Tricellular Junction Regulation, Signaling and Scaffolding

by

Zohreh Sharifkhodaei

B.Sc. Animal Biology, Shahid Beheshti University, 2006
M.Sc. Animal Physiology, Tarbiat Moallem University, 2008

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Zoology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

October 2017

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Abstract

The focus of this thesis is to understand the regulatory mechanisms of that control permeability barriers in epithelia. A key role of epithelia is to maintain permeability barriers between tissues. Epithelial junctions are formed to establish a functional barrier between the cells and to ensure cell-cell adhesion. In *Drosophila*, the tricellular junction (TCJ) generates a barrier at the contact site of three adjacent epithelial cells. Gliotactin is localized exclusively at the corner of three epithelial cells and loss of Gliotactin from the TCJ disrupts the barrier function. Conversely, overexpression of Gliotactin triggers the spread of Gliotactin away from the TCJ leading to apoptosis, delamination, overproliferation and cell migration. Therefore, the expression level of Gliotactin needs to be tightly regulated. Gliotactin protein levels are controlled by tyrosine phosphorylation and subsequent protein endocytosis and degradation. Here we found that Gliotactin expression is also tightly regulated at the mRNA level through microRNA-184. miR-184 targets the Gliotactin 3’UTR and other septate junction mRNAs including NrxIV and Mcr. Gliotactin overexpression triggers BMP signaling through inhibition of Dad, an inhibitory SMAD, and activation of the Tkv type-I receptor and Mad. Elevated level of phosphorylated MAD leads to induction of miR-184 expression. Regulation of Gliotactin at the TCJ is mediated through a Gliotactin-BMP-miR184 feedback loop. We identified a new complex at the TCJ, which regulates junction assembly and function. The scaffolding proteins Scribbled (Scrib) and Discs Large (Dlg) are in close proximity with two TCJ components, Gliotactin and Bark beetle (Bark). The presence of the Scrib PDZ1-2 and the Dlg GUK domains are required for proper formation of the TCJ complex. Loss of Bark or Gliotactin from the TCJ leads to basolateral spread of Scrib and Dlg, while Scrib or Dlg knockdown disrupts the integrity of the complex and promotes the loss of Bark or Gliotactin from the TCJ. Our proposed model suggests that Scrib
and Dlg recruit Bark to the TCJ, which in turn leads to Gliotactin recruitment to the TCJ. Overall, we propose that tricellular junction is regulated through two distinct mechanisms, signaling and scaffolding.
Lay summary

The main reason that microbes, chemicals, and fluids cannot pass through our skin or intestine is due to sealing elements. Sealing elements work like glue and tightly seal the spaces between cells. The glues are made from groups of proteins and form a junction between neighboring cells and at the corner of cells. One of the main proteins that play a role to glue the cells at the corners of cells is Gliotactin. The amount of Gliotactin needs to be regulated. Too little Gliotactin destroys the sealed region, thus, chemicals pass between and through tissues. Too much Gliotactin kills the cells, and causes various disorders similar to cancer. We identified a new way to control the levels of Gliotactin, and a new group of proteins working with Gliotactin as the glue at the cell corners.
Preface

Chapter 2: “The *Drosophila* tricellular junction protein Gliotactin regulates its own mRNA levels through BMP-mediated induction of miR-184”


For this publication I contributed to all aspects of the project, including study design, data collection, analysis and interpretation. M.P.-B contributed to data collection for the reverse transcriptase PCR experiment (Figure 2.6A). M.M.G. contributed to the generation of the Gliotactin transgenic lines (Figure 2.2). G.S. contributed to data collection for co-expression of Gliotactin with Tkv-DN experiment (Figure 2.10A-B). T.A.F. and D.V.V generated the miR-Sponge transgenic lines. V.J.A contributed study conception, design, and data interpretation.

Chapter 3: “Scribbled mediates the tricellular Junction formation in *Drosophila*”

Work in this chapter has produced a manuscript in preparation as: Sharifkhodaei Z., Gilbert M.M, and Auld V.J. Scribbled mediates the tricellular Junction formation in *Drosophila*.

For this publication I contributed to all aspects of the project, including study design, data collection, analysis and interpretation. M.M.G. determined that the Scribbled and Gliotactin proteins are in close proximity at the tricellular junction. V.J.A contributed study conception, design, and data interpretation.
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<th>Description</th>
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<tbody>
<tr>
<td>Abl</td>
<td>Abelson Kinase</td>
</tr>
<tr>
<td>ap</td>
<td>Apteronous</td>
</tr>
<tr>
<td>ALM</td>
<td>Apical margin of the lateral membrane</td>
</tr>
<tr>
<td>ATPα</td>
<td>α-Subunit</td>
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<tr>
<td>Bark</td>
<td>Bark beetle</td>
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<tr>
<td>BBB</td>
<td>Blood-brain-barrier</td>
</tr>
<tr>
<td>BCB</td>
<td>Blood-cerebrospinal fluid-barrier</td>
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<tr>
<td>BFP</td>
<td>Blue fluorescent protein</td>
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<td>Bone morphogenetic protein</td>
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<td>Basket</td>
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<tr>
<td>Cont</td>
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<td>Coracle</td>
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<tr>
<td>da</td>
<td>Daughterless</td>
</tr>
<tr>
<td>Dad</td>
<td>Daughters against dpp</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<td>Dlg</td>
<td>Discs large</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant negative</td>
</tr>
<tr>
<td>Dpp</td>
<td>Decapentaplegic</td>
</tr>
<tr>
<td>E-cad</td>
<td>E-cadherin</td>
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<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>EpCAM</td>
<td>Epithelial-specific cell adhesion molecule</td>
</tr>
<tr>
<td>ERKs</td>
<td>Extracellular signal–regulated kinases</td>
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<td>Fas3</td>
<td>Fasciclin 3</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide Exchange Factor</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<td>Gliotactin</td>
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<td>GUK</td>
<td>Guanylate kinase</td>
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<td>Description</td>
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<td>Inhibitory SMAD</td>
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<td>JAMs</td>
<td>Junctional adhesion molecules</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>l(2)gl</td>
<td>Lethal giant larvae</td>
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<td>LAP</td>
<td>Leucine-rich repeat and PDZ domain</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich repeats</td>
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<tr>
<td>LSR</td>
<td>Lipolysis-stimulated lipoprotein receptor</td>
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<td>MAD</td>
<td>Mothers against decapentaplegic</td>
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<td>MAGUK</td>
<td>Membrane-associated guanylate kinase</td>
</tr>
<tr>
<td>MAPKs</td>
<td>Mitogen-activated protein kinases</td>
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<tr>
<td>Mcr</td>
<td>Macroglobulin complement-related</td>
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<td>Mega</td>
<td>Megatrachea</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>miRNA</td>
<td>MicroRNA</td>
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<tr>
<td>NMJ</td>
<td>Neuromuscular junction</td>
</tr>
<tr>
<td>Nrg</td>
<td>Neuroglian</td>
</tr>
<tr>
<td>Nrv2</td>
<td>Nervana2</td>
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<td>NrxIV</td>
<td>Neurexin IV</td>
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<tr>
<td>Omb</td>
<td>Optomotor blind</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD-95/SAP90, Disc-large, ZO-1</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase 3 protein 2</td>
</tr>
<tr>
<td>PLA</td>
<td>Proximity ligation assay</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>pSJ</td>
<td>Pleated septate junction</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time PCR</td>
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<tr>
<td>RNAi</td>
<td>Ribonucleic acid interference</td>
</tr>
<tr>
<td>R-SMAD</td>
<td>Regulatory SMAD</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Scrib</td>
<td>Scribbled</td>
</tr>
<tr>
<td>SH3</td>
<td>src homology 3</td>
</tr>
<tr>
<td>Sinu</td>
<td>Sinuous</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small/short interfering RNA</td>
</tr>
<tr>
<td>SJ</td>
<td>Septate junction</td>
</tr>
<tr>
<td>SPmiR</td>
<td>Sponge miRNA</td>
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<tr>
<td>Src</td>
<td>Proto-oncogene tyrosine-protein kinase</td>
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<td>sSJ</td>
<td>Smooth septate junctions</td>
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<tr>
<td>SV40</td>
<td>Simian virus 40</td>
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<td>TGF-β</td>
<td>Transforming growth factor beta</td>
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<tr>
<td>TCJ</td>
<td>Tricellular junction</td>
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<tr>
<td>TJ</td>
<td>Tight junction</td>
</tr>
<tr>
<td>Tkv</td>
<td>Thick veins</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream activating sequence</td>
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<tr>
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<td>Untranslated region</td>
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<tr>
<td>Vari</td>
<td>Varicose</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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<tr>
<td>ZO</td>
<td>Zonula occludens</td>
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Acknowledgements

First and foremost I would like to express my sincere gratitude to my supervisor Dr. Vanessa Auld for her immense knowledge, precious guidance, continuous encouragement and patience. I learned from her thoughtful thinking both in science and life. I owed her a depth of gratitude that cannot be measured.

I would like to thank my committee members Dr. Shernaz Bamji, Dr. Hakima Moukhles, and Dr. Michael Gordon for their valuable suggestions and ideas over the years.

I owe particular thanks to Dr. Murry Gilbert for her kindness, without her support it would be impossible to complete this dissertation.

I thank all Auld lab members past and present for their cooperation and friendship.

Last but certainly not least, I offer my enduring gratitude to my lovely family who tolerates being far away from me throughout these years to make me a grown scientist.
To My Mom & Dad

For their endless love and support

To My Husband Javad

For his constant kindness and encouragement
**Chapter 1: Introduction**

Permeability barriers are created by specialized junctions and are essential to prevent fluid flow and restrict the diffusion of solutes, pathogens, and morphogens across tissues such as the brain, intestine, and epidermis (Adachi-Yamada et al., 1999; Rubin and Staddon, 1999). These barriers are conserved throughout all animals and are formed by adhesion of proteins that span the membrane and adhere to other proteins on neighboring cells to create tight, impermeable junctions. Permeability barriers are formed by tight junctions in vertebrates (Tsukita et al., 2001) and septate junctions in insects (Hortsch and Margolis, 2003), and have similar physiological roles and some homologous components. In addition, in many epithelia a specialized junction, called the tricellular junction, forms at the convergence of multiple tight junctions or septate junctions to create a permeability barrier at the corners of neighboring cells (Fristrom, 1982; Noirot-Timothee et al., 1982). However, when barriers break down dramatic consequences occur in vertebrates and invertebrates. Protection from toxic metabolites, regulation of nutrients, oxygen, ions exchange, and cell homeostasis are disrupted and results in lethality and disease (Abbott, 2005; Abbott et al., 1986; Begley and Brightman, 2003). The general question of how junctions are regulated and formed during development is not well known. In this thesis, we aim to understand the regulatory mechanisms that control epithelial barriers and junctions.
1.1 **Tight and septate junctions form permeability barriers in vertebrates and invertebrates**

Epithelial cells establish and maintain the permeability barrier between apical and basolateral compartments. Epithelial barriers are required to restrict solute flow and pathogen invasion into the body and between tissues. The integrity of permeability barriers is maintained by the epithelial junctions during development. Tight junctions (TJ) in vertebrates and septate junctions (SJ) in *Drosophila* regulate the formation and function of permeability barriers in epithelial cells. Tight junctions and septate junctions share physiological roles in creating permeability barriers and to some extent share a number of molecular components.

1.2 **Tight junction**

The zonula occludens is created by tight junctions (TJ), which forms a barrier in the paracellular space and restricts the movement of molecules between cells. Tight junctions mediate adhesion between epithelial cells and separate the apical and basolateral cell surface domains in the plasma membrane (Stevenson and Keon, 1998). TJs are found apical to the adherens junctions and consist of close cellular contacts or "kissing points", which are essential to block paracellular flow (Noirot-Timothee et al., 1982) (Figure 1.1, 1.2). Tight junctions are found throughout polarized epithelia including intestine, kidney and skin. Within the nervous system, tight junctions are found in endothelial cells to create the blood-brain-barrier (BBB) and at Nodes of Ranvier in the myelinating glia to block paracellular flow (Carlson et al., 2000; Tepass et al., 2001; Tsukita et al., 2001). The molecular components that form tight junctions are well established (Figure 1.2).
**Zonula occludens-1 (ZO-1)** protein first was discovered specifically in the tight junction (Stevenson et al., 1986), and later the discovery of a transmembrane protein occludin confirmed the direct role of these two main proteins in tight junction paracellular permeability barrier (Furuse et al., 1993). ZO-1 is a scaffolding protein of the MAGUK protein family and contains three PDZ domains (PSD-95/SAP90, Discs-large, ZO-1), a SRC homology 3 (SH3) domain, and guanylate kinase (GUK) domain, that lacks enzymatic function (Gumbiner et al., 1991; Jesaitis and Goodenough, 1994). There are three ZO proteins found at tight junctions, ZO-1, ZO-2, and ZO-3 all of which are MAGUK proteins. ZO-1 and ZO-2 phosphorylation leads to dissociation of ZO-1 and -2 from the Occludin and Claudin1/5 proteins respectively. This protein dissociation results in tight junction disruption and barrier dysfunction (Kundumani-Sridharan et al., 2013).

**Occludin**, a tight junction integral protein, has four transmembrane domains, two extra- and two intracellular loops. Occludin directly interacts with the zonula occludens proteins through its C-terminal domain and this interaction is essential for Occludin localization (Furuse et al., 1993). Expression of an occludin mutant lacking the C-terminal domain and thus the PDZ binding motif disrupts the paracellular permeability barrier (Balda et al., 1996). This region also plays a role in tight junction assembly during development (Chen et al., 1997). The other role of Occludin is signal transduction regulation through its extracellular domain. Overexpression of Occludin suppresses Raf-1 through its extracellular domain and results in tumor growth (Li and Mrsny, 2000; Wang et al., 2005).

**Claudins** are another transmembrane protein family found at tight junctions and contain four transmembrane domains. Through highly conserved C-terminal PDZ-binding motifs, Claudins directly interact with PDZ-domain-containing proteins like ZO-1, ZO-2 and ZO-3 (Roh et al., 2002; Tsukita et al., 2001). Studies have not shown any sequence homology between Claudins
and Occludin. Claudins play a critical role in cell-cell adhesion and tight junction formation. Claudin knockout mice die from dehydration within one day of birth due to defects in epidermal barrier function (Furuse et al., 2002). Furthermore, expression of Claudin-1 and -2 in cells lacking functional tight junctions rescues these phenotypes (Furuse et al., 1998). These findings suggest that claudins are crucial for tight junction barrier. Co-expression of Occludin and Claudins in L-fibroblast showed that Occludin was recruited along with Claudins into the tight junction. This result confirms the importance of claudins as the main components of tight junction (Furuse et al., 1999).

**Junctional adhesion molecules (JAMs)** are immunoglobulin (Ig) superfamily members. JAMS consist of an extracellular domain with two Ig-like motifs, a single transmembrane domain and a cytoplasmic tail (Kostrewa et al., 2001). Dimerization motifs in the extracellular domains are essential for both homophilic and heterophilic interactions (Bazzoni et al., 2000; Cunningham et al., 2002; Liang et al., 2002). In epithelia JAM-A and −C are localized to the tight junction (Mandell and Parkos, 2005), while JAM-B is along the lateral membrane (Aurrand-Lions et al., 2001). JAMs knockout leads to tight junction defects and barrier disruption (Liu et al., 2000). All JAMs except JAM-L, have a PDZ binding motif at their C-terminal domains, and it is likely through this domain that JAMs interact with scaffolding proteins like ZO-1 to regulate tight junction assembly (Ebnet et al., 2001; Itoh et al., 2001).

As will be outlined below, there are number of tight junction components that are conserved in the *Drosophila* septate junction. For instance, *Drosophila* Claudin-like proteins are considered homologues of vertebrate Claudins and are important for septate junction formation. Sinuous, Megatrachea, and Kune Kune proteins are essential for correct organization and assembly of septate junction and barrier function in *Drosophila* (Behr et al., 2003; Nelson et al.,
The septate junction also includes cell adhesion proteins in the Ig superfamily similar to JAMs in vertebrates. Neuroglian, Contactin, Fasciclin 3, and Lachesin play a role in the assembly and function of septate junctions (Dubreuil et al., 1996; Faivre-Sarrailh et al., 2004; Llimargas et al., 2004; Snow et al., 1989). The functional and structural homologies between vertebrate tight junction and Drosophila septate junctions, make Drosophila an ideal model to study cell junction and permeability barrier formation in vivo.

1.3 Septate junction

Although SJs and TJs have the same function they differ in their structure and subcellular location. TJs are found apical to the adherens junctions; in contrast, SJs are located just basal to the adherens junctions. TJs consists of close cellular contacts or "kissing points", which are essential to block paracellular flow (Lane et al., 1994; Tsukita et al., 2001) (Figure 1.1). On the other hand, septate junctions contain multiple-strands and form a ladder-like of electron dense septa (Tepass and Hartenstein, 1994). There are two different types of septate junctions: pleated and smooth. These two types have different structures and distributions in tissues; however, their functions are similar (Tsukita et al., 2001). Freeze fracture studies show that the pleated septate junctions (pSJ) are located in membrane grooves, but this pattern is not found for smooth septate junctions (sSJ). pSJs are observed in ectodermally derived tissue such as the hindgut, forgut, trachea, glia and imaginal discs, whereas smooth SJs are found in endodermally derived tissue such as the midgut, both pSJ and sSJ functions to maintain the permeability barriers. (Fristrom, 1982; Lane and Swales, 1982; Noirot-Timothee and Noirot, 1980; Tepass and Hartenstein, 1994). This research project will concentrate only on pleated SJs.
1.3.1 Core components of the septate junction

There are many transmembrane and scaffolding proteins that play important roles in SJ and barrier formation. A core complex of proteins contributes to the septate junction domain, and mutations in any of these proteins disrupt SJ formation and result in a loss of barrier function (Figure 1.3). A second group of SJ-associated proteins are also localized to the septate junction but are not part of the main complex. These associated-proteins are indirectly involved in the septate junction regulation and include scaffolding proteins like Scribbled and Dlg.

**Neurexin IV (NrxIV)** is a core SJ protein, a member of the Neurexin family, and the homologue of vertebrate Caspr (Peles et al., 1997). This protein is localized to the epithelial cells of ectodermal origin such as epidermis, the tracheal system, hindgut, and cells of the PNS and CNS (Baumgartner et al., 1996; Ward et al., 1998). *NrxIV* mutants lead to lack of ladder-like structure of septate junction, and mislocalization of other SJ proteins such as Coracle (Baumgartner et al., 1996). A range of phenotypes are associated with loss of *NrxIV* including dorsal closure defects and paralyzed embryos due to the breakdown of the barrier in the glial SJ. Thus, it is suggested that NrxIV is essential for the structure and formation of SJs (Baumgartner et al., 1996).

**Coracle (Cora)** is a cytoplasmic associated SJ protein, and is homologous to the vertebrate protein Band 4.1 (Ward et al., 1998). Cora is associated with NrxIV and both are localized to the SJ (Baumgartner et al., 1996; Fehon et al., 1994; Ward et al., 1998). Cora interacts with the cytoplasmic glycophorin C domain of NrxIV and is required for barrier formation. Both coracle and/or NrxIV mutants lack functional permeability barriers in a range of epithelia due to loss of septae (Genova and Fehon, 2003).
**Varicose (Vari)** is a member of MAGUK family proteins and is a homolog of PALS2 (Wu et al., 2007). In the embryo and the wing imaginal disc, Vari expresses two different isoforms. The short isoform is required for viability and the long one has a supportive role. In *Drosophila*, embryos lacking *varicose* cannot develop a functional septate junction in the tracheae and the epidermis. It is shown that Vari is colocalized with NrxIV in septate junction, and this interaction is through Vari PDZ domain and NrxIV cytoplasmic region (Bachmann et al., 2008; Moyer and Jacobs, 2008; Woods and Bryant, 1991; Wu et al., 2007).

The **Na⁺/K⁺ ATPase** as one of the core SJ proteins consists of an α subunit (ATPα) and a β (Nrv2) subunit. The Na/K ATPase is essential for permeability barrier and septate junction function. Loss of Na/K ATPase leads to disruption of septae, mislocalization of core SJ proteins, and barrier defects (Genova and Fehon, 2003). Nrv2 encodes two different transcripts Nrv2.1 and Nrv2.2; the products of these two transcripts vary in their cytoplasmic domain (Sun and Salvaterra, 1995). Nrv1 and Nrv3 are other β subunits but only Nrv2 is required for functional barrier and SJ formation (Genova and Fehon, 2003; Sun et al., 1995; Xu et al., 1999).

**Neuroglian (Nrg)**, is one of the transmembrane core SJ proteins with multiple Ig domains, and is the homologue of vertebrate four L1-CAM members including L1, CHL1, Neurofascin, and Nr-CAM (Bieber et al., 1989). Nrg, which is localized in axons, epithelial and glial cells, makes a co-dependent complex with Cora, NrxIV, Nrv2, ATPα and Cont. Similar to other core SJ proteins, Nrg is necessary for SJ formation and paracellular barrier function and loss of Neuroglian leads to SJ disruption (Charles et al., 2002; Dubreuil et al., 1997). Mutations in Nrg or any component of this complex causes reduced septa and loss of diffusion barrier, abnormal salivary gland and trachea morphology (Genova and Fehon, 2003).
**Contactin (Cont)**, a main SJ component, is a homologue of vertebrate Contactin, an Ig domain protein linked to the membrane through a GPI domain (Brummendorf and Rathjen, 1996). Contactin is part of a protein complex with NrxiIV and Neuroglian in epithelial and glial cells, and is required for a functional barrier (Banerjee et al., 2006; Faivre-Sarrailh et al., 2004).

**Macroglobulin complement protein (Mcr)** is a member of the thioester-containing proteins (TEPs) family in vertebrate that plays role in innate immune responses (Medzhitov and Janeway, 2002). Mcr is a core SJ component (Batz et al., 2014; Hall et al., 2014). Mcr is required for SJ formation and maintenance and the correct localization of Mcr is dependent on SJ proteins, however Mcr and Nrg are interdependent suggesting a subcomplex in the SJ domain ((Batz et al., 2014; Hall et al., 2014). Loss of Mcr leads to mislocalization of SJ components, permeability barrier defects, embryonic lethality, and a lack of septa in epithelial tissues (Batz et al., 2014; Hall et al., 2014).

