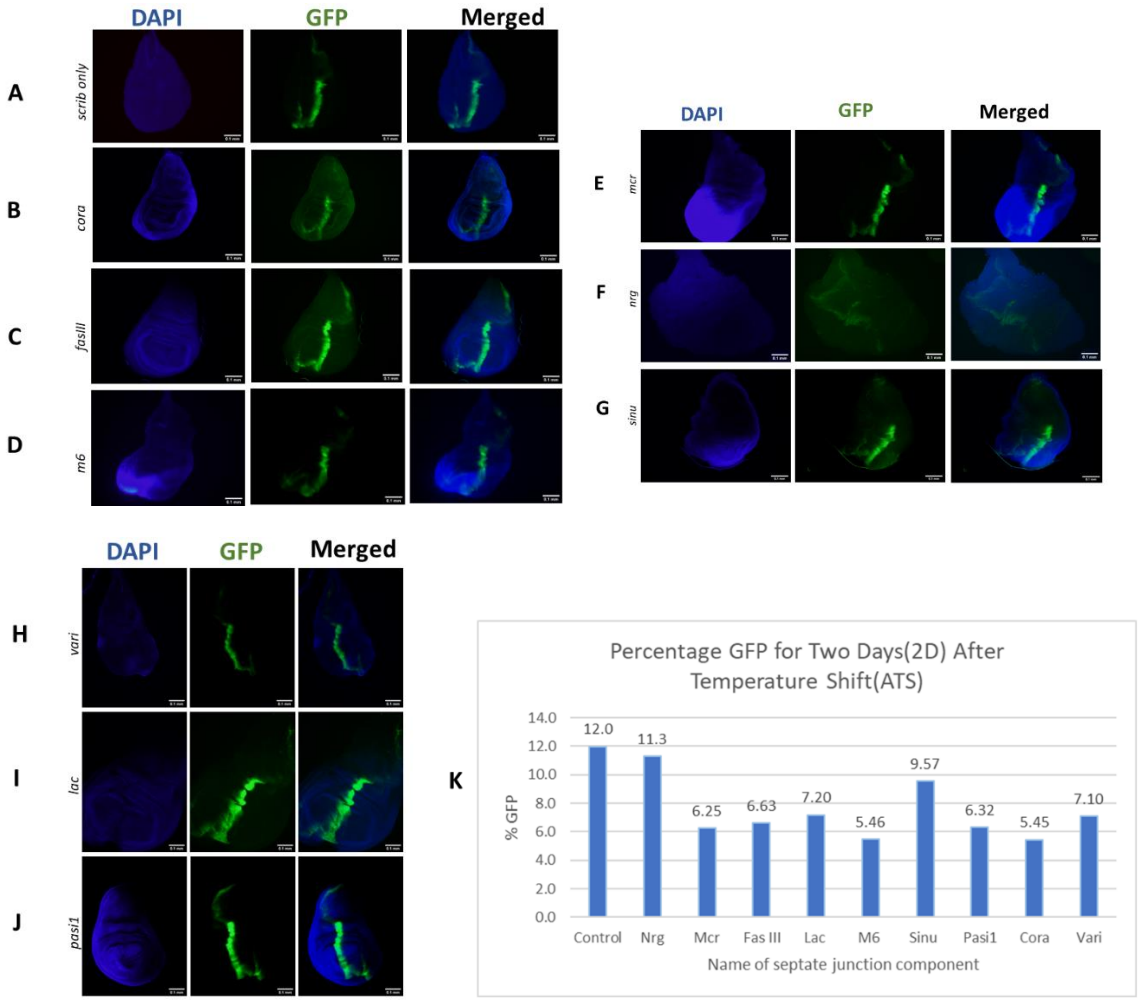

Figure 16: Images of 3rd instar wing imaginal discs. Conditional septate RNAi (ptc-GAL4, UAS-GFP, ex-lacZ/UAS-septate-RNAi; GAL80ts, UAS-scrib RNAi/ +) two days of scrib knockdown. DAPI was used to visualize DNA (blue), GFP (green), and merged image of GFP and DAPI. (A) scrib-RNAi alone (B-J) names of septate genes knocked down with *scrib* (K) graphical representation of %GFP area. Scale bar: 0.1mm.

5D ATS, the discs appeared proliferated and the GFP covered almost half of the tissue (Figure 17). Here, Mcr and FasIII appeared to have similar coverage of around 50% while the lowest was in Lac, Pasi1 and Cora (Figure 17K). Despite having lower GFP in 2D ATS, Mcr, FasIII and Sinu had increased GFP compared to the control. Sinu had the highest GFP coverage of them all(Figure 17K).