### 1.3.2 The septate junction homologues in the nervous system

Of interest is that the core complex of SJ proteins conserved both the molecular and functional levels with a complex of proteins found at the paranodal junction in the vertebrate Node of Ranvier in myelinated axons (Bhat et al., 2001). In the paranodal domain compact myelin, created by either oligodendrocytes in the CNS or Schwann cells in the PNS, forms a series of loops that wrap around the axon and a ladder-like junction (similar to septate junctions) mediates contact between the glial and axonal membranes. The paranodal junction contains the vertebrate homologues of the septate junction proteins NrxiIV, Contactin and Neuroglian (Paranodin/Caspr, Contactin and Neurofascin respectively) (Bhat et al., 2001; Charles et al., 2002; Medzhitov and Janeway, 2002). Caspr has a binding site for the cytoskeletal adaptor protein Band 4.1B, the
vertebrate homologue of Coracle. Band 4.1B is required for the interaction between the axonal cytoskeleton and the paranodal SJ. Band 4.1B along with the other main components plays a pivotal role to maintain the paranodal SJ integrity (Buttermore et al., 2011). A scaffolding protein Whirlin with PDZ domains potentially interacts with Caspr through the PDZ binding motif. Whirlin is essential for the paranodal junction stabilization and compaction (Green et al., 2013). The formation of the paranodal SJ is critical to maintaining an effective rate of nerve conduction. The paranodal junction is required to segregate voltage-gated Na\(^+\) and K\(^+\) channels into distinct domains, anchor different paranodal loops to the axon, and form an ion diffusion barrier (Bhat et al., 2001; Lyons and Talbot, 2008). Thus, paranodal junctions play a fundamental role to organize and maintain axonal domains along myelinated axons.

1.3.3 The SJ-associated components

SJ-associated proteins are a second group of proteins located at the septate junction. These proteins are not essential for the formation of the physical permeability barrier. For instance the scaffolding proteins Scribbled (Scrib) and Discs-large (Dlg) are localized to the septate junction and indirectly regulate septate junction formation and function. Scrib and Dlg are considered tumor suppressor proteins with multiple roles in apicobasal polarity, cell migration, synaptic function and cell proliferation (Albertson and Doe, 2003; Humbert et al., 2003; Nagase et al., 1995; Sans et al., 2001).

**Discs large (Dlg)** is a member of the membrane-associated guanylate kinase (MAGUK) superfamily. This family shares common domains including three PDZ (PSD-95, Dlg, ZO-1) domains in N-terminal, one central Src homology 3 (SH3) domain, and one guanylate kinase (GUK) domain at the C-terminus (Figure 1.3). These binding sites mediate the scaffolding
function of MAGUK proteins (Anderson, 1996; Woods and Bryant, 1991). The PDZ domains interact with PDZ binding motifs of variety of membrane and intracellular proteins and accelerate signal transduction. SH3 domain mediates protein-protein interaction through binding to proline-rich motifs. The GUK domain interacts directly or indirectly with multiple proteins to target cytoskeletal organization and proliferation control (Kim et al., 1995; Kim et al., 1998; Pawson and Scott, 1997). In Drosophila, Dlg interacts directly through its GUK domain with the GUK-holder protein at the neuromuscular junction (Mathew et al., 2002). Dlg localization to the SJ domain after epithelial differentiation is dependent on the SJ core complex. Loss of NrxIV or Cora from the wing imaginal disc or salivary gland leads to loss of Dlg. Similarly, loss of ATPα from the trachea causes Dlg mislocalization (Genova and Fehon, 2003; Hijazi et al., 2011; Nelson et al., 2010; Paul et al., 2003; Ward et al., 1998). It is not clear whether the Dlg interaction with SJ components is direct or which SJ component recruits Dlg to the SJ domain. However Dlg is in the same complex with Gliotactin at the tricellular junction as discussed below (Padash-Barmchi et al., 2013; Schulte et al., 2006).

**Scribbled (Scrib),** a tumor suppressor protein, is essential for epithelial polarity, cell proliferation and neuroblast spindle asymmetry (Bilder, 2004). Scrib is associated along with Dlg with the SJ in polarized epithelia and at the neuromuscular junction. Scrib consists of sixteen leucine-rich repeats (LRR) and four PDZ domains that mediate protein-protein interactions (Figure 1.3) (Apperson et al., 1996; Bilder and Perrimon, 2000). The Scrib LRR domain functions in targeting another polarity protein Lethal giant larvae (Lgl) to the epithelial membrane (Bilder et al., 2000), and the PDZ domains are necessary to target Scrib to the SJ domain (Albertson et al., 2004). Both Scrib and Dlg may play a role in the formation of septate junctions, as loss of these proteins leads to SJ disruption (Bilder and Perrimon, 2000; Bilder et
al., 2003). However as loss of these proteins also leads to the disruption of adherens junctions, the effect of Dlg and Scrib on the SJ might be indirect due to loss of epithelial polarity. The direct role of Scrib and Dlg on SJ or TCJ formation beyond polarity complex formation has not been investigated.

Scrib and Dlg are considered part of the Scrib polarity complex based on genetic studies in the epithelia (Bilder et al., 2000) and biochemical interactions at the neuromuscular junction (Mathew et al., 2002). The Scrib polarity complex is localized in the basolateral region at SJ site and consists of Scrib, Dlg, and Lgl. These three tumor suppressor proteins play important role to establish cell polarity and regulate cell growth. Cooperative activity of these genes is essential for cortical distribution of Lgl and junctional localization of Scrib and Dlg (Bilder et al., 2000). The Scrib complex is a regulator of basolateral polarity by repressing the apical Par3-aPKC-par6 polarity complex (Benton and St Johnston, 2003). Phosphorylation of Lgl by aPKC leads to Lgl dissociation from the apical domain. Thus, Lgl distribution is restricted to the basolateral domain (Betschinger et al., 2003; Kalmes et al., 1996). Since Lgl localization at the membrane is regulated by basolateral Scrib complex (Bilder et al., 2000) and its apical activity is inhibited by Par3 complex suggesting that Lgl is an important mediator between the apical and basolateral activities (Bilder, 2004).

At the neuromuscular junction, the Scrib and Dlg interaction is mediated through the GUK-holder protein. Scrib regulates synaptic vesicles distribution and cycling, and synaptic plasticity in NMJ (Roche et al., 2002). Dlg is also a central regulator of synapse development and maturation (Kornau et al., 1995; Lahey et al., 1994). Scrib and Dlg interact at the synapse through binding of the Scrib PDZ2 and Dlg GUK domains to Guk-holder, a synaptic protein. Dlg and Guk-holder distributions overlap at the bouton border to trigger bouton budding, a process of
NMJ expansion. These proteins are also colocalized with Scrib and recruit it to the postsynaptic region (Mathew et al., 2002). It is also likely that Scrib and Dlg interact at the epithelial SJ domain through a linker protein. However, whether this interaction is mediated by Guk-holder or any other protein is not known and the role of Scrib and Dlg within the SJ is not understood.

1.4 The tricellular junction and permeability barrier in vertebrates

The tricellular junction (TCJ) is formed at the convergence of three cells and their SJs or TJs. The TCJ is important for the formation of a permeability barrier at the corner of cells (Fristrom, 1982; Noirot-Timothee et al., 1982) and is critical in blocking the flow of fluids or pathogens between these gaps. In vertebrates, the TCJ is found at the corner of cells and is formed at the convergence of three tight junctions (Friend and Gilula, 1972; Staehehlin, 1973). The vertebrate TCJ, also called the tricellular tight junction, is important to maintain trans-epithelial/endothelial barriers (Ikenouchi et al., 2005) (Figure 1.4).

**Tricellulin** is a MARVEL domain protein, related to the Occludin family of proteins, and is localized uniquely to the tricellular tight junction (Ikenouchi et al., 2005). Tricellulin is expressed in all epithelial junctions throughout the body (Riazuddin et al., 2006). Loss of Tricellulin severely disrupts tight junction organization, not only at tricellular contacts but also at the bicellular tight junction. In Tricellulin knockout the bicellular junction protein Occludin has a thinner and discontinuous pattern (Ikenouchi et al., 2005). Tricellulin knockdown also leads to a decrease in transepithelial electrical resistance and an increase in paracellular permeability (Ikenouchi et al., 2005). Loss of Occludin from the TJ results in spread of Tricellulin away from the TCJ suggesting that Occludin restricts the localization of Tricellulin to the TCJ (Ikenouchi et al., 2008).
Lipolysis-stimulated lipoprotein receptor (LSR)/Angulin-1 is another vertebrate TCJ protein with a crucial role in tricellular tight junction formation (Masuda et al., 2011). LSR is an integral membrane protein with an extracellular Ig domain, transmembrane and cytoplasmic domains. The interaction between the LSR cytoplasmic domain and the Tricellulin c-terminal region results in the formation of tricellular junction strands (Masuda et al., 2011). Loss of LSR disrupts barrier function and leads to mislocalization of Tricellulin from the TCJ. However the distribution of LSR is not dependent on Tricellulin suggesting that LSR is required to recruit Tricellulin to the TCJ (Masuda et al., 2011). These findings suggest that tricellular contacts play an important role in the formation and maintenance of epithelial barrier in vertebrates, and Tricellulin and LSR are critical for this epithelial barrier.

1.5 The tricellular junction and its components in Drosophila

In *Drosophila*, the tricellular junction (TCJ) is formed at the convergence of three adjacent cells and establishes a permeability barrier to block the flow of solutes at the corners of cells. At each corner the SJ strands turn 90° and run basolaterally to form three parallel strands around the TCJ similar to central sealing elements in vertebrates. A series of diaphragms at the central canal of the cell corners creates the functional barrier at the TCJ (Noirot-Timothee and Noirot, 1980; Fristrom, 1982; Noirot-Timothee et al., 1982) (Figure 1.5). The TCJ plays a critical role in SJ maturation and function and loss of the TCJ disrupts the SJ ladder-like structure and barrier function (Schulte et al., 2003). Gliotactin, a member of the Neuroligin family, was the first protein identified in the *Drosophila* TCJ (Schulte et al., 2003). Recently, Bark-beetle (Bark) (aka; Anakonda) was identified as a new member of the TCJ (Byri et al., 2015; Hildebrandt et
al., 2015). What other proteins are localized and function at the Drosophila TCJ has not been determined.

1.5.1 Bark-beetle is a new member of the TCJ

Bark-beetle (Bark) is a transmembrane protein with a huge extracellular triple-repeat domain and an intracellular domain containing a PDZ-binding motif at the C terminus (Byri et al., 2015). It is proposed that the triple-repeat extracellular domain of Bark may localize at the triangular diaphragms in the central canal and form the physical permeability barrier (Byri et al., 2015) (Figure 1.6). The presence of Bark is essential for the formation of the TCJ. Loss of Bark results in barrier defects and TCJ disruption (Byri et al., 2015; Hildebrandt et al., 2015). Embryonic loss of Bark at stages of embryogenesis (stage 17, not stage 15) leads to basolateral spread of SJ proteins including NrxIV, Nrg, Mega, while Dlg distribution is normal. It has been shown that in embryonic loss of core SJ proteins Bark is normally localized to the TCJ. These findings suggest that Bark is dispensable for the formation of SJ however it is critical for TCJ formation. Embryonic Bark knockdown leads to the loss of Gliotactin from the TCJ, supporting a role for Bark in Gliotactin trafficking to the TCJ (Byri et al., 2015; Hildebrandt et al., 2015). However, how Bark and Gliotactin interact is still unknown and how Bark is recruited to the TCJ is also unknown.

1.5.2 Gliotactin is a critical TCJ component

Gliotactin is localized at the TCJ (Schulte et al., 2003) and is expressed in a wide range of epithelia (Schulte et al., 2006; Schulte et al., 2003) and glia (Auld et al., 1995) (Figure 1.7). Gliotactin, a member of Neuroligin Family, is a single pass transmembrane protein with an
extracellular Choline-esterase domain that lacks enzymatic activity. The intracellular domain of Gliotactin contains a conserved PDZ binding motif. The cytoplasmic domain also has two conserved tyrosine phosphorylation sites at amino acid 766 and 790 (Gilbert and Auld, 2005) (Figure 1.7F). Neuroligin 3, which is expressed in vertebrate nervous system and glia (Gilbert and Auld, 2005; Song et al., 1999), is the closest vertebrate homologue to Gliotactin. Neuroligin is well known as a post-synaptic protein that interacts with PSD95 (a homologue of Drosophila Dlg) during synapse formation and regulation (Dean et al., 2003; Scheiffele et al., 2000). Neuroligin proteins have a wide distribution in the nervous system and in endothelia (Bolliger et al., 2001; Bottos et al., 2009; Gilbert et al., 2001; Philibert et al., 2000). However, little is known about their function outside of the nervous system.

Mutations in Gliotactin result in the breakdown of the paracellular barriers and the SJ domain. Loss of Gliotactin is lethal and in epithelia causes a disruption of the TCJ and the spread of the SJ domain within the basolateral domain away from the normal position just under the adherens junction. The septa are uncompact which leads to a loss of the permeability barrier (Schulte et al., 2003) (Figure 1.7). In embryonic septate junction mutants such as NrxIV, Gliotactin is mislocalized suggesting that there is a mutual dependence between Gliotactin and SJ proteins (Gilbert and Auld, 2005; Schulte et al., 2003). During embryonic development, Gliotactin is first detectable in the ectoderm at stage 11 with a diffuse pattern similar to NrxIV. However, at stage 13 Gliotactin is located at the lateral surface of the cells; in contrast, NrxIV remains spread around the cells. By stage 15, Gliotactin overlaps with NrxIV in the tricellular domain suggesting that Gliotactin mediates the formation of the TCJ and recruits the SJ components into this complex (Schulte et al., 2006; Schulte et al., 2003). These findings suggest that Gliotactin at the TCJ consolidates the association of the SJ at the TCJ.
Gliotactin probably works as a mediator between SJ strands and tricellular diaphragms. Gliotactin could be in contact with tricellular diaphragm through its extracellular domain and with SJ through its intracellular domain (Schulte et al., 2003). It is likely that Gliotactin through its C-terminal PDZ binding motif interacts with Dlg and Scrib PDZ domains at the SJ strands. However, this hypothesis has not been investigated.

Gliotactin is also expressed in a specific class of glia, the subperineurial glia, which are responsible for the formation of the blood-brain barrier (BBB). Gliotactin mutant embryos have defects in the structure and the function of the SJ that form the BBB (Auld et al., 1995). Thus, Gliotactin and the TCJ are involved in cell-cell interactions, and form part of the junctions necessary for barrier formation in a wide range of tissues (Auld et al., 1995).

Gliotactin and the TCJ also have non-permeability barrier roles. Gliotactin plays an important role as a cell shape sensor in epithelial mitotic cells. During the cell division epithelial cells adopt a rounded shape and Gliotactin regulates microtubule pulling force and spindle orientation through the Dynein-associated protein Mud. During mitosis Mud is colocalized with Gliotactin at the TCJ, and loss of Gliotactin results in Mud reduction from the TCJ (Bosveld et al., 2016). Aged-dependent remodeling studies show that Gliotactin is localized at the TCJ in differentiated intestinal cells. Loss of Gliotactin in differentiated entrocytes disrupts the integrity of the barrier and induces intestinal stem cell proliferation and accumulation through JNK pathway signaling. This work suggest an important link between Gliotactin, stem cell, and intestinal homestatis (Resnik-Docampo et al., 2017). These findings suggest that besides a Gliotactin role in the regulation of barrier integrity, Gliotactin is critical to maintain cell homeostasis, and cell organization through interactions with other protein complexes at the TCJ.
1.5.3 Gliotactin is in a protein complex with Discs large

Since Gliotactin may function to link the tricellular diaphragms with the SJ strands, the C-terminal PDZ binding motif could be in contact with PDZ domain proteins including Scrib and Dlg. Vertebrate studies show that PSD-95 (a homologue of Dlg) is a binding partner of Neuroligin (Nlg; a homologue of Gliotactin) and this interaction is mediated by the PDZ binding motif (Irie et al., 1997; Song et al., 1999). Nlg induces the formation of excitatory and inhibitory post-synaptic contacts and PSD-95 regulates the distribution of Nlg and the specificity of Nlg-induced synapse contact (Levinson et al., 2005; Prange et al., 2004). Similar to the Nlg-PSD95 interaction, in Drosophila the MAGUK protein and SJ-associated protein Discs large (Dlg) is concentrated at the TCJ and forms a protein complex with Gliotactin (Schulte et al., 2006).

Gliotactin and Dlg interact with each other in a Ca^{2+} dependent manner and knockout of Dlg leads to the loss of Gliotactin from the TCJ (Padash-Barmchi et al., 2013). However, the binding between Gliotactin and Dlg appears to be indirect (Padash-Barmchi et al., 2013; Schulte et al., 2006), and another protein possibly mediates this association. Gliotactin overexpression results in a decrease in Dlg level at the SJ but not vice versa, and the presence of Gliotactin PDZ binding motif and phosphorylation sites are essential for co-endocytosis (Padash-Barmchi et al., 2010; Padash-Barmchi et al., 2013). This has led to the suggestion that Dlg participates in different protein interactions with Gliotactin in the TCJ versus the SJ (Schulte et al., 2006).

Scribbled could be a good candidate for this TCJ interaction, given the known association of Dlg and Scrib in the polarity complex. In chapter three, we hypothesize that there is a scaffolding protein complex at the TCJ including Dlg and Scrib which mediates TCJ formation.
1.5.4 Gliotactin protein levels and the TCJ localization are controlled by tyrosine phosphorylation and endocytosis

Gliotactin is normally tightly confined to the TCJ. Prior work determined that this localization is maintained through endocytosis of Gliotactin when the protein spreads away from the TCJ. Tyrosine phosphorylation of Gliotactin leads to ubiquitination, endocytosis and degradation in lysosomes to confine Gliotactin expression to the TCJ (Padash-Barmchi et al., 2010). There are two highly conserved tyrosine phosphorylation sites in the intracellular C-terminal domain of Gliotactin, Y766 and Y790, found in all Gliotactin homologues. Overexpression of Gliotactin or block of endocytosis using a dominant negative form of Rab5 causes Gliotactin to spread away from the TCJ into the bicellular SJ domain and both apical and basolaterally within the plasma membrane. To test the role of tyrosine phosphorylation, the two highly conserved tyrosine residues were mutated to phenylalanine (GliFF) to block phosphorylation and aspartic acid (GliDD) to mimic phosphorylation. GliDD is rapidly endocytosed creating large endocytic vesicles but GliFF is not. It is suggested a potential kinase that regulates Gliotactin phosphorylation might be Src as activated Src can phosphorylate Gliotactin in vitro (Padash-Barmchi et al., 2010), However recently it has been suggested that C-terminal Src kinase (CSK) could phosphorylate Gliotactin in vivo (Samarasekera, 2017). These results suggest that tyrosine phosphorylation and endocytosis of Gliotactin are necessary to maintain localization at the TCJ.

The conserved PDZ binding motif of Gliotactin also functions to stabilize Gliotactin at the TCJ. In cells expressing a form of Gliotactin lacking the PDZ binding motif, Gliotactin levels are reduced form the junction and Gliotactin was localized in endosomes at a much higher frequency than wild type Gliotactin (Padash-Barmchi et al., 2013). It appears that GliΔPDZ is less stable and undergoes endocytosis and recycling. Thus, the Gliotactin PDZ binding motif is required to
regulate Gliotactin endocytosis and acts to stabilize the protein (Padash-Barmchi et al., 2013). Overexpression of a Gliotactin protein lacking the PDZ binding motif does not lead to Dlg reduction, while full length Gliotactin does. Gliotactin triggers Dlg downregulation through co-endocytosis of Gliotactin and Dlg, and the Gli PDZ binding motif is required for this interaction (Padash-Barmchi et al., 2013). These findings support the hypothesis that Dlg may bind to Gliotactin and stabilize Gliotactin at the TCJ.

1.5.5 Spread of Gliotactin leads to proliferation and JNK mediated apoptosis

The existence of a mechanism to regulate Gliotactin at the protein level suggests that Gliotactin levels need to be tightly controlled. In support of this hypothesis, overexpression of Gliotactin can be harmful to epithelia cells and causes a range of deleterious phenotypes including: overproliferation, delamination, cell migration and apoptosis (Padash-Barmchi et al., 2010). Overexpressions of other SJ components including NrxIV and Nrg or Dlg do not lead to these effects (Padash-Barmchi et al., 2010). The phenotypes triggered by ectopic expression of Gliotactin are dependent on Jun kinase (JNK) signaling. These effects are blocked by a dominant negative form of JNK, and enhanced using a JNK phosphatase mutant (puckered) (Padash-Barmchi et al., 2010). Surprisingly phosphorylations of the tyrosine residues of Gliotactin are key to the triggered overexpression-phenotypes as removal of all Gliotactin tyrosine sites blocked these phenotypes (Padash-Barmchi et al., 2013). Reducing endocytosis results in the presence of Gliotactin beyond the TCJ, overproliferation, delamination and the induction of cell death (Padash-Barmchi et al., 2010) and thus it is critical that Gliotactin levels are regulated by tyrosine kinase phosphorylation and endocytosis.
1.5.6 **Gliotactin may be regulated by microRNAs**

Another common mechanism to ensure correct protein levels is through microRNA-mediated degradation of mRNAs. MicroRNAs are ~22 nucleotide dsRNA sequences that bind to complementary sequences in the 3’ UTR of multiple target mRNAs, to negatively regulate gene expression post-transcriptionally, usually resulting in either transcript degradation or translation silencing (Bartel, 2009). MicroRNAs are well conserved in both plants and animals and play important roles in regulation of various developmental and physiological processes (Chen and Rajewsky, 2007; Lee et al., 2007; Tanzer and Stadler, 2004). Due to the conserved role of miRNAs from insects to vertebrates, Drosophila is an ideal model to study the function of microRNAs in animals. In insects, microRNAs are essential regulators of many biological processes including oogenesis, embryogenesis, metamorphosis, behavior and host-pathogen interactions (Lucas et al., 2015). For instance miR-989 is essential for border cell migration during oogenesis (Kugler et al., 2013), and miR-7 plays role in wing growth during development through targeting Notch signaling pathway (Aparicio et al., 2015). In vertebrates, microRNAs play a vital role in the regulation of tight junctions and permeability barriers (Figure 1.8), however the role of miRNAs in the regulation of junctional proteins is still poorly understood. miR-122 can disrupt the TJ function through degradation of Occludin in patients with Ulcerative Colitis (Wu et al., 2008). miR-212 also triggers barrier breakdown via repression of ZO-1 (Tang et al., 2008). In intestinal dysfunction disorders, miR-874 and -155 inhibit Occludin translation (Qin et al., 2013; Zhi et al., 2014), and miR-204 blocks Occludin expression via TGFβ signaling pathway (Wang et al., 2010) (Figure 1.8). In *Drosophila*, microRNAs have the potential to regulate the level of junctional proteins (Kertesz et al., 2007). In the 3’UTR of Gliotactin, there are a number of predicted binding sites for miRNAs that have the potential to regulate the
expression level of Gliotactin. These sites are conserved across the 12 sequenced *Drosophila* genomes and include target sequences for miRNA-124, 184, 1, 1011, 190, 981, 281, and bantam. A number of these sites were partially validated using *in vitro* assays. For instance, a 3’UTR reporter construct of Gliotactin is regulated by the bantam miRNA in *Drosophila* tissue culture cells (S2 cells) (Nahvi et al., 2009). Other algorithms to predict targets of *Drosophila* miRNAs identified Gliotactin as one of the major targets of miRNA-124 (Robins et al., 2005). Similarly many SJ components including Gliotactin were predicted to be targets of miR-184 and *in vitro* luciferase assays confirmed that Gliotactin, NrxIV, Cont, Cora, Megatrachea, Sinuous, and Kune-kune are potential targets of miR-184 (Kertesz et al., 2007)(Figure 1.9). Of interest in the analysis of miR-184 was the extensive overlap of genes that are expressed in the SJ, including the Claudin-like proteins plus the core SJ proteins and Gliotactin. This suggests that miR-184 may regulate Gliotactin in concert with other SJ proteins. Therefore, in chapter two of the thesis, we hypothesize that Gliotactin and SJ components are regulated by miRNAs to ensure the expression at the correct time and place during development.

### 1.6 *Drosophila* as a model system to study the TCJ

In this thesis we used the wing imaginal disc to study the function of Gliotactin in the formation of the TCJ in *Drosophila melanogaster*. The adult wings originate from an undifferentiated epithelium called the wing imaginal disc, which is an ideal tissue for studying the TCJ. Wing imaginal disc is developed from the embryonic ectoderm. Early in the development, blastoderm epithelium is emerged from cellularization, then polarity is established in the epithelial cells which in turn leads to the formation of apical adherent junction and later the formation of basolateral septate junction and tricellular junction (Tepass et al., 2001). The wing imaginal disc
has just two different cell layers: the peripodial and columnar epithelia (Figure 1.10). In both cell types, the SJ and TCJ are basal to the adherens junction, and columnar cell layer mainly contributes to the formation of adult wing. There are several advantages to use *Drosophila* as a model to study the development and maintenance of cellular junctions. *Drosophila* has a simple genome and rapid generation time with a wide array of genetic approaches. Through the use of the Gal4/UAS system, target genes or transgenes can be expressed in any tissue of interest (Figure 1.10). In the wing imaginal disc, tissue specific enhancers drive the expression in specific regions, for example using the apterous-GAL4 driver will allow expression only in the dorsal half of the wing imaginal disc. This has the added advantage that the ventral half of the disc serves as an internal wild type control to compare side by side with the experimental one. It has been shown that apterous gene expression starts from the L2 larvae stage, and in all experiments we tested the SJ and TCJ formation and function at L3 larvae stage. Overall, *Drosophila* is an excellent model to study the formation and function of epithelial permeability barriers.

### 1.7 Hypothesis

The following two hypotheses are tested in the thesis.

**Hypothesis 1:** We hypothesize that control of Gliotactin at the mRNA level is mediated by microRNAs at the 3'UTR.

The working model is that Gliotactin organizes a protein complex that mediates the formation of TCJs. It is already known that control of Gliotactin levels occurs at the protein level through phosphorylation, endocytosis and lysosome-mediate degradation (Padash-Barmchi et al., 2010). In chapter two of the thesis, we tested whether Gliotactin is also regulated at the mRNA level.
through microRNA control, specifically miR-184. We also examined whether miR-184 controls a cohort of TCJ and SJ proteins. Finally, we tested the mechanisms underlying microRNA control of Gliotactin and the cellular consequences of loss of regulation.

**Hypothesis 2:** We hypothesize that there is a Scrib scaffolding protein complex at the TCJ, which mediates Gliotactin and the TCJ formation and function.

In chapter three of the thesis, we tested the role of Scrib at the TCJ to determine whether Scrib interacts with the TCJ protein Gliotactin and the other scaffolding protein Dlg. We examined whether Scrib localization is dependent on Gli or vice versa. Finally, we investigated which Scrib domains are required for this novel interaction with Gliotactin.
Figures for chapter one:

**Figure 1.1: Comparison between the tight junction and the septate junction**

In vertebrates, the tight junction is generated from a structure with multiple kissing points and is located above the adherens junction. While in *Drosophila*, the septate junction with a ladder-like structure is located below the adherens junction (This figure is reproduced from Tepass, 2003).
Figure 1.2: The tight junction structure and its components

(A) The protein-protein interactions that occur between the tight junction components are shown. Some of the interactions are essential for structural integrity, and some are important for signaling pathways. ZO1-3 proteins are in contact with Occludin, Claudin, and JAM proteins. The actin cytoskeleton interacts with some TJ components including ZO1-3 (This figure is reproduced from Shin et al., 2006). (B) TEM of the tight junction with the multiple kissing point structure (arrows) is shown in the epithelia membrane (This figure is reproduced from Stevenson and Keon, 1998).
Figure 1.3: The septate junction structure and its components

(A) A ladder-like structure of the septate junction is shown with a TEM from the wing columnar epithelia (This figure is reproduced from Genova and Fehon, 2003). (B) A cartoon demonstrates the interaction between the core and associated components of the septate junction. ATP-α and Nrv2, two subunits of Na/K ATPase along with NrxiIV, Contactin and Neuroglian are located at the septate junction domain. Coracle, Dlg, and Scrib are localized in intracellular surface of the cells. The dotted lines show the potential interactions. Gliotactin is also shown at the corner of the cells (This figure is reproduced from Hortsch and Margolis, 2003).
Figure 1.4: The tricellular junction and its components in vertebrate

(A) A tricellular tight junction is shown in a freeze fracture SEM of mouse epithelial cells. Tricellular contacts are labeled with an anti-LSR antibody and immunogold labeling. The sealing elements at the TCJ between two neighboring cells are indicated with arrowheads. (B) A cartoon represents the formation of the tricellular tight junction at the convergence of three adjacent epithelia cells. (C) LSR (green) and Tricellulin (red) are localized at the tricellular tight junction, and ZO-1 (blue) is distributed along the tight junction in a mouse epithelia (This figure is reproduced from Masuda et al., 2011) (http://jcs.biologists.org/content/124/4/548).
Figure 1.5: The tricellular junction structure in *Drosophila*

(A) Invertebrate TCJ (arrow) is shown in freeze fracture SEM of epithelia (This figure is reproduced from Noirot-Timothee et al., 1982). The bicellular septate junction septae run 90 degrees at the tricellular junction (arrows). (B, C) Schematic diagrams of the TCJ. At the corner of the three neighboring epithelial cells, the TCJ is formed. Arrows show the bicellular and tricellular junctions. In C, SJ strands (green) run basolaterally and a series of diaphragms fill the TCJ (orange) (This figure is reproduced from Flores-Benitez et al., 2015).
Figure 1.6: Model of the TCJ with Bark beetle as a new member of the TCJ

(A) Bark beetle (Aka) (dark blue) with a huge triple-repeat extracellular domain fills the TCJ central canal, and Gli (yellow) is localized at the TCJ. The geometry of the tricellular junction with the 120 degree are shown. The septate junction strands turn 90 degree and form lateral limiting strands (LS). (B) At stage 16 of embryogenesis, Bark beetle (Aka) (green) is colocalized with Gliotactin (magenta) at the TCJ of the embryonic epithelia (This figure is reproduced from Byri et al., 2015).
Figure 1.7: Gliotactin is required for the formation and function of the TCJ

(A) Wild type TCJ from a TEM of embryonic epithelia. (B) In a Gliotactin mutant, the ladder-like structure of the SJ is disrupted (arrowhead) (This figure is reproduced from Schulte et al., 2003). (C) Gliotactin is localized at the TCJ, and Dlg is distributed along the septate junction in the wing imaginal disc (This figure is reproduced from Schulte et al., 2006) (http://jcs.biologists.org/content/119/21/4391.long). Injection of rhodamine-dextran dye into the body cavity of an embryo is excluded from the salivary gland (green) in wild type embryos (D). The permeability barrier is disrupted in a Gliotactin mutant (E) and the dye penetrates inside the lumen of the salivary gland (This figure is reproduced from Schulte et al., 2003). (F) A cartoon showing a Gliotactin-dimer with the conserved protein domains. Gliotactin is a single transmembrane (blue) protein with an extracellular choline-esterase like domain, two conserved tyrosine phosphorylation sites and one PDZ-binding motif.
Figure 1.8: The tight junction regulation through microRNAs

A diagram indicating the currently known effects of microRNAs on tight junction components. A single TJ protein can be a target of multiple microRNAs and one microRNA can regulate a suite of junctional proteins. For instance, Occludin is regulated by a group of miRNAs, and miR-212 regulates ZO-1 and Occludin (This figure is reproduced from Cichon et al., 2014).
Figure 1.9: miR-184 target genes

*In vitro* interaction of miR-184 with the wide range of targets is shown through the luciferase assay. Many septate junction genes are targets of miR-184 (shown in red). The expression of 3’UTRs containing miR-184 complementary binding sites (N) are in green and the constructs containing the miR-184 target site tested experimentally in the pro-apoptotic reaper (rpr) sequence (N>rpr) are in blue (This figure is reproduced from Kertesz et al., 2007). The greater the degree of mRNA degradation, the lower the normalized luciferase ratio.
Figure 1.10: The Gal4/UAS system in the wing imaginal disc

(A) The wing imaginal disc has two different cell layers, the peripodial layer and columnar layer. The apterous enhancer-Gal4 drives the expression of target gene in the dorsal part of the wing imaginal disc (red) in the columnar epithelia but not the peripodial layer. (B, D) The expression of UAS-mCD8::GFP in the dorsal side of the wing imaginal disc is driven through apterous-Gal4. (C) The Gal4 transcription factor under the control of a tissue-specific enhancer drives the expression of UAS-target gene (GFP in this example). Gal4 binds to the upstream activator sequence (UAS) and leads to transcription of the downstream target gene in a tissue-specific manner (Brand and Perrimon, 1993; Phelps and Brand, 1998).
Chapter 2: The *Drosophila* tricellular junction protein Gliotactin regulates its own mRNA levels through BMP-mediated induction of miR-184

2.1 Synopsis

Epithelial bicellular and tricellular junctions are essential for establishing and maintaining permeability barriers. Tricellular junctions are formed by the convergence of three bicellular junctions at the corners of neighboring epithelia. Gliotactin, a member of the Neuroligin family, is located at the *Drosophila* tricellular junction, and is crucial for the formation of tricellular and maturation of septate junctions as well as permeability barrier function. Gliotactin protein levels are tightly controlled by phosphorylation at tyrosine residues and endocytosis. Blocking endocytosis or overexpressing Gliotactin results in the spread of Gliotactin from the tricellular junction, resulting in apoptosis, delamination, and migration of epithelial cells. We show that Gliotactin levels are also regulated at the mRNA level by micro (mi) RNA-mediated degradation and that miRNAs are targeted to a short region in the 3’UTR that includes a conserved miR-184 target site. miR-184 also targets a suite of septate junction proteins, including NrxIV, coracle and Mcr. The miR-184 expression is triggered when Gliotactin is overexpressed, leading to activation of the BMP signaling pathway. Gliotactin specifically interferes with Dad, an inhibitory SMAD, leading to activation of the Tkv type-I receptor and activation of Mad to elevate the biogenesis and expression of miR-184.
2.2 Introduction

Permeability barriers are essential to prevent fluid flow and to restrict the diffusion of molecules and pathogens across tissues, such as the epidermis, intestine, and brain. Permeability barriers are formed by tight junctions in vertebrates and septate junctions in insects, and the barriers have similar physiological roles and homologous components (Tsukita et al., 2001). Pleated septate junctions form a ladder-like array of electron-dense septa located just below the adherens junction (Tepass and Hartenstein, 1994). Septate junctions comprise an interdependent protein complex, including Neurexin IV (NrxIV) (Baumgartner et al., 1996), Coracle (Fehon et al., 1994), Na+/K+ ATPase and Neuroglian (Genova and Fehon, 2003; Paul et al., 2003), Contactin (Faivre-Sarrailh et al., 2004) and Mcr (Batz et al., 2014; Hall et al., 2014). In many epithelia, a specialized junction, the tricellular junction (TCJ), forms at the convergence of the tight junctions or septate junctions from three cells to create a permeability barrier at the corners of neighboring cells. In Drosophila, the TCJ is formed in a range of epithelia and is crucial for septate junction maturation and permeability barrier formation (Fristrom, 1982; Noirot-Timothée et al., 1982; Schulte et al., 2003). Gliotactin, a single-pass transmembrane protein and a member of the Neuroligin family, is specifically localized to the Drosophila TCJ (Schulte et al., 2003). Loss of Gliotactin results in a disruption of the TCJ, the basolateral spread of septate junction proteins and a loss of the permeability barrier (Schulte et al., 2003).

Gliotactin protein levels and TCJ localization are tightly controlled by phosphorylation of tyrosine residues and endocytosis (Padash-Barmchi et al., 2010). Overexpressed Gliotactin spreads away from the TCJ and is deleterious, leading to apoptosis, delamination, overproliferation and cell migration in the wing imaginal disc (Padash-Barmchi et al., 2010). Mutation of two conserved tyrosine residues to phenylalanine in Gliotactin disrupts the
phosphorylation, endocytosis and subsequent degradation of Gliotactin (Padash-Barmchi et al., 2010). Beyond control at the protein level, Gliotactin has been identified as a potential target of micro (mi)RNAs, with multiple conserved miRNA-target sites predicted in the 3'UTR (Kertesz et al., 2007). Target sequences for one of these microRNAs, miR-184, have been identified in the 3'UTR of other genes coding for septate junction proteins, including NrxIV and Coracle (Kertesz et al., 2007), and have been predicted to exist in a range of other septate junction genes, including the Pasiflora genes (Deligiannaki et al., 2015). However, the role that miR-184 plays in the regulation of Gliotactin and the septate junction in vivo has yet to be determined.

Here, we show that Gliotactin mRNA levels are controlled by miR-184-targeted degradation through the 3'UTR. In addition, miR-184 can control the expression level of the core septate junction proteins NrxIV and Coracle, as well as Mcr. We found that overexpressed Gliotactin is the trigger for increased miR-184 expression through activation of the BMP type-1 receptor, Thickveins (Tkv). Moreover, Gliotactin activation of Tkv is through a block of the Tkv inhibitor Daughter against dpp (Dad). We conclude that Gliotactin leads to a feedback loop to control Gliotactin mRNA levels thorough Tkv signaling and induction of miR-184.

2.3 Results

2.3.1 Overexpression of Gliotactin causes self-downregulation

Gliotactin localization is normally restricted to the TCJ within the columnar epithelia of the wing imaginal disc (Figure 2.1A). Overexpression of a full-length Gliotactin transgene causes spread away from the TCJ and around the cell. When driven with apterous- GAL4, the pattern of expression is uniform across the dorsal half of the wing imaginal disc (Schulte et al., 2006; Padash-Barmchi et al., 2010). In contrast, when an EP insertion (GliEP) with an upstream
activating sequence (UAS) element in the 5’ end of Gliotactin was driven using *apterous*-GAL4, Gliotactin expression occurred in patches within the dorsal side of the wing disc (Figure 2.1B, C). The EP insertion drives the expression of the endogenous Gliotactin mRNA, including the entire 3’UTR, whereas our original UAS Gliotactin transgene lacked the complete 3’UTR, leading to the hypothesis that the differences in expression patterns might reside in the 3’UTR. Using Target Scan Fly (Kheradpour et al., 2007), eight miRNA targets are predicted within the Gliotactin 3’UTR that are highly conserved across Drosophilidae species (Figure 2.1L). This suggested an additional level of Gliotactin regulation controlled by the 3’UTR. To test this, we expressed a range of transgenes with cherry-tagged Gliotactin and different 3’UTR lengths (Figure 2.2). These included the entire 3’UTR (Gli::Ch-L.UTR), a short 3’UTR lacking the miR-184- and miR-1011-target sites (Gli::Ch-S.UTR), and finally the SV40 3’UTR lacking all miRNA target sites (Gli::Ch-no.UTR). Transgenic lines overexpressing the entire 3’UTR (Gli::Ch-L.UTR) had regions with reduced levels of Gliotactin protein (Figure 2.1D, E) that when measured across different regions in the dorsal side (Figure 2.1J) were significantly different (Figure 2.1K). This mirrored the patchy pattern observed with the GliEP insertion, although the degree of downregulation was not as strong (Figure 2.1K). Transgenes lacking the miR-184- and miR-1011-binding sites (Gli::Ch-S.UTR) (Figure 2.1F, G) or the whole 3’UTR (Gli::Ch-no.UTR) (Figure 2.1H, I) showed no significant reduction in Gliotactin levels across the apterous expression area (Figure 2.1K). These results suggest that the 3’UTR and, specifically, miR-184 and/or miR-1011 control Gliotactin levels.
2.3.2 miR-184 controls septate junction proteins and Gliotactin

miR-184 has been identified *in vitro* as a potential regulator of other septate junction proteins, including NrxIV, Coracle and Mcr, in addition to Gliotactin (Kertesz et al., 2007). miR-184 is expressed throughout embryogenesis (Aboobaker et al., 2005; Li et al., 2011) in larval imaginal discs (Li et al., 2011) and plays a role in the regulation of the female germline (Iovino et al., 2009). To test the role of miR-184 in the control of Gliotactin and septate junction proteins, we overexpressed miR-184 in the wing imaginal disc using *apterous*-GAL4. Elevated miR-184 expression caused a downregulation of Gliotactin (Figure 2.3A, B) and a reduction of the septate junction proteins NrxIV (Figure 2.3C, D), Mcr (Figure 2.3E, F) and Coracle (Cora) (Figure 2.3G,H). The expression of other junctional proteins, Disc Large (Dlg) (Figure 2.3A), E-cadherin (E-cad) and Fasciclin 3 (Fas3) (Figure 2.3I, J) were unaffected. The degree of downregulation of each septate junction protein or Gliotactin was insufficient to trigger loss of polarity or to trigger cell death, and we observed no disruption to the epithelium. To test the specificity of the miRNA control, we individually expressed miR-1011 and the miRNA bantam, which are also predicted to target the Gliotactin 3′UTR (Figure 2.1L). Neither miR-1011 nor bantam expression had any effect on Gliotactin (Figure 2.3K, L). These results suggest that miR-184 can control Gliotactin and a subset of septate junction mRNAs.

2.3.3 Overexpression of the extracellular domain is the trigger for self-downregulation

The role of miR-184 in Gliotactin control was unexpected because miR-184 mutants are viable with no disc abnormalities (Iovino et al., 2009). Overexpression of Gliotactin is deleterious, leading to cell death, overproliferation and cell migration (Padash-Barmchi et al., 2010), suggesting that mechanisms have evolved to ensure protein levels are tightly controlled. We
hypothesized that the overexpression of Gliotactin could trigger elevated miR-184 expression for an additional level of control. To test this hypothesis, we expressed a Gliotactin transgene that lacked the 3’ UTR and in which the intracellular domain had been replaced with a blue fluorescent protein (BFP) tag (Gli::EBFP) (Figure 2.2C). With this Gliotactin construct, tyrosine phosphorylation and subsequent endocytosis cannot occur, and endogenous Gliotactin can be analyzed using a monoclonal antibody specific to the intracellular domain. When expressed with the *apterous*-GAL4 driver, Gli::EBFP did not alter the subcellular localization of endogenous Gliotactin nor lead to cell death or disruption of the epithelia, but did result in the overall downregulation of endogenous Gliotactin (Figure 2.4A). To confirm these results, we overexpressed a Gliotactin transgene containing the entire protein-coding sequence but lacking the 3’UTR (GliWT) (Figure 2.2) in a background with endogenous Gliotactin tagged with YFP (Gli::YFP) (Figure 2.4B). Here too, endogenous Gliotactin was downregulated when the transgene was overexpressed. Expression of the Gli::EBFP or GliWT transgenes also led to the downregulation of Mcr and NrxIV (Figure 2.5C, D), suggesting that overexpression of Gliotactin leads to elevated miR-184 expression. Overexpression of Gli::EBFP did not downregulate Dlg (data not shown). To determine whether this phenomenon is specific to Gliotactin, we overexpressed NrxIV using an EP insertion in Nrx-IV (NrxIV-EP) under the control of *apterous*-GAL4. NrxIV overexpression failed to trigger self-downregulation (Figure 2.5A) and failed to downregulate Gliotactin or Mcr (Figure 2.5B). These results suggest that the overexpression of the Gliotactin extracellular domain is sufficient to trigger the downregulation of Gliotactin and other septate junction proteins, and that this effect is specific to Gliotactin and not a general property of septate junction proteins.
We next tested to see whether Gli::EBFP triggered downregulation of endogenous Gliotactin through miRNA control of the 3'UTR. To do this, we generated a series of transgenic lines that expressed Cherry-tagged Gliotactin under the control of the Gliotactin promoter and with different 3'UTRs: full-length, short and no UTR (Figure 2.2B). The Gliotactin promoter was used in an attempt to match the normal levels of expression. Each Cherry-tagged protein was trafficked to the TCJ with some spread away from the corners (Figure 2.5E–G). When Gli::EBFP was overexpressed in these backgrounds, we observed that only the Gliotactin with the entire 3'UTR (GP-Gli::Ch-L.UTR) was significantly downregulated (Figure 2.4C, F). Those with the short 3'UTR (GP-Gli::Ch-S.UTR) or no 3'UTR (GP-Gli::Ch-no.UTR) were unaffected (Figure 2.4D–E) and showed no significant downregulation in protein levels (Figure 2.4F). These findings suggest that the overexpression of Gliotactin triggers a feedback loop that specifically targets mRNAs with the long 3'UTR for degradation. Because this region contains the conserved miR-184 recognition sequence, this further indicated that control of Gliotactin mRNA is mediated by miR-184.

2.3.4 Gliotactin mRNA is downregulated and miR-184 increased with Gliotactin overexpression

To confirm that Gliotactin downregulation was at the mRNA level, we used daughterless-GAL4 (da-GAL4) to overexpress a full-length Gliotactin with the entire 3'UTR (GliEP) and quantified mRNA levels using RT-PCR analyses (Figure 2.6). The EP insertion drives the expression of the endogenous Gliotactin mRNA with the full 3'UTR and, when overexpressed with the strong da-GAL4 driver, Gliotactin mRNA levels were not significantly higher than those of control (Figure 2.6A). If there was no miRNA-mediated degradation of the Gliotactin mRNA, we expected to
see significantly more Gliotactin mRNA when EP was expressed. To test if overexpression of Gliotactin was driving the degradation of endogenous mRNA, we used da-GAL4 to express the Gli::EBFP transgene that lacks the entire intracellular domain and the 3'UTR. Using primers targeted to the intracellular domain, we examined the levels of endogenous Gliotactin mRNA. We found that the mRNA levels for endogenous Gliotactin were reduced compared to those in control when Gli::EBFP was overexpressed (Figure 2.6A), confirming that the downregulation of endogenous Gliotactin occurs at the mRNA level.