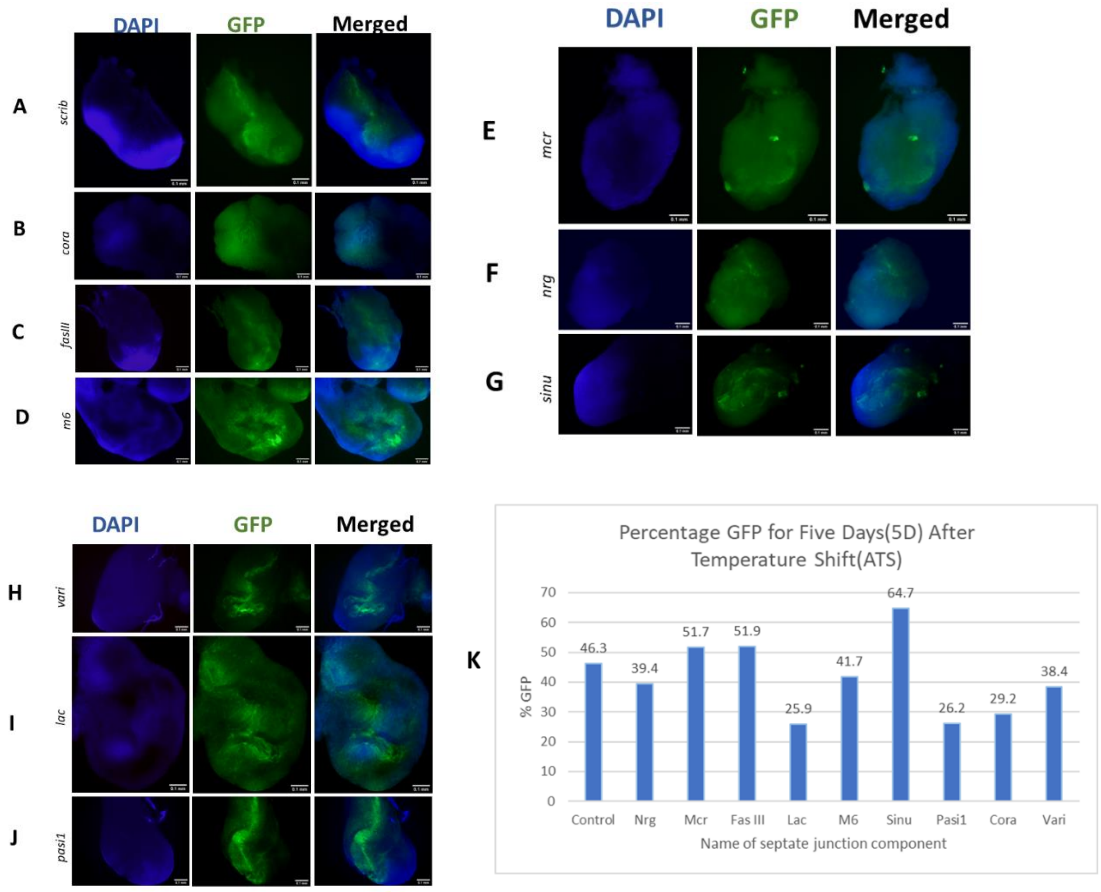

Figure 17: Images of 3rd instar wing imaginal discs. Conditional septate RNAi (ptc-GAL4, UAS-GFP, ex-lacZ/UAS-septate-RNAi; GAL80ts, UAS-scrib RNAi/ +) five days of *scrib* knockdown. DAPI were used to visualize DNA (blue), GFP (green), and merged image of GFP and DAPI. (A) scrib-RNAi alone (B-J) names of septate genes knocked down with scrib (K) graphical representation of %GFP area. Scale bar:0.1mm

Additionally, FasIII was added to observe how the wing tissue looks like upon conditional knockdown of FasIII and Scrib (Figure 18). The disc two days after knockdown appears to have a hollow (unstained) AP region that has expanded slightly towards the anterior end.

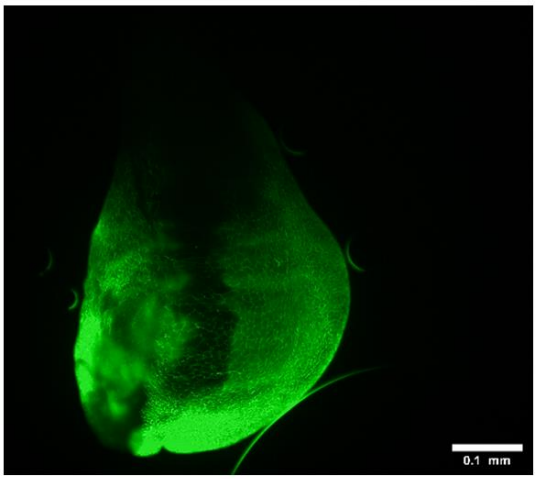

Figure 18: FasIII and Scrib knockdown in third instar wing disc, 2D ATS. FasIII (green) stained to observe the distribution of the septate protein in the knockdown tissue. The *ptc* AP boundary is unstained indicating FasIII proteins are absent in the region. The FasIII knockdown has affected the anterior flanking cells. Anterior is left and posterior is right. Scale bar:0.1mm

3.3 DISCUSSION

3.3.1 Conditional Knockdown of Scrib

Scribble (Scrib) is an apicobasal polarity determinant and loss-of- function of *scrib* leads to loss of epithelial cell polarity, proliferation and overgrowth of tissue (Bilder & Perrimon, 2000). Previous studies report that *scrib* knockdown not only lead to loss of apicobasal polarity in an autonomous manner but also to neighbouring flanking cells in a non-autonomous manner(Gui et al., 2021). This results in expansion of loss of polarity and overgrowth across the tissue over time. The initial narrow GFP stripe can be interpreted as *scrib* knockdown cells within the *ptc* region. However, about four days to five days after knockdown, there is intercellular regressive alignment of polarity where the disc cells change from WT optimal to suboptimal conditions. Interestingly, it was found that AJ component α-catenin can bind to core SJ component, Kune, to regulate basolateral localization of Scrib (Gui et al., 2021). Knockdown of Kune, alone, also showed loss of Scrib but loss of Scrib was greater when α-catenin and Kune were both co-knocked down.