Our results suggest that Gliotactin mRNA degradation is mediated by miR-184 and that overexpression of Gliotactin leads to an increase in miR-184 levels. To test this hypothesis, we used quantitative real-time (qRT)-PCR to quantify changes in the pre-miR-184 RNA (Figure 2.6C) (Enderle et al., 2011). We determined that overexpression of either full-length Gliotactin (GliEP) or Gli::EBFP using apterous-GAL4, triggered a significant increase in miR-184 levels compared to those of control (Figure 2.6B). Our findings confirmed that induction of miR-184 levels results from overexpression of Gliotactin and thereby mediates Gliotactin mRNA levels in vivo.

2.3.5 A miR-184 sponge blocks Gliotactin downregulation

As the long 3'UTR of Gliotactin was necessary for the downregulation of the Gliotactin mRNA, this pointed to a role for either miR-184 or miR-1011. We found that miR-184 but not miR-1011 led to the downregulation of Gliotactin and other septate junction proteins (Figure 2.3). Next we investigated whether miR-184 was solely responsible. miRNA sponges are an effective means to sequester miRNAs within a cell (Loya et al., 2009). We utilized a sponge specific to miR-184 (SPmiR184) and expressed it using apterous-GAL4 within the wing imaginal disc. The sponge
alone had no significant effect on endogenous Gliotactin expression (Figure 2.5H). However, when two copies of the sponge were expressed along with UAS-miR-184, the downregulation of Gliotactin was blocked (Figure 2.7A). To test the effect of the sponge on Gliotactin overexpression, we co-expressed SPmiR-184 with Gli::EBFP and observed that the downregulation of endogenous Gliotactin was blocked (Figure 2.7B). To test the specificity of the miRNA, we expressed a bantam sponge (Herranz et al., 2012) along with Gli::EBFP. The bantam sponge did not prevent the downregulation of Gliotactin (Figure 2.7C) and the levels of Gliotactin were not significantly different from those in controls expressing Gli::EBFP alone (Figure 2.7D). Our findings strongly suggest that miR-184 regulates Gliotactin mRNA levels.

2.3.6 miR-184 expression is triggered through the BMP signaling Pathway

Our next aim was to determine the mechanism linking overexpression of Gliotactin to the induction of miR-184. miRNA levels are known to be controlled through a number of different pathways, including those involving MAPKs, BMP and TGF-β (Saj and Lai, 2011). There are three MAPKs in Drosophila melanogaster, Basket (Bsk; a JNK homologue), p38 (with three isoforms) and rolled (an ERK homologue). We did not observe any changes in the Gli::EBFP-induced reduction of endogenous Gliotactin when Bsk or p38 were blocked using dominant-negative transgenes [Bsk-DN (Petzoldt et al., 2013) and p38-DN (Adachi-Yamada et al., 1999), respectively] or with RNA interference (RNAi)-mediated knockdown of rolled (Biteau and Jasper, 2011)(Figure 2.8A–F). These results excluded MAPK signaling as the trigger for miR-184 transcription.

We next tested the role of TGF-β and BMP signaling. Co-expression of Gli::EBFP with dominant-negative Baboon protein (Parker et al., 2006) to block the TGF-β type-I receptor had
no effect on the Gli::EBFP-mediated downregulation of Gliotactin (Figure 2.8G, H), eliminating possible TGF-β involvement. To block BMP signaling, we co-expressed Gli::EBFP and a dominant-negative form of the BMP type-I receptor, Thickveins (Haerry et al., 1998). Expression of dominant-negative Tkv reduced the Gli::EBFP-mediated downregulation of endogenous Gliotactin (Figure 2.9A, B, E), whereas dominant-negative Tkv alone had no effect on endogenous Gliotactin levels (nor any additional deleterious effects), suggesting that Tkv was an integral part of the Gliotactin triggered miR-184 expression.

Ligand activation of Tkv and the type-II BMP receptor Punt leads to the phosphorylation and activation of Mad, a homologue of vertebrate SMAD (Derynck et al., 1996; Newfeld et al., 1996). We observed that expression of Gli::EBFP led to a significant increase in immunolabeling of phosphorylated Mad and to a greater spread of phosphorylated Mad across the dorsal side of the apterous boundary in the wing pouch (Figure 2.8I–K). To test whether Mad activation played a role in the miR-184-mediated reduction in Gliotactin levels, we co-expressed Gli::EBFP and a dominant-negative Mad transgene that lacked the DNA-binding domain (Mad-DN; (Takaesu et al., 2005). We found that dominant-negative Mad reduced the downregulation of endogenous Gliotactin (Figure 2.9C, D, E). Overall blocking of BMP-dependent signaling in conjunction with Gli::EBFP expression significantly inhibited Gliotactin downregulation compared to that with Gli::EBFP alone or with the negative control dominant-negative Bsk (Figure 2.9E). To confirm that inhibition of the BMP pathway blocked the increase in miR-184 transcription, we co-expressed Gli::EBFP with dominant-negative Mad and assayed the levels of miR-184 by using qRT-PCR. The level of miR-184 was the same as that in control (apterous-GAL4 alone) when the BMP signaling pathway was blocked and was significantly lower than when Gli::EBFP
was expressed alone (Figure 2.6B). These results suggest that excess Gliotactin triggers an increase in miR-184 through BMP-dependent signaling.

Two further direct tests of the role of Tkv activation in Gliotactin regulation were performed. Activation of Tkv and Mad leads to the induction of transcription of spalt and optomotor blind (omb/bifid) in the wing disc (Blair, 2007). In the first test, we used omb-lacZ (Sun et al., 1995) as a reporter of Tkv activation in the presence of increased Gliotactin (Gli::EBFP). We measured the region of omb-lacZ induction on the dorsal side compared to that on the ventral side along the apterous-GAL4 boundary. The pattern of omb-lacZ expression in control discs was evenly matched on both sides of the boundary (Figure 2.9F). Overexpression of Gliotactin led to a significant spread of omb-lacZ expression on the apterous-dorsal side relative to that on the ventral side (Figure 2.9G, H), indicating an increase in Tkv signaling. For the second test, we utilized a fluorescent reporter of Tkv activation (Tkv-TIPF), under the control of UAS, to confirm that the Tkv receptor itself was activated. The Tkv-TIPF reporter emits YFP fluorescence upon receptor activation and displacement of activated FKBP12 (also known as FK506-bp2; (Michel et al., 2011). We co-expressed Gli::EBFP with the Tkv-TIPF reporter using apterous-GAL4 (Figure 2.9J) and compared fluorescence levels to those upon expression of Tkv-TIPF alone (Figure 2.9I). Gli::EBFP triggered a significantly higher level of Tkv-TIPF fluorescence compared to that in controls (Figure 2.9K). These tests confirm that elevated Gliotactin activates the Tkv receptor, Mad and downstream transcription targets.

2.3.7 Overexpression of Gliotactin activates Tkv signaling through inhibition of Dad

If the BMP receptor mediates the increase in miR-184 transcription, we hypothesized that blocking this pathway enhances the phenotypes observed with overexpression of Gliotactin.
Overexpression of Gliotactin with *apterous*-GAL4 leads to the spread of Gliotactin away from the TCJ, resulting in deleterious consequences that include apoptosis, delamination of the columnar epithelia and spread of cells from the dorsal-apterous side into the ventral wild-type side of the wing disc (Figure 2.10A) (Padash-Barmchi et al., 2010). When both GliWT and dominant-negative Tkv were co-expressed in the wing imaginal disc, we observed that blocking Tkv signaling significantly enhanced the spread of the Gliotactin-overexpressing cells into the ventral half of the disc (Figure 2.10B, E). However, when both GliWT and an activated form of Tkv (Tkv-Act) were co-expressed, we observed that increased Tkv activation completely blocked the cell migration (Figure 2.10C, E). When GliWT was co-expressed with the miR-184 sponge, this increased migration of the Gliotactin-overexpressing cells (Figure 2.10C, E). However, simultaneous expression of Gliotactin and miR-184 significantly reduced the cell migration (Figure 2.10I, E). Taken together, these results show that loss of Tkv signaling and block of miR-184 lead to increased levels of endogenous Gliotactin expression and enhance the deleterious effects resulting from Gliotactin overexpression. Increased Tkv signaling and miR-184 expression suppressed the Gliotactin phenotypes.

We next wanted to establish the mechanism by which increased Gliotactin leads to activation of Tkv. To determine whether Tkv activation is through the ligand Deacapentaplegic (Dpp), GliWT and Dpp (UAS-Dpp::GFP) (Teleman and Cohen, 2000) were co-expressed in the wing imaginal disc. An increase in Dpp did not block the migration of cells overexpressing Gliotactin into the ventral half of the disc (Figure 2.10F arrow, J). In control discs, the overexpression of Dpp::GFP, driven by *apterous*-GAL4, had no effect on endogenous Gliotactin expression (data not shown; n=10 discs). Therefore, increased Dpp expression was not the cause of increased Tkv activation by Gliotactin. An alternative route to activation of Tkv could be through inhibition of
Dad, a Tkv inhibitor. We expressed GliWT in a Dad heterozygous mutant (Dad[j1E4]/+), which completely blocked the migration of GliWT cells into the ventral side of the disc (Figure 2.10G, J). Co-expression of GliWT and an EP (UAS) insertion in Dad (DadEP), to increase Dad expression, resulted in enhanced cell migration (Figure 2.10H, arrow and J). These results suggest that the activation of Tkv by Gliotactin is through inhibition of Dad. Our combined results indicate that the feedback loop to control Gliotactin mRNA works through activation of the BMP signaling pathway (Figure 2.11). Activation occurs through inhibition of Dad, resulting in increased Tkv receptor signaling and phosphorylation of Mad, leading to increased transcription of miR-184.

2.4 Discussion

Gliotactin, a TCJ protein, is crucial for the formation of septate junctions, the TCJ and the function of permeability barriers. At the protein level, Gliotactin is tightly controlled by tyrosine phosphorylation and endocytosis, and overexpression of Gliotactin leads to spread beyond the TCJ, with deleterious consequences including delamination, cell migration and apoptosis (Padash-Barmchi et al., 2010). We found that Gliotactin is also regulated at the mRNA level, specifically by miR-184-mediated degradation through a conserved miR-184-target site in the 3′UTR. Furthermore, we demonstrate that miR-184 controls other septate junction proteins, including NrxIV, Coracle and Mcr. We propose that excessive Gliotactin acts as the trigger to induce miR-184 expression through the activation of BMP signaling and the Tkv type-I BMP receptor. Activation of Tkv is not through elevated levels of its ligand Dpp, but through inhibition of Dad, an inhibitory SMAD.
2.4.1 Gliotactin overexpression acts as the trigger for miR-184 induction

miR-184 is expressed throughout *Drosophila* embryogenesis in the brain, ventral nerve cord (Li et al., 2011), mesoderm, endoderm (Aboobaker et al., 2005) and in imaginal discs at larval stages (Li et al., 2011). miR-184 regulates germline development, where loss of miR-184 leads to oogenesis defects (Iovino et al., 2009). However, miR-184 null mutants are viable with normal morphology, leading to the suggestion that maternally deposited miR-184 might persist through embryogenesis, allowing development into larval stages and beyond (Iovino et al., 2009). An alternative explanation is that miR-184 is expressed at basal levels throughout development, but increased expression and function occurs through a trigger. Our findings suggest that overexpression of Gliotactin is a trigger for increased miR-184 transcription and that this in turn leads to the downregulation of a suite of other septate junction proteins. *In vitro* luciferase assays have previously identified Gliotactin, NrxIV, Contactin, Coracle, Megatrachea, Sinuous and Kune-Kune as potential targets of miR-184 (Kertesz et al., 2007). Our *in vivo* results show that miR-184 regulates Gliotactin at the mRNA level as part of a feedback loop. miRNAs play a key role in the regulation and maintenance of a range of vertebrate epithelia. For instance, miR-200 is enriched in breast and ovarian epithelia, and can suppress the epithelia-to-mesenchymal transition (Gregory et al., 2008; Park et al., 2008). miR-122a regulates intestinal tight junction permeability by targeting Occludin mRNA (Ye et al., 2011). miR-184 is expressed at high levels in the vertebrate eye, including the retina pigmented epithelia, cornea and lens (Kapsimali et al., 2007; Ryan et al., 2006), and regulates the blood–retina barrier (Wang et al., 2010) as well as angiogenesis (Yu et al., 2008). Collectively, these findings reveal a role of miRNAs in the control and maintenance of epithelial cells.
In the *Drosophila* imaginal disc, miR-184 controls the levels of a suite of septate junction proteins and the TCJ protein Gliotactin. During imaginal disc development, there is a dynamic reorganization of the septate junctions as cells rearrange to generate the structures of the adult wing (Fristrom, 1982). Intact septa either become extended or compacted in response to these cellular movements in order to maintain the transepithelial barrier. Thus, miR-184 might function to coordinate septate junction domain proteins during imaginal epithelial morphogenesis to ensure that cells move and rearrange more easily as the wing structures develop. However, expression of the miR-184 sponge did not generate defects in wing morphogenesis, and mir-184 mutants are viable with no defects in epithelial morphogenesis, suggesting that increased miR-184 expression is transient and localized. Because the septate junction complex is highly stable (Oshima and Fehon, 2011), it is likely that basal levels of miR-184 have little impact on the septate junction domain. Rather, a localized increase in miR-184 in response to a trigger would be necessary to change the stability of this complex, as might occur during dynamic processes such as morphogenesis, proliferation, migration or apoptosis. miRNAs in both *Drosophila* and vertebrates can regulate apoptosis by controlling the levels of pro-apoptotic or anti-apoptotic genes (Jovanovic and Hengartner, 2006). For instance, the *Drosophila* death genes Drice, hid, reaper, grim and sickle are targeted either individually or as a group by miRNAs, including miR-2, miR-6, miR-2/13, miR-11, miR-14 and miR-308 (Leaman et al., 2005; Stark et al., 2003; Xu et al., 2003). In particular, the bantam miRNA promotes cell proliferation through regulation of hid (Brennecke et al., 2003) and is controlled by the Dpp pathway (Doumpas et al., 2013; Martin et al., 2004; Oh and Irvine, 2011; Zhang et al., 2013). Thus, an increase in miR-184 might be triggered by Tkv activation during the processes that underlie the apoptosis stimulated by overexpression of Gliotactin.
2.4.2 BMP signaling pathway induces miR-184 activation

Our analysis points to the BMP signaling pathway as part of the miR-184 trigger downstream of Gliotactin overexpression. In vertebrates, BMP or TGF-β signaling pathways play an important role in the transcription and biogenesis of miRNAs (Kato et al., 2009; Winbanks et al., 2011). BMP4 and TGF-β elevate the levels of 20 different species of miRNAs and recruit receptor-regulated SMADs (R-SMADs) to enhance Drosha processing of pri-miRNAs (Davis et al., 2008; Harris and Ashe, 2011; Saj and Lai, 2011). BMP control of microRNA biogenesis and processing has been shown to control the proliferation and maintenance of vertebrate epithelia (Saj and Lai, 2011; Wang et al., 2010). For instance, TGF-β activation of miR-204 and miR-211 (miR 204/211) leads to targeting of SNAIL2, which normally represses Claudin transcription and other proteins crucial for maintaining epithelial structure (Carrozzino et al., 2005). miR-204 in retinal pigment epithelium inhibits TGF-βR2, Smad3 and SNAIL1 and SNAIL2 to protect tight junction integrity (Wang et al., 2010). These findings suggest that the expression of miR-204/211 and involvement of TGF-β signaling in a feedback loop is an important signal for integrity and maintenance of tight junctions (Kato et al., 2009; Xu et al., 2009).

In Drosophila, Dpp signaling and Mad can control cell proliferation through transcriptional control of the bantam microRNA through different transcription factors, including omb, brinker and yorkie (Doumpas et al., 2013; Martin et al., 2004; Oh and Irvine, 2011; Zhang et al., 2013). Our data show that overexpression of Gliotactin elevated Mad phosphorylation and increased transcription of omb, but we found no evidence that the bantam miRNA was involved in Gliotactin or septate junction protein control. The predicted bantam-targeted site was retained within the short Gliotactin 3'UTR, and neither the expression of bantam miRNA nor the bantam sponge had an effect on Gliotactin levels.
We propose that Gliotactin activates the BMP signaling pathway to increase pri-miR-184 transcription, acting through Tkv and Mad. Co-expression of Gliotactin with dominant-negative Mad blocked the increase in transcription of miR-184 and prevented the resulting downregulation of endogenous Gliotactin. It is likely that Gliotactin acts upstream of Mad through activation of the Tkv receptor. The increased fluorescence intensity of the Tkv-TIPF reporter upon Gliotactin overexpression confirms that the Tkv receptor itself is activated in response to overexpression of Gliotactin, either indirectly or directly. Gliotactin might increase the BMP ligand–receptor interaction, perhaps through increased membrane retention, increased ligand levels or increased capacity for ligand–receptor interactions. Alternatively, Gliotactin might interfere with an inhibitor of Tkv, such as the inhibitory Smad, Dad. Dad is stably associated with Tkv and negatively regulates phosphorylation of Mad, probably by competing with Mad for Tkv binding (Bokel et al., 2006; Inoue et al., 1998). Our results favor the latter explanation, that Gliotactin increases Tkv signaling through disruption of Dad. Increased Dpp expression did not rescue the effects of overexpressed Gliotactin in the wing disc. Conversely, a reduction of the Dad gene dose by 50% completely suppressed the Gliotactin overexpression phenotypes, whereas increased Dad expression enhanced those phenotypes. These results point to a feedback loop by which Gliotactin reduces the association of Tkv and Dad to increase Tkv activation. Whether this interaction is intra- or extracellular, or indirect remains to be determined. Gliotactin interacts with the MAGUK scaffolding protein Dlg (Padash-Barmchi et al., 2010); however, the signaling complex formed between Gliotactin and Dlg is mediated by the PDZ-binding motif (Padash-Barmchi et al., 2013). Because Gli::EBFP lacks this domain, it is unlikely that the activation of miR-184 is mediated by the binding of a PDZ protein. Rather, the
extracellular domain is likely to mediate the interactions that trigger Tkv activation. However, if there are other triggers of Tkv and miR-184 production, these have yet to be determined. The activation of Mad by Tkv leading to increased miR-184 expression could be due to the direct transcriptional activation of miR-184 by Mad or through the removal of an inhibitor such as brinker. The growth-promoting function of Dpp works through the repression of the transcription-repressor brinker (Schwank et al., 2008). Over 1000 genes have been identified as potential brinker targets, including the bantam miRNA (Doumpas et al., 2013; Oh and Irvine, 2011). However, miR-184 has not been identified as a brinker target or any other BMP components. Regardless of the transcription mechanism, our model proposes that increased Gliotactin leads to displacement of Dad from Tkv, increased Mad activation and miR-184 transcription, which reduces Gliotactin mRNA levels within the cell. Thus, Gliotactin is subject to two levels of regulation – at the protein level through tyrosine-mediated phosphorylation and endocytosis and at the mRNA level through miR-184 mediated-degradation that is stimulated by Tkv receptor signaling.

2.5 Material and methods

2.5.1 Fly stocks

The following fly strains were used: Gli[EP2306], NrxIV[EP604] (Rorth, 1996), UAS-GliWT (Schulte et al., 2006), Gli::YFP (Kyoto DGRC), NrxIV::GFP (Buszczak et al., 2007), UAS-mCD8::RFP (Bloomington), UAS-miR-184 (Iovino et al., 2009), UAS-miR-1011 (Bejarano et al., 2012), UAS-sponge-bantam (Herranz et al., 2012), UAS-bantam (Yang et al., 2008), UAS-Bsk-DN (Petzoldt et al., 2013), UAS-p38-DN (Adachi-Yamada et al., 1999), UAS-rolled-RNAi (Biteau and Jasper, 2011), UAS-Tkv-Act (Haerry et al., 1998) and UAS-Tkv-DN (Haerry et al.,
1998), UAS-Tkv-TIPF (Michel et al., 2011), UAS-Mad-DN (Takaesu et al., 2005), UAS-Baboon-DN (Parker et al., 2006), UAS-Dpp::GFP (Teleman and Cohen, 2000), Dad[j1E4] (Ogiso et al., 2011), Dad[EP3196] (Bellen et al., 2004), omb-lacZ (Sun et al., 1995) and *apterous–GAL4* (Bloomington *Drosophila* Stock Center).

**2.5.2 Generation of transgenic lines**

For the generation of the Gliotactin 3'UTR transgenic lines, the entire coding sequence of Gliotactin was amplified from a Gliotactin cDNA (AE27.41) (Auld et al., 1995). The Gliotactin coding sequence, an mCherry tag and one of three different UTRs [no 3'UTR (SV40), short 3'UTR (AE27.41, Auld et al., 1995) and long 3'UTR (RE15719, *Drosophila* Genomics Resource Center)] were amplified using the following primers:

- **extracellular-Gliotactin5**: 5'-TCTAGATCATCATGATGCAC-3' (XbaI site) and extracellular-Gliotactin3: 5'-GAATTCCAGGGTCAACGAATC-3' (EcoRI site);
- **mCherry5**: 5'-GAATTCATGGTGAGCAAGGGAGAG-3' (EcoRI site) and mCherry3: 5'-AAGCTTCTTGTACAGCTCGTCCATG-3' (HindIII site);
- **Gliotactin-transmembrane5**: 5'-AAGCTTGATGTATTCGTGACCAC-3' (HindIII site) and Gliotactin-transmembrane3:
  - 5'-CGTACGCACATGATGCAGATG-3' (BsiWI); C-terminal-Gliotactin5:
  - 5'-CGTACGCACATGATGCAGATG-3' (BsiWI site) and
  - C-terminal-Gliotactin3: 5'-GACGTCTTTATACGGATGTCTGAGGAGGAG-3'; no-3'UTR (SV40)5: 5'-GACGTCTTTATACGGATGTCTGAGGAGGAG-3' (AatII site) and no-3'UTR (SV40) 3: 5'-CTCGAGGATCCAGACCAGCGAAG-3' (XhoI site); short-3'UTR5: 5'-GACGTCTTTATACGGATGTCTGAGGAGGAG-3'; short-3'UTR3: 5'-CTCGAGGATCCAGACCAGCGAAG-3' (XhoI site) and short-3'UTR3:
  - 5'CTCGAGTCTTTATACGGATGTCTGAGGAGGAG-3' (XhoI site); long-3'UTR5:
5′-GACGTCGTAACAGCTCTCTAAGCAGCAG-3′ (AatII site) and long-3′UTR3:
5′-CTCGAGATTTATTTATGGACAAATACATAAAAC-3′ (XhoI site).

The Gliotactin promoter was amplified using the following primers:

Gliotactin-promoter5: 5′-CACTTGGATCCTTATTTACAATCAGC-3′ (BamHI site) and
Gliotactin-promoter3: 5′-GATGATCTAGAAAAATATTCAAAAGTAG-3′ (XbaI site).

PCR products were subcloned into the pβSΔF′ vector and sequenced for verification. The UTR constructs (no, short and long 3′UTR) were subcloned into pUAST-linker vector or 3′ to the Gliotactin promoter (3.7 Kb) in the pCasper 5 vector. To generate the pUAS-Gli::EBFP line, the enhanced BFP (Addgene) was PCR amplified and introduced into the GliNter construct, as previously described (Padash-Barmchi et al., 2010) using the SpeI and EcoRI restriction sites. Each construct was sequenced, and multiple independent transgenic lines were generated (Genetic Services).

The miR-184 sponge (SPmiR-184) construct contains 20 repetitive seed complementary sequences separated by variable four-nucleotide linkers, assembled as previously described (Loya et al., 2009) and cloned into the 3′UTR of mCherry between NotI and XbaI sites in a modified pWALIUM10-moe vector (Ni et al., 2009), carrying the white+ marker and flanking insulator sequences (Bejarano et al., 2012); the combined miRNA and linker sequences were checked against every mature miRNA sequence in the Drosophila genome to prevent off-target effects. Transgenic strains were generated using phiC31 site-specific genomic integration on the second (attP40) and third (attP2) Drosophila autosomes (Genetic Services).
2.5.3 RNA analysis

2.5.3.1 Reverse transcriptase PCR

Embryos were collected and dechorionated in 50% bleach. Total RNA was extracted using Trizol (Invitrogen). 2 µg of total mRNA was used to synthesize the cDNA using random hexamers and Superscript II reverse transcriptase (Applied Biosystems). Synthesized cDNA was amplified using AE2XI and AE2XII primers from the C-terminus of Gliotactin. AE2XI primer: 5′-GCGTGGAGTGGACACATTGCCC-3′; AE2XII primer: 5′-GGAATTCCGCTGACTCCC-3′. Actin primers were used as an internal control. The expression data were normalized to the levels of actin. Band intensity was measured using ImageJ, and data were analyzed using one-way ANOVA and GraphPad Prism6.

2.5.3.2 qRT-PCR

RNA was extracted from 60 third instar wing discs, and RNA was extracted using QIAGENE RNeasy kit. 1 µg of total RNA was used to generate cDNA with random primers and Superscript III reverse transcriptase (Applied Biosystems). SYBR GREEN-based qRT-PCR (Bio-Rad) was performed for miR-184 and actin. qRT-PCR conditions were used for apterous-GAL4, GliEP, Gli::EBFP and Mad-DN lines using primers designed for pre-miR-184 (Enderle et al., 2011). Forward primer sequence: 5′-ATGCACATGAGTTGGCAGACAGC-3′, reverse primer sequence: 5′-CTTACCCCACGTTTCGATTACGC-3′. qRT-PCR cycling conditions were 95°C for 30s, 40× (95°C for 5 s, 60°C for 30 s). qRT-PCR data were analyzed by using the comparative ΔΔCt method (Livak and Schmittgen, 2001). qRT-PCR products were sequenced to confirm primer specificity.
2.5.4 Statistical analyses

All statistical tests were calculated and graphed with GraphPad Prism 6. Immunofluorescence levels were quantified using ImageJ (Schneider et al., 2012). Fluorescence intensity of UAS-Gli::Ch constructs was measured across two areas on the apterus side (Figure 2.1J). The intensity ratio was calculated, and significance was calculated using a one-way ANOVA with Tukey’s post-hoc test. For all other figures, the mean intensity was calculated using the same fixed-size square in the pouch region of the apteres and the wild-type sides of wing imaginal disc (Figure 2.4). The ratio of intensity in the apteres side compared to intensity in the wild-type side was calculated. The migration of Gliotactin-overexpressing cells (Figure 2.10) from the apteres side into the wild-type side of the wing imaginal disc was measured, and the distance of spread was calculated as a ratio of the length of the ventral, or wild-type, side of the disc. Significance was determined using a one-way ANOVA with Tukey’s post-hoc test.

For Tkv-TIPF quantification (Figure 2.9I, J), fluorescence intensity was measured in a set area with ImageJ on the apteres side with or without Gli::EBFP. Discs from both genotypes were analyzed at the same time, images were collected with the same exposure time and neutral density filters, and were deconvolved using the same protocol. Significance was determined using an unpaired t-test.

2.5.5 Immunolabeling

Third instar larval imaginal discs were stained as described previously (Schulte et al., 2006). Primary antibodies were: mouse anti-Gli 1F6.3 at 1:200 (Auld et al., 1995), rabbit anti-Gli at 1:300 (Venema et al., 2004), mouse anti-Dlg 4F3 at 1:200 (Developmental Studies Hybridoma Bank) (Parnas et al., 2001), rat anti-DE-Cadherin at 1:50 (Developmental Studies Hybridoma
Bank), mouse anti-Coracle (9C and C615-16B cocktail) at 1:100 (Developmental Studies Hybridoma Bank) (Fehon et al., 1994), rabbit anti-NrxIV at 1:500 (Baumgartner et al., 1996), guinea pig anti-Mcr at 1:800 (Hall et al., 2014), mouse anti-Fasciclin 3 at 1:100 (Developmental Studies Hybridoma Bank), rabbit anti-phosphorylated Mad at 1:200 (Cell Signaling). DAPI was used at 1:1000 (Thermo Scientific). Secondary antibodies were used at 1:300 – goat anti-rabbit (conjugated to Alexa Fluor 647 or Alexa Fluor 568), goat anti-mouse (conjugated to Alexa Fluor 647, Alexa Fluor 488 or Alexa Fluor 568), goat anti-rat (conjugated to Alexa Fluor 647 or Alexa Fluor 568) (Molecular Probes).

2.5.6 Imaging

Image stacks were collected with a DeltaVision Spectris microscope (Applied Precision, Issaquah, WA) with a 20× air- or 60× oil-immersion lens (NA1.4) and CoolSnap HQ digital camera. Deconvolution of 0.2 m z-sections with SoftWorx (Applied Precision) used a point-spread function measured from 0.2-μm beads conjugated with Alexa dyes (Molecular Probes) mounted in Vectashield (Vector Labs). Side projections were created using the SoftWorx program. Images were exported to Photoshop (Adobe Systems) for compilation.
Figure 2.1: Overexpression of Gliotactin causes self-downregulation
(A) The expression pattern of Gliotactin in a wild-type wing imaginal disc, immunolabelled for Gliotactin (green, A’) and Dlg (red, A”). Gliotactin was concentrated at the tricellular junction (arrow) and Dlg at the septate junction. (B–H) *apterous*-GAL4 driven expression of Gliotactin transgenes within the wing imaginal disc. The dashed line marks the dorsal–ventral boundary with the dorsal apterous side of the wing imaginal disc to the left. (B, C) High- (B) and low- (C) resolution images of a wing imaginal disc with overexpression of endogenous Gliotactin (green, B’, C’) from an EP (UAS) insertion in Gliotactin. Gliotactin was downregulated in a patchy manner, whereas Dlg was not (red, B”). (D) Overexpression of a Gliotactin transgene with the full-length 3’ UTR (Gli::Ch-L.UTR) (green, D’) was downregulated (arrow) in regions within the apterous side, whereas Dlg (red, D”) was not. (F–I) Overexpression of Gliotactin with the short 3’ UTR (Gli::Ch-S.UTR) or a Gliotactin transgene without a 3’ UTR (Gli::Ch-no.UTR). Neither Gliotactin (green, F’, H’) nor Dlg (red, F”, H”) were downregulated. (E, G, I) Side views of the corresponding panels above. (J) Diagram of wing imaginal disc. Fluorescence intensity was measured in two separate areas (squares) in the wing pouch of the apterous side of the imaginal disc (green). A side view of the disc shows the position of the columnar epithelia within the wing pouch. (K) The fluorescence intensity ratio (apterous to wild-type side) of Gli::Ch-L.UTR and GliEP was significantly downregulated in regions across the apterous expression domain compared to the lack of downregulation of Gli::Ch-S.UTR or Gli::Ch-no.UTR (n=10 discs, ****P<0.0001, mean±s.d., one-way ANOVA). (L) The Gliotactin 3’ UTR, indicating the conserved miRNA-target sites. Sites for miR-281, bantam, miR-981, miR-190, miR-124, miR-1011 and miR-184 are shown, and the dotted line indicates the border of the short and full-length of 3’ UTRs observed in Gliotactin-expressed sequence tags. Scale bars: 15 μm (all images except C); 50 μm (C).
Figure 2.2: Gliotactin transgenic lines

(A) Three Gliotactin transgenes under the control of UAS. Gliotactin is tagged with Cherry in the extracellular domain. Each transgene has different 3’UTRs: a long UTR (UAS-Gli::Ch-L.UTR), short (UAS-Gli::Ch-S.UTR) and no UTR (UAS-Gli::Ch-no.UTR). Gliotactin has two different polyadenylation sites leading to two mRNA isoforms with differing 3’UTRs. The cDNA with the short 3’UTR isoform is AE27.41 and the cDNA with the long 3’UTR is RE15719. (B) Three Gliotactin transgenes under the control of the cloned Gliotactin promoter are shown with full length, short and no 3’UTR. The cloned Gliotactin promoter extended 3.7 Kb 5’ of the translation start site. (C) Two Gliotactin transgenes used for overexpression. GliWT contains the entire coding region but lacks the 3’UTR. Gli::EBFP also lacks the 3’UTR and has the entire intracellular domain deleted (including the conserved tyrosine sites necessary for endocytosis and the PDZ motif) and replaced with enhanced blue fluorescent protein (EBFP). Both transgenes include the SV40 polyadenylation signal.
Figure 2.3: miR-184 controls a suite of septate junction proteins

In all panels, apterous-GAL4 was used to drive expression in the wing disc. The dashed line indicates the dorsal–ventral boundary with the apterous side on the left. (A–J) The expression of miR-184 led to the downregulation of Gliotactin (green, A′), NrIV (green, C′), Mcr (green, E′) and Coracle (green, Cora, G′) but not Dlg (red, A″–E″), E-cadherin (green, Ecad, I′) or Fasciclin 3 (red, Fas3, I″). Side projections for each panel are shown below. (K, L) Expression of miR-1011 (K, blue) or bantam miRNA (L, blue) did not reduce the levels of Gliotactin (green, K′, L′) or Dlg (red, K″, L″). All xy panels represent a single z slice. Scale bars: 15 μm.
Figure 2.4: Gliotactin overexpression triggers self-downregulation through 3’UTR

apterous-GAL4-driven expression in the wing disc. Dashed lines indicate the dorsal-ventral boundary with the apterous side on the left. (A) Expression of Gli::EBFP, lacking the intracellular and 3’UTR domains and tagged with BFP (red, A″), led to downregulation of endogenous Gliotactin (green, A’). (B) Expression of GliWT, a full-length Gliotactin lacking the 3’ UTR (red, B″), caused downregulation of endogenous Gliotactin tagged with YFP (Gli::YFP) (green, B’). (C–E) Expression of Gli::EBFP (red, C″–E″) led to downregulation of GP-Gli::Ch-L.UTR (green, C’) but not of GP-Gli::Ch-S.UTR (green, D’) or GP-Gli::Ch-no. UTR (green, E’). (F) Statistical analysis of the Gliotactin fluorescence intensity on the apterous side (black square in cartoon) normalized to that of the wild-type non-apterous side (white square in cartoon) confirmed that Gli::EBFP significantly downregulated GP-Gli::Ch-L.UTR compared to GP-Gli::Ch-S.UTR or GP-Gli::Ch-no.UTR (n=10 discs, ****P<0.0001, mean±s.d., one-way ANOVA). All panels represent a single z-slice. Scale bars: 15 μm.
Figure 2.5: Gliotactin, not NrxIV, triggers other SJ protein downregulation

In all panels *apterous*-GAL4 (*ap*-GAL4) was used to drive expression in the wing disc. The dashed line indicates the dorsal-ventral boundary with the apterous side on the left. (A-B) Overexpression of NrxIV did not induce self-downregulation. (A) Expression of NrxIV-EP under the control of *ap*-GAL4 driver did not show self-downregulation (green) and did not affect Dlg levels (red). (B) Expression of NrxIV-EP driven with *ap*-GAL4 (blue) did not affect Gliotactin (green) or Mcr (red) expression. (C-D) Gliotactin overexpression resulted in NrxIV and Mcr downregulation. (C) Expression of Gli::EBFP transgene under the control of *ap*-GAL4 lead to downregulation of Gliotactin (green) and Mcr (red). (D) GliWT (green) expression under ap-Gal4 resulted in NrxIV::GFP (red) downregulation. (E-G) Expression of GP-Gli::Ch lines alone did not show Gliotactin downregulation. GP-Gli::Ch-L.UTR (green, E), GP-Gli::Ch-S.UTR (green, F) and GP-Gli::Ch-no.UTR (green, G) are expressed at levels that lead to protein spread beyond the cell corners but were not sufficient to trigger downregulation. Immunolabeling for Dlg (red) labeled the septate junction. (H) Expression of the miR-184 sponge alone does not alter Gliotactin expression. Expression of two copies of the miR-184 sponge (red) under the control of the *ap*-GAL4 driver did not change endogenous Gliotactin expression levels (green). All en face panels represent a single Z slice. Scale bars: 15 μm.
Figure 2.6: Gliotactin overexpression leads to reduced Gliotactin mRNA and increased miR-184 expression

(A) RT-PCR analysis of Gliotactin expression in embryos expressing daughterless-GAL4 (da-GAL4, da) alone or driving GliEP or Gli::EBFP (da>GliEP and da>Gli::EBFP, respectively). Actin was used as internal control, and the mRNA level of each genotype was normalized against actin level in that genotype (n=3, *P<0.05, one-way ANOVA). (B) qRT-PCR analysis of Gliotactin expression in wing imaginal discs isolated from third instar larvae expressing apterous-GAL4 alone (ap-GAL4), or driving either GliEP or Gli::EBFP. miR-184 in GliEP and Gli::EBFP wing discs was significantly higher than that in control (ap-GAL4) (n=8, **P<0.01, ****P<0.0001, one-way ANOVA). Expression of dominant-negative Mad (MAD-DN) with Gli::EBFP blocked the expression of miR-184, and there was no significant (NS) difference compared to the control (n=8, P>0.5, one-way ANOVA). Data are mean±s.d. (C) qRT-PCR primers were designed and sequenced for pre-miR-184.
**Figure 2.7: A miR-184 sponge blocks the Gliotactin downregulation**

*apterous*-GAL4-driven expression in the wing disc. Dashed lines indicate the dorsal–ventral boundary, with the apterous side on the left. (A) Co-expression of a miR-184 construct with two copies of the miR-184 sponge (SPmiR-184×2) tagged with mCherry (blue, A) blocked the downregulation of Gliotactin (green, A'). Dlg expression was normal (A''). (B) The miR-184 sponge co-expressed with Gli::EBFP. Two copies of the miR-184 sponge (red, B'') blocked the downregulation of endogenous Gliotactin (green, B'). (C) Co-expression of two copies of a bantam sponge with Gli::EBFP (red, C''). Endogenous Gliotactin (green, C') was still downregulated. (D) The double miR-184 sponge significantly blocked the downregulation of endogenous Gliotactin (n=10 discs, ****P<0.0001, mean±s.d., one-way ANOVA) relative to the expression of Gli::EBFP alone, whereas the double bantam sponge (SPbant×2) did not. All image panels represent a single z slice. Scale bars: 15 μm.
Figure 2.8: The Gliotactin feedback loop is not mediated by MAPK or TGFβ signaling

In all panels ap-GAL4 was used to drive expression in the wing imaginal disc. Side projections are shown for each corresponding en face panel with the dashed line indicating the dorsal-ventral boundary. (A-F) The MAPK signaling pathway is not involved. Expression of Gli::EBFP and three different constructs to reduce MAPK signaling. Expression of Gli::EBFP (red) and Bsk-DN (A), p38-DN (C) and rolled-RNAi (E) did not block the reduction of endogenous Gliotactin (green, A’-E’). (G-H) The TGF-β signaling pathway is not involved. Co-expression of Gli::EBFP (red) with Baboon-DN did not block the reduction of Gliotactin (green, G’). (I-J) Gli::EBFP leads to increased phospho-Mad Comparison of the fluorescent intensity of phospho-Mad in control discs (I) and ap>Gli::EBFP (J) shows increased phospho-Mad in the dorsal half of the wing disc. (J”) Quantification of fluorescent intensity of phospho-Mad in control versus Gli::EBPF showed a significant increase in the ratio of dorsal versus ventral fluorescence (n=16 discs, p<0.0001, mean±SD). All en face panels represent a single Z slice. Scale bars: 15 μm.
Figure 2.9: BMP receptor Tkv signaling is key to Gliotactin regulation
apterous-GAL4-driven expression in the wing disc. Dashed lines indicate the dorsal–ventral boundary with the apterous side on the left. Side projections are shown for each corresponding xy panel in A and C. (A–D) Co-expression of Gli::EBFP (red, A”-D”) with dominant-negative Tkv (Tkv-DN, A) or dominant-negative Mad (MAD-DN, C) blocked the downregulation of endogenous Gliotactin (green, A’-D’). B and D show side views. (E) The block in downregulation of endogenous Gliotactin by Tkv-DN and Mad-DN was significant when compared to that upon expression of Gli::EBFP alone (n=10 discs, ****P<0.0001, mean±s.d., one-way ANOVA). In contrast, dominant-negative Basket (Bsk-DN) had no effect on Gliotactin downregulation. (F) Expression of omb-lacZ (red, F”) in a control disc. omb-lacZ expression spanned the dorsal–ventral boundary (arrow dorsal and arrowhead ventral), with equal distribution on either side. (G) Expression of Gli::EBFP (green, G’) triggered the spread of omb-lacZ (red, G”) along the dorsal–ventral boundary on the apterous side (arrow dorsal and arrowhead ventral). (H) The region of omb-lacZ expression was significantly greater with GliWT compared to wild type (n=10 discs, ****P<0.0001, mean±s.d., one-way ANOVA). (I–K) Comparison of the fluorescence intensity of Tkv-TIPF (green, I’, J’) alone or co-expressed with Gli::EBFP shows increased activation of Tkv by Gli::EBFP. Gliotactin immunolabeling (red, I”, J”). (K) Quantification confirmed a significant increase in the fluorescence intensity of Gli::EBFP with Tkv-TIPF compared to Tkv-TIPF alone (n=10 discs, ****P<0.0001, mean±s.d., t-test). (A–D) All xy panels represent a single z slice. Scale bars: 15 μm. Scale bars: 50 μm (F–J).
Figure 2.10: Gliotactin phenotypes can be enhanced or suppressed by changing Tkv signaling levels

apterous-GAL4-driven expression in the wing imaginal disc. The dashed line indicates the dorsal–ventral boundary with the apterous side on the left. (A) Overexpression of GliWT (green, A’) triggered apoptosis and the migration of delaminated cells into the non-apterous (ventral) side of the disc (arrow) (Dlg, red, A”). (B) Co-expression of GliWT (green, B’) and a dominant-negative Tkv construct (Tkv-DN) increased the migration of cells into the non-apterous side of the disc (arrow) (Dlg, red, B”). (C) Co-expression of GliWT (green, C’) and activated Tkv (Tkv-
Act) blocked the spread of cells (Dlg, red, C″). (D) Co-expression of GliWT (green, D′) and two copies of the miR-184 sponge (SPmiR-184X2, red, D″) increased cell migration and enhanced the effects of Gliotactin overexpression. (E) The degree of migration was quantified as illustrated in the wing disc cartoon. The distance that GliWT cells migrated from the apertous into the non-apertous side (red arrow) was compared to the distance from the apertous boundary to the distal wing edge (black arrow). For A–D, I, co-expression of Tkv-DN or the miR-184 sponge (SPmiR-184) significantly increased the migration of GliWT cells, whereas co-expression of Tkv-Act (Tkvact) or miR-184 significantly decreased migration. (For all: n=10 discs, ****P<0.0001, mean±s.d., one-way ANOVA.) (F) Co-expression of GliWT (green, F′) and Dpp::GFP (red, F″) enhanced cell migration. (G) Expression of GliWT (green, G′) in a heterozygous Dad mutant (Dad[ j1E4]/+) blocked migration (Dlg, red, G″). (H) Co-expression of GliWT (green, H′) with an EP insertion in Dad (DadEP) to increase Dad expression enhanced migration of Gliotactin-expressing cells (Dlg, red, H″). (I) Co-expression of GliWT (green, I′) and miR-184 prevented the migration of GliWT cells (Dlg, red, I″). (J) The degree of migration in F–H was quantified. Cell migration in GliWT-, Dpp::GFP- and GliWT-, DadEP-expressing wings was significantly increased, whereas migration in GliWT, Dad[ j1E4]/+ was significantly decreased compared to GliWT alone. (For all: n=10 discs, ****P<0.0001, mean±s.d., one-way ANOVA) Scale bars: 50 μm.
Figure 2.11: Gliotactin auto-regulation model

Increased Gliotactin expression leads to the dissociation of Dad from Tkv, resulting in increased Tkv activation and increased phosphorylation of Mad. Activated Mad leads to increased miR-184 transcription, which in turn binds to target sequences on the 3’UTR of Gliotactin and reduces Gliotactin mRNA levels. P: phosphorylation.
Chapter 3: Scribbled mediates tricellular junction formation