This study takes a step further by determining if other SJ components genetically interact with Scrib. Here, the influence of SJ components on the non-cell autonomous loss of Scrib to neighbouring cells is determined. The AP boundary of the wing imaginal disc contains both loss-of-function of Scrib and specific SJ component. The effect of the double knockdown on the area of GFP stripe is studied two days and five days after the knockdown begins.

3.3.2 Narrowing the Candidates

By five days of conditional *scrib* knockdown, the GFP area reached up to 47%. This coverage reached above 50% in double knockdown with Mcr, FasIII, Sinu or Cora. From the 11 candidates tested, these four candidates reveal to be important in regulating basolateral polarity.

Based on the increased GFP area in *cora*/*scrib* knockdown discs, it may genetically be involved in regulating Scrib in the basolateral domain. Although this wasn't the case with Cora knockdown from the second chromosome.

While double knockdown of Mcr, FasIII or Sinu with *scrib-RNAi* had low GFP area two days after knockdown, this coverage increased after five days. This indicates that these proteins may not be necessary to localize Scrib until after majority of the cells have lost Scrib. Figure 19 summarizes the interplay involved in intercellular loss of Scrib and the septate proteins that deem most important in this interaction.

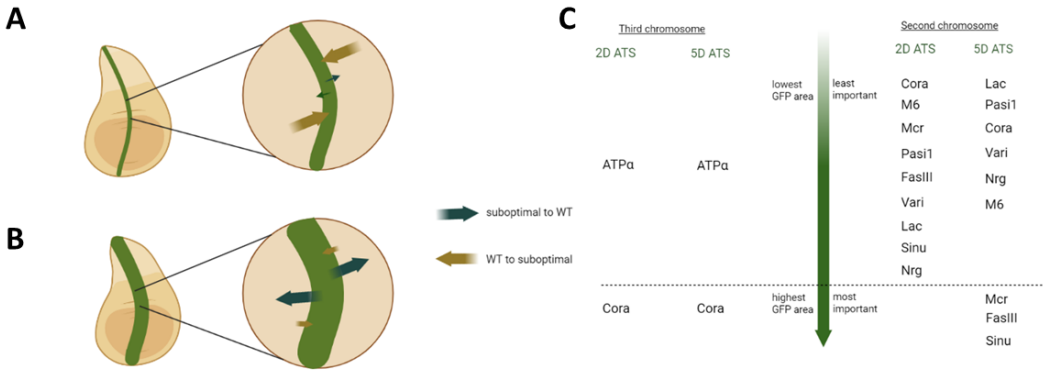

Figure 19: Schematic diagram representing the cell competition between wildtype (WT) cells and *scrib-septate* knockdown cells in the *ptc* (patched) region which also expresses GFP. (A) Scenario where WT cells have more advantage over knockdown cells resulting in the prevention of GFP-associated loss of Scrib to neighbouring cells. (B) Represents a scenario where the suboptimal knockdown cells have an advantage in outcompeting the WT cells and Scrib is lost in neighbouring cells as denoted by the expansion of GFP. (C) Shows septate junction proteins in order of importance in suppressing loss of polarity to neighbouring cells. For the genes expressed in the third chromosome, the hierarchy is the same for both conditions. For the genes expressed in the second chromosome, only three candidates are present.

Finally, this study also shows that FasIII(Figure 18) expression is significantly suppressed after *scrib* knockdown, indicating that genetic interactions between Scrib and FasIII might be key for the process. Since previous studies also suggest genetic interactions between Scrib and Kune / Nrx-IV, cooperation between the Scrib complex and septate junction complex may be a conserved role during epithelial morphogenesis. Future studies will unveil these interactions in tissue growth and homeostasis.

SUMMARY

This study aims to identify septate junction components which could possibly interact with basolateral polarity determinant, Scribble (Scrib), to regulate intercellular coordination between optimal and suboptimal cells. By employing conditional knockdown using UAS/GAL4/GAL80^{ts} system, septate junction component and Scrib are co-knocked down in the wing imaginal disc in a spatiotemporal manner. In this study, 18 septate junction components have been tested. Among them 7 were eliminated based on low fecundity of the flies. From the remaining 11 candidates, Coracle was found to suppress the loss of Scrib to the neighbouring cells. Macroglobulin, Fasciclin III, and Sinuous appear to be important in later stages after polarity loss. In conclusion, this study identifies previously undetermined roles for cell polarity regulation and tissue homeostasis of the septate junction components through intercellular communication.

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