3.1 Synopsis

A scaffold protein Scribble that in human is encoded by the SCRIB plays role in cell polarity, migration and proliferation in epithelial cells. In Drosophila Scribble (Scrib), Disc large (Dlg) and Lethal giant larvae (l(2)gl) are tumor suppressor genes and act as key regulators of apicobasal polarity. Scribble complex components control many cell functions besides cell polarity including assembly and maintenance of cell junctions, and protein trafficking. The Scribble leucine-rich repeats (LRR) domain is essential to maintain the polarized epithelial monolayer and control proliferation, while the PDZ domains are required for septate junction (SJ) formation independent of Scribble’s role in apicobasal polarity. Here we show that there is a novel Scrib complex at the TCJ. We found that Scrib is in close proximity with Gliotactin and Bark, along with Dlg at the TCJ. We propose that Scribble PDZ domains 1-2 are essential for the recruitment of Gliotactin and Bark to the TCJ. Moreover, Bark and Gliotactin knockdown lead to the basolateral spread of Scribble and Dlg, and SJ proteins knockdown including NrxIV and ATPα result in the basolateral spread and upregulation of Gliotactin, Bark and Scrib at the TCJ. We also found that Scrib and Dlg knockdown led to the loss of Scrib, Dlg, Bark, and Gliotactin from the TCJ, however, the last region that these proteins are retained before loss from the membrane is at the TCJ. We also suggest that the Dlg GUK domain plays role in Bark trafficking a different mechanism from Gliotactin.
### 3.2 Introduction

Permeability barriers play important roles in preventing fluid flow and in restricting the diffusion of microscopic objects both in vertebrate and invertebrate. Permeability barriers are formed by tight junctions in vertebrates and septate junctions in insects and have similar physiological functions and homologous components (Tsukita et al., 2001). *Drosophila* septate junctions are basolateral junctions and form permeability barriers below adherens junctions in epithelial cells (Tepass and Hartenstein, 1994; Tsukita et al., 2001). SJ core components, including Neurexin IV (NrxIV) (Baumgartner et al., 1996), Coracle (Cora) (Fehon et al., 1994), the NA+/K+ ATPase (the alpha subunit (ATPα) and β subunit, Nervana2 (Nrv2) and Neuroglian (Nrg) (Genova and Fehon, 2003; Paul et al., 2003), are required for the formation and function of SJ. However the SJ is key for the formation of permeability barriers between two neighboring cells, an addition junction the tricellular junction (TCJ) is necessary to create a barrier in the corners between cells. The TCJ is formed by the convergence of three biccicular junctions at the corners of neighboring epithelia to block the flow of fluids and maintain permeability barrier at the corners (Fristrom, 1982; Noirot-Timothee et al., 1982; Schulte et al., 2003). Gliotactin, a member of Neuroligin family, is one of the main TCJ components (Schulte et al., 2003). Gliotactin plays an important role to maintain permeability barrier and is critical for the formation of the TCJ and maturation of the SJ (Kikuchi et al., 2010; Schulte et al., 2003). Gliotactin loss of function disrupts the TCJ function and leads to the basolateral spread of the SJ proteins in polarized epithelia (Schulte et al., 2003). Recently, Bark-beetle (Bark) (also known as Anakonda) was identified as a new member of the TCJ (Byri et al., 2015; Hildebrandt et al., 2015). Bark is a transmembrane protein with a huge extracellular triple-repeat domain, which is proposed to form the tricellular plug (Byri et al., 2015). Bark is required for the formation of the TCJ but not the bicellular SJ and loss
of Bark results in barrier defects and disruption of the TCJ (Byri et al., 2015; Hildebrandt et al., 2015). Bark knockdown leads to the loss of Gliotactin from the TCJ, supporting a role for Bark in Gliotactin trafficking to the TCJ (Byri et al., 2015; Hildebrandt et al., 2015). However, how Bark and Gliotactin interact is still unknown. Bark and Gliotactin both have highly conserved PDZ binding motifs at the C-terminal end suggesting that recruitment maybe mediated by PDZ binding proteins. Gliotactin interacts extensively with the PDZ scaffolding protein Discs large (Dlg), but the role of Dlg at the TCJ in forming the permeability barrier is not known. The scaffolding protein Scribbled (Scrib) has major roles in cell polarity, cell migration and cell proliferation in epithelial cells (Dow et al., 2007; Humbert et al., 2003; Nagase et al., 1995). The Scrib polarity complex consists of Scrib, Dlg and Lethal giant larvae (l(2)gl) in Drosophila melanogaster. Scrib, Dlg and Lgl are highly conserved tumor suppressor genes and are critical to establish apicobasal polarity by restricting apical proteins from the basolateral surface in epithelial cells (Bilder, 2004; Bilder et al., 2000; Jacob et al., 1987; Woods and Bryant, 1991). In the columnar epithelia of the Drosophila imaginal disc, loss of Scrib leads to disruption of the epithelial organization, loss of polarity, overproliferation, and disc overgrowth. The leucine-rich repeat (LRR) domain of Scrib is essential to maintain the polarized epithelial monolayer and control proliferation, while the PDZ domains are thought to be required for septate junction (SJ) formation independent of Scrib’s role in apical polarity (Zeitler et al., 2004). Therefore, a possible new role for Scrib could be in controlling the TCJ formation and regulating permeability barrier formation.

Here, we show that there is a novel Scrib complex at the tricellular junction. Scrib is in close proximity with the TCJ proteins Gliotactin and Bark, along with Dlg. The Scrib PDZ domains 1-2 are essential for the recruitment of Bark and Gliotactin to the TCJ. Loss of Gliotactin and Bark
lead to spread of Scrib and Dlg basolaterally, and in Scrib and Dlg downregulation both Scrib and Dlg were lost from the SJ domain first before their loss from the TCJ. We propose that the Dlg GUK domain plays role in Bark membrane trafficking in a different mechanism from Gliotactin. In summary, our findings suggest that the formation of the Scrib TCJ complex is required for TCJ assembly and function.

3.3 Results

3.3.1 Scrib is colocalized with Gliotactin and Bark at the TCJ

We hypothesized that there is a scaffolding protein complex at the TCJ, which mediates TCJ formation and function and interacts with Gliotactin and Bark. Gliotactin and Bark both contain conserved PDZ binding motifs. While these motifs are not necessary for protein localization (Byri et al., 2015; Schulte et al., 2006), we suggest that Gliotactin and Bark PDZ binding motifs interact with PDZ scaffold protein Scrib, and this interaction facilitates the formation of the TCJ. To test this hypothesis first we checked the distribution of TCJ proteins and potential scaffolding proteins. We used a Bark (Bark: also known as Anakonda) tagged with GFP (Byri et al., 2015). Gliotactin and Bark, as expected, are restricted to the TCJ within the epithelia of the wing imaginal disc including the large ribbon-like TCJ of the peripodial epithelia and the smaller TCJ of the columnar epithelia (Figure 3.1A-D). Here we used a Proximity Ligation Assay (PLA) to show that Gliotactin and Bark are in close proximity at the TCJ in the peripodial (Figure 3.1E; arrow) and columnar epithelia (Figure 3.1F, G; arrow). To test the specificity of the PLA, we used apterous-Gal4 to drive Bark-RNAi in the dorsal side of the wing imaginal disc. Knock down of Bark led to the loss of the PLA between Gliotactin and Bark (Figure 3.1H). Thus, our result confirms that Gliotactin and Bark are in close proximity at the TCJ. Using a combination
of immunolabeling or Scrib endogenously tagged with GFP (Scrib::GFP), we observed that Scrib is localized throughout the bicellular junction and concentrated at the TCJ with Gliotactin and Bark in both the peripodial (Figure 3.1A-B; arrows) and columnar (Figure 3.1C-D; arrows) epithelia of the wing imaginal disc. These observations suggest that Scrib, Bark, and Gliotactin could form a complex at the TCJ.

3.3.2 Scrib is in close proximity with the TCJ proteins, Gliotactin and Bark

To identify how Scrib interacts with the two TCJ proteins Bark and Gliotactin, we used a proximity ligation assay (PLA) in the wing imaginal disc. We found that Scrib is in close proximity with Gliotactin (Figure 3.2A-D; arrows) and Bark (Figure 3.2E-H; arrows) in both peripodial (Figure 3.2A, E; arrows) and columnar (Figure 3.2B, F; arrows) epithelia. We also confirmed the specificity of this close proximity through RNAi-mediated knockdown. We used apterous-Gal4 to knock down Bark or Gli in the dorsal side of the wing disc, and the PLA between Scrib and Gliotactin (Figure 3.2D), and Scrib and Bark (Figure 3.2H, I) disappeared. These findings suggest that the presence of Bark and Gli are required for the formation of a complex with Scrib at the TCJ.

3.3.3 Scrib and Bark are in close proximity with Dlg

We next wanted to identify whether the PDZ scaffolding protein Dlg is also part of the TCJ complex. Gliotactin has previously been shown to form a complex with Dlg (Schulte et al., 2006) and Scrib and Dlg are well known to form a complex (Bilder and Perrimon, 2000). Here we used PLA to show that Scrib is in close proximity with Dlg throughout the SJ and TCJ domains (Figure 3.3A-C) in both peripodial (Figure 3.3A; arrow) and columnar (Figure 3.3B; arrow)
epithelia. We also found that Bark and Dlg are in close proximity at the TCJ (Figure 3.3D-F) of both peripodial (Figure 3.3D; arrow) and columnar (Figure 3.3E; arrow) epithelia. Overall, our PLA results suggest that there is a novel TCJ complex including scaffolding proteins Scrib and Dlg with PDZ domains and Gliotactin and Bark beetle with PDZ binding motifs.

### 3.3.4 Bark and Gliotactin knockdowns lead to the basolateral spread of SJ proteins

To test the interactions between Scrib and other components of the TCJ we carried out RNAi-mediated knockdown experiments. Embryonic Bark knockdown has previously been shown to result in loss of Gliotactin from the TCJ of embryos (Byri et al., 2015). Here we showed that Bark downregulation in the apterous side of the wing imaginal disc led to basolateral spread of Scrib, Dlg (Figure 3.4A-B, I), and other components of septate junction including Nrv2.1 (Figure 3.4C-D, I), and Cora (Figure 3.4I). Using apterous-Gal4 paired with Gli-RNAi to knock down Gliotactin, we found that Gliotactin downregulation caused a basolateral spread of Scrib, and Bark (Figure 3.4E-F), similar to Nrv2.1 and Cora (Figure 3.4G) however Bark remained localized to the TCJ (Figure 3.4E-G). We observed that Scrib was reduced more from the TCJ in Bark-RNAi (Figure 3.4H’) compared to Gli-RNAi (Figure 3.4H). These findings suggest that Bark is necessary for accumulation of Gliotactin and Scrib at the TCJ, and loss of both Bark and Gliotactin from the TCJ results in spread Scrib and Dlg along with other SJ components. Thus, the presence of both TCJ proteins Bark and Gliotactin are essential to hold the septate junction in the normal location just basal to the adherens junction.
### 3.3.5 The tricellular junction formation is independent the SJ

Next, to determine the role of SJ proteins on TCJ formation, we knocked down two core SJ components, NrxIV and the alpha subunit of the Na/K ATPase pump (ATPα), in the apterous side of the wing imaginal disc. ATPα RNAi-mediated knockdown led to the upregulation and basolateral spread of Scrib, Gliotactin (Figure 3.5A-C, M), and Bark (Figure 3.5D-F, M).

Surprisingly, we found that Scrib, Gliotactin, and Bark remained concentrated at the corner of the cells in the basal region, away from the septate junction (Figure 3.5C, F). Similar to ATPα, we knocked down NrxIV and the SJ scaffolding protein Varicose in the dorsal side of the wing imaginal disc. Reduction of both NrxIV and Vari (data not shown) proteins resulted in the basolateral spread of Scrib, Gliotactin (Figure 3.5G-I, M) and Bark (Figure 3.5J-L, M). Also we showed that Scrib, Gliotactin and Bark were localized to the TCJ in the basal part of the cells, away from the SJ (Figure 3.5I, L). Our findings suggest that even in the absence of septate junction components, TCJ proteins are concentrated at the corner of the cells. Thus, the formation of the TCJ is not dependent on the localization of septate junction proteins.

### 3.3.6 Loss of Scrib and Dlg from the SJ does not disrupt the TCJ

To test the interactions between Scrib, Dlg, Gliotactin and Bark at the TCJ, we used Scrib RNAi-mediated knockdown. We regulated the degree of RNAi expression through temperature shifts to 29°C for 24 or 48 hrs to knock down Scrib without disrupting epithelial polarity. Loss of Scrib immunolabeling from the entire SJ and TCJ region resulted in the loss of Gliotactin, Dlg (Figure 3.6A-C) and Bark (Figure 3.6D-F) from the TCJ. Dlg-RNAi also has previously been shown to downregulate Gliotactin at the TCJ (Padash-Barmchi et al., 2013). We then used RNAi to knockdown Dlg to a level that did not interfere with polarity but did remove Dlg.
immunolabeling from both the TCJ and SJ domains. Loss of Dlg led to downregulation of Scrib (Figure 3.6G-H) and Bark (Figure 3.6I-K) at the TCJ. In attenuating the RNAi we observed examples where Dlg and Scrib were lost from the SJ but still retained at the TCJ (Figure 3.6C; arrows). In these instances, when Scrib or Dlg were still concentrated at the TCJ (but not the SJ domain), Gliotactin, Bark and Dlg or Scrib were also retained (Figure 3.6C, F, K; arrows). These results suggest that the TCJ formation is independent of the Dlg/Scrib complex within the SJ domain. However these results also point to a key role for both Dlg and Scrib in mediating the formation of the TCJ.

3.3.7 The presence of Scrib and Dlg are required for the SJ localization

To identify the role of Scrib and Dlg in the control of septate junction proteins, we knocked down Scrib and Dlg using apterous-Gal4. As above knockdown of Scrib or Dlg using RNAi was carried to the point where Scrib or Dlg immunolabeling was absent from the SJ and TCJ, without triggering loss of polarity. We assayed the SJ protein Nrv2, which is the beta subunit of the Na/K ATPase, and a core component of the SJ. Loss of Scrib and Dlg caused the reduction and basolateral spread of Nrv2 (Figure 3.7A-C; D-F). The expression of the adherens junction protein E-cad was unaffected (Figure 3.7A’,’’, B’’,’’, D’’,’’, E’’). When knock down was slightly less efficient this again resulted in the retention of Scrib and Dlg at the TCJ and the loss of immunolabeling from the SJ domain. In these regions, Nrv2 is localized normally in the SJ domain around the columnar epithelia (Figure 3.7C, F; arrows). These results suggest that the presence of Scrib and Dlg at the TCJ is required for the correct localization of the septate junction complex basal to the adherens junction.
3.3.8  **Scrib PDZ1-2 domains are essential for Scrib interactions at the TCJ**

To determine which domains of Scrib might be important for Scrib control of the TCJ complex, we utilized previously characterized Scrib alleles that lack one or more of the PDZ domains. Scrib contains a series of Leucine Rich repeats in the N-terminal half of the protein and four PDZ domains in the C-terminal half (Figure 3.11A). In the *scrib*[^6] mutant, which retains the LRR domains but lacks all four PDZ domains the distribution of the TCJ protein complex including Dlg, Gliotactin (Fig 3.8A-C) and Bark (Fig 3.8A) was disrupted. However in the *scrib*[^12] mutant, which lacks the 3rd and 4th PDZ domains and retains the 1st and 2nd PDZ domains (Figure 3.11A), Dlg and Gliotactin (Figure 3.8D-F) were correctly localized at the TCJ. Of note, Bark (Figure 3.8D) was localized to both the bicellular and TCJs. Our findings suggest that the presence of the PDZ 1-2 domains is essential for Scrib interactions and the recruitment of the TCJ protein complex.

3.3.9  **The Dlg GUK domain is required for the TCJ protein localization and Bark trafficking**

Dlg is also a scaffolding protein with multiple interaction domains (Figure 3.11B) and to test how Dlg interacts with the TCJ complex, we made use of loss of function mutants in Dlg. Dlg is a member of the membrane-associated guanylate kinase (MAGUK) family and contains PDZ, Src homology 3 (SH3), and guanylate kinase (GUK) domains (Woods and Bryant, 1991). Loss or disruption of the PDZ domains leads to a complete disruption of epithelia polarity (Hough et al., 1997; Woods and Bryant, 1991) and thus it was not possible to assess junctions in wing discs of these mutants. Both the SH3 and GUK domains can interact with other protein complexes and the interaction between the SH3 and GUK domains regulates the accessibility of the GUK.
domain to binding with other proteins (Newman and Prehoda, 2009; Shin et al., 2000). We used a point mutation in the Dlg SH3 domain, $dlg1[m30]$, which interferes with the SH3-GUK interaction, opens up the GUK domain for more binding and blocks the ability of the SH3 to bind to other proteins. In this mutant the GUK is present and previous research has suggested that the GUK domain still binds partners (McGee and Bredt, 1999), however it could bind to new partners and also possibly not be regulated properly. In the $dlg1[m30]$ wing discs, Gliotactin and Bark were mislocalized throughout the bicellular junction. Gliotactin and Bark were present in the regions where Scrib was more concentrated at the junction, however it is hard to detect whether these regions are at the TCJ as the polarity is severely disrupted (Figure 3.9A-B; arrows). Next, we tested $dlg1[6]$, which lacks one-third of GUK domain including the C-terminus. The $dlg1[6]$ mutant is a strong allele and affects polarity (Woods and Bryant, 1991). We observed that in these discs Scrib was recruited to the bicellular junction (Figure 3.9C-E). We next tested the distribution of the TCJ proteins Bark and Gliotactin. Bark trafficking appeared to be disrupted with Bark retained in large intracellular vesicles throughout the epithelium (Figure 3.9C-E). We used E-cad to define the cell membrane and observed the Bark concentrated regions corresponded to intracellular vesicles (Figure 3.9C-E). In the $dlg1[6]$ mutant, Gliotactin was not colocalized with Bark and rather appeared to be concentrated in smaller intracellular vesicles at more apical levels. It has previously been shown that Gliotactin localization to the TCJ is controlled by endocytosis and lysosome-mediated degradation (Padash-Barmchi et al. 2010). In $dlg1[6]$ wing discs, Gliotactin colocalized with the early endosomal marker, Rab5, and late endosomal marker, Rab7 (Figure 3.9F). However neither Rab5 (data not shown) nor Rab7 (Figure 3.9F) was colocalized with the Bark intracellular vesicles. To confirm these observations Dlg-RNAi tested the effect of global Dlg knockdown and also led to retention
of Bark inside intracellular vesicles (Figure 3.9 G; arrow). Our results suggest that loss of Dlg and the GUK domain, in particular, affects the recruitment of Bark and Gliotactin to the membrane, and Bark membrane trafficking is through different mechanism than Gliotactin. Given the localization of Gliotactin in endocytotic vesicles, our results also suggest that the role of Dlg is to stabilize Gliotactin at the TCJ.

Our combined results indicate there is a novel Scrib complex at the TCJ (Figure 3.10). We found that Scrib and Dlg are in close proximity with Bark and Gliotactin at the TCJ, and the presence of Scrib PDZ 1-2 domains and Dlg GUK domain are essential for this interaction and recruitment of the TCJ protein complex.

3.4 Discussion
The scaffolding protein Scribbled (Scrib) is required for cell polarity, migration and proliferation of epithelial cells. The apicobasal Scrib complex controls many cell functions besides cell polarity including assembly and maintenance of cell junctions, and protein trafficking. Here we show that Scrib forms a complex at the tricellular junction (TCJ) of epithelial cells. Scrib is in close proximity with Gliotactin, Bark beetle (Bark) and Discs large (Dlg) at the TCJ, and loss of each component from the TCJ disrupts the integrity of the complex. The presence of the PDZ1-2 domains of Scrib is essential for this interaction. We also show that knockdown of both TCJ proteins Gliotactin and Bark result in a spread of Scrib, Dlg, and other SJ components, and knockdown of ATPα and NrxIV the two main SJ proteins leads to an upregulation and concentration of Gliotactin, Bark and Scrib at the TCJ. We found that reduced levels of Scrib and Dlg proteins lead to loss of Bark, Gliotactin, and Scrib or Dlg, however the last detectable region where Scrib and Dlg are detected before the entire loss from the cell membrane is at the TCJ.
Our data lead to a model where Scrib and Dlg form a complex to recruit Bark and Gliotactin to the TCJ.

### 3.4.1 Scribble as a junctional regulator

Scribble protein regulates the formation and assembly of junctions in wide range of animals. Mammalian Scribble1, a member of the LAP (leucine-rich repeat and PDZ domain) protein family, plays role in establishment of apicobasal polarity and is closely related to *Drosophila* Scribbled and *C.elegans* LET-413 (Bilder and Perrimon, 2000; Legouis et al., 2000). *Drosophila* Scrib is localized to the septate junction domain while LET-413 has basolateral distribution and is required for adherent junction assembly. Disruption of either protein leads to epithelial organization defects and mislocalization of junctional proteins (Bilder and Perrimon, 2000; Legouis et al., 2000). In vertebrate epithelia, Scrib is an important regulator of tight junction function (Ivanov et al., 2010). Disruption of the barrier in inflamed mucosa leads to Scrib downregulation and mislocalization in intestinal epithelial cells (Ivanov et al., 2010). siRNA-mediated knockdown of Scrib also disrupts the epithelial barrier and inhibits the TJ reassembly such that the tight junction proteins ZO-1 and occludin have discontinuous expression patterns at cell-cell contacts (Ivanov et al., 2010). The interaction of Scrib with ZO-1 is essential for Scrib recruitment to the TJ and TJ regulation (Ivanov et al., 2010). The trimeric Scrib complex (Scribbled, Lethal giant larvae, Dlg1) is dispensable for the TJ assembly in human intestinal epithelia, however the Scrib-ZO-1 complex plays a unique role to control the barrier integrity and the TJ organization (Ivanov et al., 2010). Thus, Scrib independent of Lgl and Dlg is important for the TJ assembly, however the role of Scrib at the tricellular tight junction and its interaction with TCJ proteins Tricellulin or LSR has not been tested. These findings suggest that
Scrib is involved in the regulation of tight junction in different protein complex from the polarity complex.

Similar to the tight junctions in vertebrates, Scrib is also localized to the *Drosophila* septate junction, however the functional interplay between scaffolding Scrib protein and the tricellular junction remains poorly understood. The similarity between mammalian Scrib1 and the *Drosophila* Scrib suggests a role for Scrib in the septate and TCJ formation. We identified a new Scrib complex at the tricellular junction of the imaginal disc epithelia that consists of Scrib, Dlg and the two TCJ proteins Gliotactin and Bark beetle. We found that loss of any component disrupts the integrity of the complex at the TCJ. The TCJ complex also regulates the organization of the bicellular septate junctions (SJ). Knockdown of Gliotactin and/or Bark beetle lead to the basolateral spread of Scrib, Dlg and other SJ proteins suggesting that the TCJ components hold the SJ. This mirrors prior work where the loss of Gliotactin and Dlg in embryonic epithelia leads to the basolateral spread and mislocalization of the SJ components within the membrane (Oshima and Fehon, 2011; Schulte et al., 2006)

On the other hand, when core septate junctions are knocked down the Scrib TCJ complex remained concentrated at the TCJ and spread within this domain basolaterally. Our results suggest that the TCJ formation or stabilization is independent of the presence of the SJ. Furthermore, knock down of Scrib or Dlg resulted in the loss of Bark, Gliotactin, and Dlg from the TCJ. Significantly the last domain that Scrib and Dlg were detected was in the TCJ, in other words, both Scrib and Dlg were lost from the SJ domain first. When the last detectable region for Scrib and Dlg was the TCJ, we observed both Gliotactin and Bark were also retained. Other SJ components are normally localized to the bicellular junction when the remaining Scrib and Dlg was detected at the TCJ. This finding suggests that the presence of both Scrib and Dlg are
required for TCJ formation. In summary, our results suggest that the TCJ formation is critical for proper SJ organization while the TCJ formation is independent of SJ formation.

3.4.2 Scrib PDZ1-2 domains are required for its localization to the junction

Scribble PDZ domains play a role in SJ organization and Scrib interactions with SJ proteins. *Drosophila* Scrib forms a complex with Dlg in a number of different contexts. For instance Scrib along with Dlg and Lgl are components of Scrib polarity complex in the formation of apicobasal polarity in epithelia. Dlg is required to stabilize the Scrib association with the membrane and Scrib is essential to recruit Lgl to the cortical domain (Bilder et al., 2000). In embryonic epidermis, mutations in Dlg or Lgl result in Scrib mislocalization (Bilder et al., 2000) and loss of Scrib leads to misdistribution of apical proteins to basolateral region, and the *scrib* embryos fail to polarize Dlg to the apical margin of the lateral membrane and show the same basolateral misdistribution (Bilder and Perrimon, 2000).

Scrib also forms a complex with Dlg at the neuromuscular junction (NMJ). Dlg is thought to bind through its GUK domain to a linker protein called GUK-holder which in turn binds to the Scrib PDZ2 domain (Albertson et al., 2004; Albertson and Doe, 2003; Mathew et al., 2002), however the role of GUK-holder as a linker protein in septate junction is still unknown. This finding suggests that Scrib complex components are mutually dependent for proper localization. It has been proposed that the Scrib LRR domain is required to control polarity and organize the basolateral epithelial membrane while the Scrib PDZ domains recruit Scrib to the septate junction (Albertson et al., 2004; Zeitler et al., 2004). Lack of the Scrib LRR domain results in loss of epithelial polarity and adherens junction. However in Scrib mutants lacking the PDZ1-2 domains, apical proteins and adherens junction markers are normally localized but the septate
junction organization is disrupted and the ladder-like structure of the SJ absent (Zeitler et al., 2004). Loss of the Scrib PDZ2 domain leads to a cytoplasmic distribution suggesting that PDZ2 is required for Scrib recruitment to the lateral membrane and septate junctions of epithelial cells (Albertson et al., 2004). Furthermore, in C. elegans the Let-413 PDZ domains are dispensable for the organization of apicobasal polarity (Legouis et al., 2000). These findings highlight that the Scrib PDZ domains are mainly essential for efficient localization to the septate junction and junction formation, and are not involved in the establishment of polarity.

Here we show that Scrib PDZ domains are essential for the function of Scrib at the septate and tricellular junction. We found that the Scrib PDZ1-2 domains play an important role for proper localization and interaction at the TCJ of epithelial cells. Loss of all Scrib PDZ domains disrupts Gliotactin, Bark and Dlg distribution while in Scrib alleles retaining the 1st and 2nd PDZ domains, Gliotactin and Dlg proteins are localized to the TCJ and Bark is localized to the bicellular and TCJ. Our results suggest that PDZ 1-2 domains are essential for Scrib interaction and the recruitment of the TCJ protein complex, however it is unknown if the interaction with Dlg and the two TCJ proteins is direct or indirect through a mediator protein like GUK-holder (Albertson et al., 2004; Albertson and Doe, 2003; Mathew et al., 2002). Both SH3 and GUK domains of Dlg function to interact with binding proteins. SH3-GUK intramolecular association regulates complex assembly and accessibility of GUK domain to the ligands. Mutations in SH3-GUK interaction disrupt the complex assembly (McGee and Bredt, 1999; Nix et al., 2000), and dysregulate the binding of proteins to the GUK domain like a constitutive association of GUK-holder to the GUK domain (Qian and Prehoda, 2006). However, SH3-GUK intramolecular interaction is not required for the localization of Scrib and Dlg (Newman and Prehoda, 2009). Similar to the previous studies, we also show that in dlg[m30] which interferes with SH3 and
GUK interaction, Dlg and Scrib are localized to the membrane. However, Gliotactin and Bark are mislocalized through the bicellular junction probably due to the dysregulation of complex assembly at the TCJ and disruption of GUK domain interaction with binding ligands. An alternative function for the Scrib TCJ complex could involve the trafficking and targeting of Bark to the TCJ. Scrib plays a role in retromer-dependent trafficking and regulates the endosomal trafficking of Crumbs, an apical polarity protein (de Vreede et al., 2014). Scrib also plays important role in NMDA receptors (NMDARs) recycling in the synapses (Piguel et al., 2014). NMDARs are required for synapse development and transmission (Traynelis et al., 2010). Scrib interacts with a PDZ binding domain of NMDARs and regulates the NMDAR internalization through interaction with Ap2 adaptor (Piguel et al., 2014).

The Scrib complex could be involved in Bark trafficking since our results show that in the dlg1[m30] mutant, Bark is present at the TCJ in those cases when Scrib also found at the TCJ. It is likely Scrib also requires Dlg to recruit Bark, as in the dlg1[6] mutant lacking the Dlg GUK domain Bark trafficking was disrupted and Bark was not trafficked to the membrane. It has been shown that Bark initiates the TCJ assembly by recruiting Gliotactin to the TCJ, however, Bark PDZ binding motif is dispensable for Bark and Gliotactin localization to the TCJ (Byri et al., 2015). On the other hand, the Gliotactin PDZ binding motif is important for Gliotactin distribution at the TCJ. Lack of the Gliotactin PDZ binding motif triggers extensive endocytosis and formation of large intracellular vesicles, and it is thought that the Gliotactin-Dlg interaction plays a role in stabilization of Gliotactin and endocytosis (Padash-Barmchi et al., 2013). These findings show that unlike Gliotactin, Bark stability at the membrane and the TCJ is not dependent on the PDZ binding motif. Bark expression level is regulated at the TCJ through
endocytosis. It has been shown that Bark is colocalized with endosomal and lysosomal markers (Hildebrandt et al., 2015), however Bark is not localized in the endocytic vesicles in dlg[6] which lacks the GUK domain. This finding suggests that Bark endocytosis is mediated through its interaction with Dlg GUK domain.

We propose that Bark recruitment to the membrane is dependent on both Scrib and Dlg, and that in turn recruits Gliotactin to the TCJ. It has been suggested that Bark localization to the TCJ is required for Gliotactin recruitment (Byri et al., 2015). Thus, Bark and Scrib form a complex with Gliotactin at the TCJ and Dlg is a key mediator for this interaction.

In summary, we suggest that there is a unique Scrib complex at the TCJ. Scrib and Dlg play important role in recruiting Bark to the junction, and both scaffolding proteins with PDZ domains are in close proximity with Gliotactin with PDZ binding motif at the TCJ, and the presence of Scrib PDZ1-2 are required for this interaction.

### 3.5 Material and methods

#### 3.5.1 Fly stocks

The following fly strains were used: Bark [L200], Bark::GFP, Df(2L)Excel6009, Bark::GFP (Byri et al., 2015), and Scrib[dt12] (Zeitler et al., 2004). UAS-Scrib-RNAi (v105412), UAS-Dlg-RNAi (v41134), UAS-Bark-RNAi (v52608) UAS-Nrx-IV-RNAi (v8353) and UAS-ATPα-RNAi (v12330) from the Vienna Drosophila Resource Center (VDRC). UAS-Gli-RNAi (NIG3903R-3) and dlg[6] (DGRC106785) from Kyoto Drosophila Stock Center. UAS-Vari-RNAi (Bl:35193), UAS-Gli-RNAi (Bl:31869), dlg[1/m30] (Voelker et al., 1985), scrib[dt6]
(Bl:29013, Zeitler et al., 2004), *scrib[673]* (Bl:41775, Zeitler et al., 2004), Scrib::GFP (Morin et al., 2001) and *apterous–GAL4* from Bloomington *Drosophila* Stock Center.

### 3.5.2 Statistical analyses

All statistical tests were calculated and graphed with GraphPad Prism 6. Distance spread was quantified using ImageJ (Schneider et al., 2012).

The distance spread of junctional proteins were measured in the side projection figures (Figure 3.5). The basolateral spread was calculated as a ratio (apterous side over control side), and significance was determined using a one-way ANOVA with Tukey’s post-hoc test.

### 3.5.3 Immunolabeling

Third instar larval imaginal discs were stained as described previously (Schulte et al., 2006).

Primary antibodies were: mouse anti-Gli 1F6.3 at 1:200 (Auld et al., 1995), rabbit anti-Gli at 1:300 (Venema et al., 2004), mouse anti-Dlg 4F3 at 1:200 (Developmental Studies Hybridoma Bank) (Parnas et al., 2001), rat anti-DE-Cadherin at 1:50 (Developmental Studies Hybridoma Bank)(Oda et al., 1994), guinea pig anti-Mcr at 1:800 (Hall et al., 2014), rabbit anti-Scrib at 1:2500 (Albertson and Doe, 2003), rabbit anti-Nrv2.1 at 1:1000 (Abcam), rabbit anti-GFP at 1:500 (Life Technologies), mouse anti-GFP at 1:300. DAPI was used at 1:1000 (Thermo Scientific). Secondary antibodies were used at 1:300 – goat anti-rabbit (conjugated to Alexa Fluor 647 or Alexa Fluor 568), goat anti-mouse (conjugated to Alexa Fluor 647, Alexa Fluor 488 or Alexa Fluor 568), goat anti-rat (conjugated to Alexa Fluor 647 or Alexa Fluor 568) (Molecular Probes).
Proximity Ligation Assay (PLA) protocol was carried to detect whether the distance between two desired proteins is less than 40 nm (Wang et al., 2015). In order to do PLA, imaginal wing discs were isolated, fixed and incubated overnight with the following antibody pairs: Rb anti-GFP+ mAb Gli; mAb GFP+ Rb anti-Gli; mAb GFP+ Rb anti-Scrib; Rb anti-GFP+ mAb Dlg. Next, the PLA anti Rb/mouse minus and plus probes were added. The probes bind to the primary antibodies and functions as primers. Then, through the ligation phase a circular DNA was made, and finally the products were fluorescently tagged and amplified by amplification reaction. For the control experiments a single primary Ab was added or RNAi-mediated knockdown was performed.

PLA protocol modifications included: all incubation steps were performed on an orbital shaker; samples were kept in the mounting solution (Vectashield) for 2-3 days at 4°C before slide preparation.

3.5.4 Imaging

Image stacks were collected with a DeltaVision Spectris microscope (Applied Precision, Issaquah, WA) with a 20× air- or 60× oil-immersion lens (NA 1.4) and CoolSnap HQ digital camera. Deconvolution of 0.2 µm z-sections with SoftWorx (Applied Precision) used a point-spread function measured from 0.2-µm beads conjugated with Alexa dyes (Molecular Probes) mounted in Vectashield (Vector Labs). Side projections were created using the SoftWorx program. Images were exported to Photoshop (Adobe Systems) for compilation.
Figures for chapter three:

Figure 3.1: Scrib is colocalized with Gliotactin and Bark at the TCJ
A-B) The expression pattern of the TCJ components in the peripodial epithelia of the wing imaginal disc, immunolabelled for Gliotactin (green, A’), Bark (blue, A’’) and Scrib (red, A’’’). Scrib was all around the cells and it was concentrated at the TCJ with Bark and Gli (arrows). (B) The zoom-in view of the same region in A. Gli (green, B’), Bark (blue, B’’) and Scrib (red, B’’) were colocalized at the TCJ (arrows).

C-D) The expression of Gli (green, B’), Bark (blue, B’’’) and Scrib (red, B’’) at the TCJ in the columnar epithelia of the wing imaginal disc. (D) The zoom-in view of the same region in C (1.5X magnification). Gli (green, D’), Bark (blue, D’’’) and Scrib (red, D’’’) were colocalized at the TCJ (arrows).

E) The proximity ligation assay (PLA) between Gliotactin and Bark in the peripodial epithelia with Bark::GFP + Gli PLA in red (E’’) and Bark::GFP in green (E’). Gliotactin and Bark::GFP PLA is in red (E’’) and Bark::GFP is in green (E’). (E’’) The zoom-in view of the same region in E (2X magnification). Gliotactin and Bark PLA was at the TCJ of the peripodial epithelia (arrow).

F) The PLA between Gliotactin and Bark in the columnar epithelia. Gliotactin and Bark::GFP PLA is in red (F’’), and Bark::GFP is in green (F’’). (F’’) The zoom-in view of the same region in F (1.5X magnification). Gliotactin and Bark PLA was at the TCJ of the columnar epithelia (arrow).

G) Side projections for each panel are shown below. The PLA between Gliotactin and Bark (red, G’’) was at the junctional level and was colocalized with Bark::GFP (green, G’). (H) apterous-GAL4-driven expression of Bark-RNAi within the wing imaginal disc. The dashed line marks the dorsal–ventral boundary with the dorsal apterous side of the wing imaginal disc to the left. In the side projection view the PLA between Gliotactin and Bark (red, H’’) was reduced in the dorsal side compared to the control side. Bark::GFP (green, H’) was reduced in the apterous side of the wing. All xy panels represent a single z slice. n=10 discs. Scale bars: 15 μm.
Figure 3.2: Scrib is in close proximity with the TCJ proteins, Gliotactin and Bark

(A) The proximity ligation assay (PLA) between Scrib and Gliotactin in the peripodial epithelia with Scrib::GFP + Gli PLA in red (A”) and Scrib::GFP in green (A’). (A”’) The zoom-in view of the same region in A (2X magnification). Scrib and Gliotactin PLA is concentrated at the TCJ (arrow). (B) PLA between Scrib and Gliotactin in the columnar epithelia with Scrib::GFP + Gli
PLA in red (B”), and Scrib::GFP in green (B’). (B’’) The zoom-in view of the same region in B (1.5X magnification). Scrib and Gliotactin PLA is concentrated at the TCJ (arrow). (C) Side projections for each panel are shown below. The PLA between Scrib and Gli (red, C”) colocalized with Scrib::GFP (green, C’) at the SJ. (D) apterous-GAL4-driven expression of Bark-RNAi within the wing imaginal disc. The dashed line marks the dorsal–ventral boundary with the dorsal apterous side to the left. The PLA between Scrib and Gli (red, D”) was reduced in the dorsal side compared to the control side. Bark::GFP (green, D’) was downregulated in the apterous side of the wing. (E) The PLA between Scrib and Bark in the peripodial epithelia with the Scrib + Bark::GFP PLA in red (E”) and Bark::GFP in green (E’). (E’’) The zoom-in view of the same region in E (2X magnification). The Scrib and Bark PLA was concentrated at the TCJ (arrow). (F) The PLA between Scrib and Bark in the columnar epithelia with the Scrib + Bark::GFP PLA in red (F”) and Bark::GFP in green (F’). (F’’) The zoom-in view of the same region in F (1.5X magnification). The Scrib and Bark PLA was concentrated at the TCJ (arrow). (G) Side projections for each panel are shown below. The PLA between Scrib and Bark (red, G”) colocalized with Bark::GFP (green, G’) at the level of the SJ. (H) apterous-GAL4-driven expression of Bark-RNAi within the wing imaginal disc. The dashed line marks the dorsal–ventral boundary with the dorsal apterous side to the left. The PLA between Scrib and Bark (red, H”) was reduced in the dorsal side compared to the control side. Bark::GFP (green, H’) was downregulated in the apterous side of the wing. (I) apterous-GAL4-driven expression of Gli-RNAi within the wing imaginal disc. The dashed line marks the dorsal–ventral boundary with the dorsal apterous side to the left. The PLA between Scrib and Bark (red, I”) was reduced in the apterous side of the wing disc, while Bark::GFP (green, I’) expression was normal. All xy panels represent a single z slice. n=8 discs. Scale bars: 15 μm.
Figure 3.3: Scrib and Bark are in close proximity with Dlg

(A) The PLA between Scrib and Dlg in the peripodial epithelia with Scrib::GFP + Dlg PLA in red (A”) and Scrib::GFP in green (A’). (A”’) The zoom-in view of the same region in A (2X magnification). The Scrib and Dlg PLA was distributed around the entire cell (arrow). (B) The PLA between Scrib and Dlg in the columnar epithelia with Scrib::GFP + Dlg PLA in red (B”), and Scrib::GFP in green (B’). (B”’) The zoom-in view of the same region in B (1.5X magnification). The Scrib and Dlg PLA was at the level of SJ domain (arrow). (C) Side projections for each panel are shown below. The PLA between Scrib and Dlg (red, C”) was colocalized with Scrib::GFP (green, C’) at the SJ domain. (D) The PLA between Bark and Dlg in the peripodial epithelia with the Bark::GFP + Dlg PLA in red (D”) and Bark::GFP in green (D’). (D”’) The zoom-in view of the same region in D (2X magnification). The Bark and Dlg PLA
was concentrated at the TCJ (arrow). (E) The PLA between Bark and Dlg in the columnar epithelia with Bark::GFP + Dlg PLA in red (E’’), and Bark::GFP in green (E’). (E’’) The zoom-in view of the same region in E (1.5X magnification). Bark and Dlg PLA was concentrated at the TCJ (arrow). (F) Side projections for each panel are shown below. The PLA between Bark and Dlg (red, F’’) was colocalized with Bark::GFP (green, F’) was at the SJ level. All xy panels represent a single z slice. n=8 discs. Scale bars: 15 μm.
Figure 3.4: Bark and Gliotactin knockdowns lead to the basolateral spread of SJ proteins

In all panels, *apterous*-GAL4 was used to drive expression in the wing disc. The dashed line indicates the dorsal–ventral boundary with the apterous side on the left. (A-D) Expression of Bark-RNAi led to the reduction of Gli (green, A’, C’) and basolateral spread of Scrib (red, A”, C”) Dlg (blue, A’’’) and Mcr (blue, C’’’). (B, D) Side projections for each panel are shown below. (E-G) Gli-RNAi mediated Gliotactin knockdown (green, E’) led to basolateral spread of Scrib (red, E”’) and Bark (blue, E’’’). (F) Side projections for each panel are shown below. (G) Side projection views indicating the basolateral spread of Bark (G’, green), Nrv2.1 (G”, red), and Cora (G’’’, blue). (H) Scrib spread along the z-axis and loss of signal intensity was more pronounced at the TCJ in Bark-RNAi (H) compared to Gli-RNAi (H’). Arrows show the border between apterous (left) and non-apterous (Begley and Brightman) sides.
of the wing discs. (I) Statistical analysis of basolateral spread of junctional proteins on the apterous side normalized to that of the control non-apterous side. The degree of Scrib, Dlg, Nrv and Cora basolateral spread was significant compared to *apterous*-Gal4 line alone (n=5 discs, ****P<0.0001, ***P<0.001, *P<0.05, mean±SD, one-way ANOVA). All xy panels represent a single z slice. Scale bars: 15 μm.
Figure 3.5: The tricellular junction formation is independent the SJ

In all panels, apterous-GAL4 was used to drive expression in the wing disc. The dashed line indicates the dorsal–ventral boundary with the apterous side on the left. (A-F) ATPα-RNAi mediated knockdown led to downregulation of Nrv (blue, A’’, D’’) and basolateral spread of Gli (green, A’, D’), Bark (red, D’’) and Scrib (red, A’’). (B, E) Side projections for each panel are shown below. (C, F) Gli (green, C’, F’), Bark (red, F’’) and Scrib (red, C’’) were concentrated to the TCJ at the level of the SJ domain (Z=9, Z=16) and still retained in the TCJ in more basal regions (Z=24 and Z=56), away from the septate junction. (G-L) NrxIV knockdown through NrxIV-RNAi led to downregulation of Nrv (blue, G’’’) and basolateral spread of Gli (green, G’), Scrib (red, G’’, J’’), and Bark (red, J’). (H, K) Side projections for each panel are shown below. (I, L) Gli (green, I’), Scrib (red, I’’, L’’), and Bark (L’’) were concentrated to the corner of the cells in the basal side (Z=50, Z=41), away from the SJ (Z=34, Z=26). (M) Statistical analysis of basolateral spread of junctional proteins on the apterous side normalized to that of the control non-apterous side. The degree of basolateral spread of Scrib, Gliotactin and Bark was significant compared to the control in ATPα- and NrxIV-RNAi. (n=6 discs, ****P<0.0001, mean±SD, one-way ANOVA). All xy panels represent a single z slice. Scale bars: 15 μm.
Figure 3.6: Loss of Scrib and Dlg from the SJ does not disrupt the TCJ

In all panels, *apterous*-GAL4 was used to drive expression in the wing disc. The dashed line indicates the dorsal–ventral boundary with the apterous side on the left. (A-C) Scrib RNAi-mediated Scrib knockdown (red, A’’) led to loss of Gli (green, A’) and Dlg (blue, A’’’) from the SJ. (B) Side projections for each panel are shown below. (C) The zoom-in view of the selected region in A (1.6X magnification). Scrib (red, C’’’), Gli (green, C’) and Dlg (blue, C’’’’) were retained to the corner of the cells (arrows). (D-F) Scrib knockdown (red, D’’) with Scrib-RNAi resulted in loss of Bark (blue, D’’’). (E) Side projections for each panel are shown below. (F) The zoom-in view of the selected region in D (1.6X magnification). Bark was remained to the TCJ (blue, F’’’’, arrows) along with Scrib (red, F’’) and Gliotactin (green, F’’). (G-K) Dlg RNAi-mediated Dlg knockdown (blue, G’’’’, I’’’’) led to the loss of Scrib (red, G’’’), Gli (green, G’, I’) and Bark (red, I’’’). (K) The zoom-in view of the selected region in I (1.4X magnification). Bark (red, K’’, arrows), Gli (green, K’), and Dlg (blue, K’’’’) were restricted to the corner of the cells. (H, J) Side projections for each panel are shown below. All xy panels represent a single z slice. n= 8 discs. Scale bars: 15 μm.
Figure 3.7: The presence of Scrib and Dlg are required for the SJ localization

In all panels, *apterous*-GAL4 was used to drive expression in the wing disc. The dashed line indicates the dorsal–ventral boundary with the apterous side on the left. (A-C) Scrib knockdown (green, A’) with Scrib-RNAi led to basolateral spread of Nrv (red, A”) while E-cad expression was unaffected (blue, A’’’). (B) Side projections for each panel are shown below. (C) The zoom-in view of the selected region in A (2.4X magnification). Nrv was localized normally to the junction (red, C”; arrows) when Scrib retained at the TCJ (green, C’). (D-F) Dlg-RNAi mediated knockdown resulted in downregulation of Scrib (green, D’) and Nrv (red, D”), while E-cad expression was normal (blue, D’’’). (E) Side projections for each panel are shown below. (F) The zoom-in view of the selected region in D (2.4X magnification). When Scrib was retained at the TCJ (green, F’), Nrv was localized normally to the junction (red, F”; arrows). All xy panels represent a single z slice. n=4 discs. Scale bars: 15 μm.
Figure 3.8: Scrib PDZ1-2 domains are essential for Scrib interactions at the TCJ

(A-C) A wing imaginal disc from a scrib[dt6]/scrib[673] mutant larvae. Loss of all four PDZ domains disrupted the localization of Gli (green, A’, B’), Bark (red, A”), Dlg (blue, A’’; red, B”) and E-cad (Blue, B’’’) from the TCJ in the columnar epithelia. (C) Side projections for each panel are shown below. (D-F) A wing imaginal disc from a scrib[dt12]/scrib[673] mutant larvae. The presence of the Scrib PDZ1-2 domains led to the normal localization of Gli (green, D’, E’), Bark (red, D”), Dlg (blue, D’’; red, E”) and Mcr (blue, E’’’) to the junction at the columnar epithelia. (F) Side projections for each panel are shown below. All panels represent a single z slice. n=6 discs. Scale bars: 15 μm.
Figure 3.9: The Dlg GUK domain is required for the TCJ protein localization and Bark trafficking

(A-B) A mutant *dlg[m30]* wing imaginal disc with the peripodial (A) and columnar (B) epithelia. The mutant Dlg is mislocalized (blue, A'''') but Bark (green, A’, B’) and Gli (blue, B''') were present when Scrib (red, A”, B”) was recruited and concentrated to the plasma membrane (arrows). (C-F) A mutant *dlg[6]* wing imaginal disc with the peripodial (C) and columnar (D-F) epithelia. (Eisenbach et al.) The mutant dlg mutant is mislocalized and (blue, D'''') led to the disruption of Bark trafficking. Bark was retained in large intracellular vesicles (green, D’; arrows) that were prevalent in the basal region of the columnar epithelia (green, E’; arrows) (Z=117) while Scrib (red, D”) was frequently recruited to the bicellular junction. Due to loss of polarity E-cad was localized through the junction at the apical (blue, D’’’’) and basal levels (blue, E’’’’). (F) Bark (green, F’, arrows) was not colocalized with Rab7 (red, F”’) and Gli (F’’’, blue), while Gliotactin was colocalized with Rab7 (arrows) (Z=59). (G) In Dlg-RNAi knock down, Bark was also concentrated to large intracellular vesicles (G’; arrows) that were not positive for Gliotactin (G”; arrows). All panels represent a single z slice. n=6 discs. Scale bars: 15 μm.
**Figure 3.10: The tricellular junction complex model**

Proposed model of the Scrib complex at the tricellular junction (TCJ). Both TCJ proteins Bark and Gliotactin are in close proximity (less than 40 nm) with Scrib and Dlg at the corner of the cells.
Figure 3.11: Scrib and Dlg domains and the location of mutations

(A) Scribbled (Scrib) protein consists of 16 Leucine Rich repeats (LLR) and four PDZ domains. Arrows indicate the location of premature stop codon in scrib[673], scrib[dt6] and scrib[dt12].

(B) The Discs large (Dlg) protein includes three PDZ domains, one Src homology 3 (SH3), and a guanylate kinase (GUK) domain. Arrows show the location of the dlg[m30] and dlg[6] mutations.
Chapter 4: Discussion

Permeability barriers restrict diffusion of fluid flow between cells and are created by specialized junctions between cells and at the corners of cells. In *Drosophila*, the formation of septate junction is critical to maintain the integrity of the permeability barrier and effectively blocks paracellular flow. A specialized junction, the tricellular junction (TCJ) forms at the convergence of three adjacent cells. At this point, the ribbon-like structures of the SJ make a hairpin turn and the TCJ barrier is created by a series of diaphragms that span the junction (Fristrom, 1982; Noirot-Timothee et al., 1982). Gliotactin and Bark beetle (Bark) are two TCJ components and the presence of both is required for the formation and maturation of the TCJ, as well as the function of the permeability barrier (Byri et al., 2015; Hildebrandt et al., 2015; Schulte et al., 2003). Gliotactin protein levels are tightly regulated by tyrosine phosphorylation and endocytosis. Gliotactin phosphorylation at two highly conserved tyrosine residues results in ubiquitination, endocytosis, and lysosome degradation to maintain Gliotactin protein localization to the TCJ (Padash-Barmchi et al., 2010). Blocking endocytosis or overexpression of Gliotactin leads to spread of Gliotactin from the TCJ, apoptosis, delamination, and migration of Gliotactin overexpressing cells (Padash-Barmchi et al., 2010). Thus, tight regulation of Gliotactin level is critical for the proper formation and function of TCJ.

4.1 Regulation of epithelial barriers through microRNAs

In chapter two of the thesis, we show that Gliotactin levels are controlled at the mRNA level by microRNA-mediated degradation. We found that miRNA-184 is targeted to the short conserved site at the 3’UTR of Gliotactin. miR-184 also regulates a subset of septate junction proteins including NrxIV, Mcr, and Cora. miRNAs function in regulation of various developmental and
physiological processes, and dysregulation of microRNAs triggers initiation and progression of many diseases (Ambros, 2004; Bartel, 2009). In vertebrates, microRNAs are required to regulate and maintain the integrity of a variety of epithelial cells. A wide range of miRNAs regulates barrier function in intestinal epithelial cells and changes in the expression level of miRNAs can lead to impairment of epithelial barrier function. miRs-184, 187, 192, 200a/200b, 204/211, and 221/222 are enriched in vertebrate epithelia and loss disrupts epithelia integrity or barrier function (Gregory et al., 2008; Kapsimali et al., 2007; McKenna et al., 2010; Park et al., 2008; Ryan et al., 2006; Wang et al., 2010). miRNAs also play crucial roles in the regulation of tight junction formation in intestinal epithelial cells (Yang et al., 2013). miR-122a is required to control intestinal permeability through targeting Occludin mRNA degradation and an inhibition of miR-122a results in an increase in the tight junction permeability (Ye et al., 2011). Similarly, elevated levels of miR-21 cause the depletion of occludin and impairment of tight junction (Takagi et al., 2010). Up-regulation of miR-212 through inhibition of ZO-1 translation leads to disruption of tight junction (Tang et al., 2008). miR-874 indirectly regulates the expression of junctional proteins Occludin and Claudin-1 through inhibition of aquaporin-3, an enhancer of intestinal permeability (Zhi et al., 2014). It also has been shown that miR-204 and -155 regulates the expression level of Claudin-10/-19 and Claudin-1 respectively (Qin et al., 2013; Wang et al., 2010). These findings suggest that miRNAs are critical to regulate tight junction formation and barrier function in intestinal epithelial cells.

In Drosophila, we show that miR-184 represses the expression level of its target Gliotactin at the tricellular junction through BMP signaling pathway. miR-184 also controls the expression of other septate junction components including NrxIV, Mcr, and Cora. Thus, miR-184 probably plays role to regulate a collection of junctional proteins and coordinate the SJ stability. However,
the loss of miR-184 shows no defect on epithelial cell organization and morphogenesis. One explanation could be due to the synergistic effects of a suit of miRNAs. miRNA networks are very complicated and work in synergistic and antagonistic manners to regulate the target genes (Grimson et al., 2007; Saetrom et al., 2007). miR-184 possibly functions along with multiple microRNAs to control the expression level of protein complexes at the junction. To test this hypothesis, the function of miR-184 in combination with other miRNAs like bantam could be suppressed simultaneously through miR-sponges and their effect on junctional proteins localization will be investigated. Secondly, it is possible that the basal level of miR-184 has no effect as the level of Gliotactin is also controlled through tyrosine phosphorylation. Indeed, the elevated level of miR-184 in response to a trigger plays an important role to regulate Gliotactin along with other proteins at the septate junction domain. To test this idea, Gliotactin phosphorylation sites need to be mutated and the effect of loss of miR-184 on Gliotactin localization in the absence of tyrosine phosphorylation investigated. As discussed above, in vertebrates it has been shown that tight junction proteins Occludin, Claudin family proteins, and ZO-1 are regulated at mRNA level through microRNAs. However the role of miR-184 in the regulation of tight junction and tricellular contacts of vertebrate epithelial cells has not been investigated. It is essential to understand whether similar to SJ, miR-184 functions to stabilize the TJ domain and control junctional proteins like Tricellulin and LSR. In vitro studies suggest that miR-203 potentially inhibits Tricellulin expression and leads to blood-cerebrospinal fluid barrier (BCB) leakage (Su et al., 2015) however the role of microRNAs in the TCJ formation and function to block the permeability at the corner of cells has not been clearly answered. In summary, it has become clear that a common feature in all animals is that a suit of microRNAs is
required to control junctional proteins, maintain the integrity of the junctions and generate functional epithelial permeability barriers.

### 4.2 Gliotactin interaction with BMP pathway components

In chapter two of the thesis, we show that overexpressed Gliotactin auto-regulates itself through BMP-dependent induction of miR-184. However, how excessive Gliotactin activates the BMP receptor is not well addressed. Bone morphogenic proteins (BMPs), a member of the transforming growth factor-β (TGF-β) family, have been found in both vertebrates and invertebrate. In epithelial cells, the BMP pathway regulates cell proliferation, differentiation and apoptosis (He et al., 2004; Miyazono et al., 2010; Walsh et al., 2010). BMP-2 and 4 ligands bind to type-I/II receptors and transduce a signal from the plasma membrane to the nucleus through the phosphorylation of R-SMAD and the formation of R-SMAD/SMAD complex (Heldin et al., 1997). Inhibitory SMADs (I-SMADs) also interact with R-SMAD and regulate its function (Hayashi et al., 1997).

In *Drosophila*, a range of ligands including Glass bottom boat-60A, Screw, (Newfeld and Takaesu, 1999), and Decapentaplegic (Dpp) (Padgett et al., 1987) mediate mothers against decapentaplegic (MAD) phosphorylation through activation of Thick vein (Tkv) type-I receptor (Newfeld and Takaesu, 1999; Sekelsky et al., 1995). The only identified I-SMAD, daughters against decapentaplegic (Dad; an inhibitory SMAD) interferes with Tkv activation and inhibits BMP signaling pathway (Inoue et al., 1998; Tsuneizumi et al., 1997). Dad physically interacts with Tkv receptor to repress the phosphorylation of MAD and inhibit the formation of Mad-Medea complex (Kamiya et al., 2008). In chapter two of the thesis, we propose that overexpression of Gliotactin triggers Tkv activation through disruption of Dad to induce miR-
Gliotactin interferes with the Tkv-Dad association and blocks the inhibitory effect of Dad on Tkv activation. However, whether this interaction is direct or through a mediator remains to be determined. To answer this question, the physical interaction between Gliotactin-Tkv, Gliotactin-Dad, and Tkv-Dad in normal and Gliotactin overexpressed cells needs to be investigated through PLA and Immunoprecipitation. If excessive Gliotactin disrupts the Dad/Tkv interaction we expect to see less association of Dad/Tkv (less PLA or protein complex isolation) and more association of Gli/Dad in overexpressed Gliotactin cells compared to the wildtype. In addition, a potential indirect effect of extracellular BMP antagonists could be tested. To check this idea, the involvement of SARA (Smad anchor for receptor activation) in BMP signaling activation through immunostaining and PLA could be investigated. Next, the level of SARA expression will be changed and its effect on Gliotactin-BMP-miR-184 axis induction will be tested.

An open question from chapter 2 is: What is the physiological role of the interaction between Gliotactin and Tkv/Dad? Previous work has been shown that loss of core SJ proteins like NrxIV and ATPα results in the spread of Gliotactin away from the TCJ (Biln, 2016). Loss of core SJ proteins also leads to apoptosis and delamination of cells from the epithelium. Whether spread of Gliotactin in this context triggers the induction of miR-184 via Tkv signaling and leads to great loss of SJ proteins targeted by miR-184 such as NrxIV and Mcr, needs further investigation. Increased miR-184 would lead to faster death and delamination. How might Tkv and miR-184 be necessary to control the SJ and TCJ? To address these questions, in NrxIV knockdown cells, the activity of Tkv receptor and phosphorylated Mad and the level of miR-184 through qRT-PCR could be measured. Induction of Tkv-TIPF activity and increased level of miR-184 would suggest the involvement of Tkv/miR-184 pathway. Next, the function of miR-184 and
Tkv could be repressed by the miR-sponge (or in the miR-184 null mutant) and Tkv dominant-negative in cells lacking NrxIV, and their effect on Gliotactin spread and other SJ proteins localization tested. To investigate the role of miR-184 in cell delamination after apoptosis in NrxIV mutant cells, cell death in combination with miR-184 function needs to be inhibited by Bsk-dominant negative and miR-184 sponge. Lack of cell delamination and presence of the SJ proteins would confirm a role for miR-184 in cell delamination after apoptosis.

It has been shown that Gliotactin is localized to the TCJ of the intestinal differentiated cells (Resnik-Docampo et al., 2017). Loss of Gliotactin promotes stem cell proliferation, represses differentiation of intestinal progenitor cells, and as a consequence leads to activation of inflammatory pathway and disruption of intestinal permeability barrier. Inhibition of the JNK pathway in Gli-depleted cells blocks the increased proliferation but could not rescue the permeability barrier defects (Resnik-Docampo et al., 2017). Therefore, the regulatory mechanism of permeability barriers in differentiated cells still remains unknown. It is likely that Gliotactin-BMP-miR184 axis plays role in regulating the permeability barrier in the TCJ of intestinal stem cells. To test this idea, the level of miR-184 and Tkv receptor activation in normal and Gli-depleted cells could be measured. Next, BMP signaling pathway and miR-184 could be activated/suppressed separately in normal and Gli-depleted cells, and the effect on permeability barrier at the TCJ of stem cells investigated. Improvements in barrier function would support the involvement of BMP pathway along with miR-184 in the TCJ regulation and intestinal homeostasis.
4.3 Regulation of junction formation and assembly through Scrib

In chapter three of the thesis, we aimed to understand how the TCJ proteins Bark and Gliotactin interact with the PDZ binding proteins Scrib and Dlg at the TCJ. We conclude that both Scrib and Dlg are required to recruit Bark to the TCJ, which leads in turn to Gliotactin/Dlg recruitment. The formation and the integrity of all cell-cell junctions are tightly regulated through specific proteins interactions, and scaffolding proteins such as Scribbled are key to junction formation (Madara, 1998). In vertebrates, Scrib maintains and assembles the adherens junction (AJ) and tight junction (TJ) in epithelial cells. Scrib regulates cell adhesion and cell migration through stabilizing the interaction between E-cadherin and β-catenin (Qin et al., 2013). Scrib interacts directly with ZO-1 (Ivanov et al., 2010) and ZO-2 (Metais et al., 2005) to control the integrity and function of the TJ in epithelial cells. Loss of Scrib from the TJ disrupts the epithelial barrier function and reassembly of the junction (Ivanov et al., 2010). Scrib is a regulator of blood-testis-barrier in Sertoli and germ cells and is colocalized with Occludin and ZO-1. Loss of the Scrib complex interferes with TJ integrity through reorganization of actin filament network, and upregulation of Occludin and β-catenin at the barrier (Su et al., 2012a). Scrib is also essential in the paranodal junction of the myelinated vertebrate axon in the CNS and PNS, the paranodal junction being highly conserved with the Drosophila septate junction. During myelination, Scrib is localized to the paranodal region (Jarjour et al., 2015) and mirrors the localization of the oligodendrocyte (CNS glial) protein Neurofascin (the Drosophila Neuroglian homologue). Neurofascin and Scrib are localized to points of axo-glial contact, opposite to the axonal Caspr protein (the Drosophila NrxIV homologue) which binds along with Contactin to Neurofascin (Eisenbach et al., 2009). Scrib regulates the differentiation of the oligodendrocytes, the initiation of peripheral and central myelination, and myelin thickness.
(Bolis et al., 2009; Cotter et al., 2010; Jarjour et al., 2015) and loss of Scrib results in the disruption of paranodal axo-glial junction (Jarjour et al., 2015). Thus, oligodendroglial expression of Scrib is critical for normal paranodal axo-glial adhesion. However, the proteins that Scrib is binding to and scaffolding at the paranode is not known.

Similar to the tight and paranodal junctions, we show that Drosophila Scrib plays an important role in the formation and regulation of the Drosophila tricellular junction. Like the Scrib-ZO1 complex, Scrib forms a TCJ complex with Glitactin, Bark, and Dlg in epithelial cells of the wing imaginal disc, and the presence of the Scrib PDZ 1-2 domains are essential for this interaction. The Scrib PDZ domains play critical roles through interactions with variety of protein complexes and are required to assemble protein complexes. For instance, Scrib PDZ domains interact with Vangle2 (Vang-like protein 2) (Kallay et al., 2006; Montcouquiol et al., 2003) and NOS1AP (Nitric oxide synthase 1 adaptor protein) (Anastas et al., 2012; Richier et al., 2010) to control planar cell polarity and cell migration respectively. Scrib PDZ domains bind β-PIX (β-p21-activated kinase-interacting exchange factor) and form a complex that is essential to regulate the localization of the small GTPase Cdc42 and promote cell polarization and organization in both epithelial and neuronal tissues (Audebert et al., 2004; Osmani et al., 2006). The Scrib PDZ domains also control SJ organization and recruit Scrib to the septate junction through interaction with other junctional proteins (Albertson et al., 2004; Zeitler et al., 2004). Unlike the LRR domain, loss of Scrib PDZ 1-2 disrupts septate junction formation and function, however, adherens junction organization is normal (Zeitler et al., 2004). Scrib protein lacking the PDZ2 domain fails to localize to the septate junction suggesting that PDZ2 is required for Scrib recruitment to the SJ (Albertson et al., 2004). PDZ2 is also necessary for Scrib to interact with the Dlg GUK domain through a mediator protein GUK-holder at the NMJ (Albertson et al., 2004).
It has been shown that Guk-holder as a Scrib-Dlg binding partner is required for Dlg hook domain interaction with Dishevelled to control spindle orientation in neuroblast (Garcia et al., 2014). It is likely that the Scrib PDZ2 domain interacts with Dlg GUK domain via a linker protein Guk-holder at the SJ, however, the role of Guk-holder at the septate junction has not been investigated. To test this idea, the effect of Guk-holder mutants or RNAi mediated-knockdown in the wing imaginal disc on the distribution and interactions of Scrib/Dlg, and Bark or Gliotactin recruitment to the TCJ could be investigated.

Our findings show that lack of all four Scrib PDZ domains interferes with Bark, Gliotactin, and Dlg localization at the TCJ and SJ, however in the presence of the Scrib PDZ 1-2 domains, Gliotactin and Dlg are localized to the TCJ and Bark is distributed through bicellular and tricellular junctions. We propose that Scrib PDZ1-2 domains play important role to recruit Bark to the junction and then Bark localization at the TCJ triggers Gliotactin recruitment. From the model of Bark function (Byri et al., 2015), it is likely that the presence of three Bark proteins at the TCJ is sufficient to allow the formation of the TCJ plug and thus recruits Gliotactin. However, it is likely that Scrib PDZ3-4 are also required for tight localization of Bark at the TCJ. The Scrib PDZ 3 and 4 domains have a front to back interdomain interaction and form a tight supermodule. This interaction functions to provide binding sites for target proteins and mutations affecting the PDZ3-4 interaction disrupt target protein binding (Ren et al., 2015). In vitro studies show that ZO-2 directly binds to the Scrib PDZ3-4 domains (Metais et al., 2005; Su et al., 2012b). In Drosophila, it also has become clear that the Scrib PDZ3 domain functions to establish planar cell polarity through its physical interaction with Strabismus/Van Gogh (Courbard et al., 2009). We also suggest that Bark localization at the TCJ is tightly regulated.
through a Scrib PDZ3-4 interaction with other mediators given the lack of concentrate of Bark at the TCJ in the absence of the Scrib PDZ3-4 domains. To test this hypothesis, different Scrib constructs with specific deletions of the PDZ3, PDZ4, and PDZ3-4 domains could be made. Next, the effect of each construct on Bark localization at the TCJ tested. Finally, through the rescue experiments, we could conclude whether Scrib PDZ3-4 domains are required for tight localization of Bark at the TCJ. To test whether both Scrib PDZ1 and 2 domains are required, Scrib constructs with deletions in PDZ1, PDZ2 could be made and the localization of Bark, Gliotactin and Dlg will be compared to the control constructs which lack or contain all PDZ domains. Thus, we could conclude which PDZ domains are essential for proper localization of Scrib complex components at the TCJ.

It is important to determine whether Gliotactin recruitment to the TCJ is dependent directly on Bark or through an intermediate protein. To test this idea through the rescue experiment, in Scrib null background, the Gliotactin recruitment to the TCJ needs to be tested in cells that are mutated for Bark and retains Scrib PDZ1-2 domains. If Gliotactin stays in cytoplasmic vesicles and fails to localize to the TCJ suggests that Bark in combination with Scrib are required for Gliotactin recruitment to the TCJ. However, if Gliotactin is localized to the TCJ in Bark-depleted cells suggests that Bark is not directly involved and Scrib PDZ1-2 domains are critical to recruit Gliotactin to the TCJ.

We propose that Scrib and Dlg as scaffolding proteins with PDZ domains are in close proximity with Gliotactin and Bark due to the PDZ binding motifs in both proteins. Scrib along with Dlg are essential for TCJ assembly and recruitment of Bark and Gliotactin to the tricellular junction. It has been suggested that Bark localization at the TCJ is essential to recruit Gliotactin to the TCJ (Byri et al., 2015). Our proposed model suggests that the formation of Bark/Scrib
complex at the TCJ, recruits Gliotactin to the TCJ and Dlg is a key mediator to form this TCJ complex. To test this hypothesis, the direct interaction between these proteins through immunoprecipitation needs to be tested in wildtype and cells lacking Scrib PDZ1-2, Dlg GUK domain, Bark, or Gliotactin. It is expected that the interaction between the TCJ complex components and the isolation of members of this protein complex will be disrupted in these cells compared to the control. Next, to test whether Gliotactin PDZ binding motif is required for its interaction with Scrib and Dlg with PDZ domains, the Gliotactin PDZ binding motif needs to be deleted. Then, the Gliotactin interaction with Scrib and Dlg at the TCJ will be tested through PLA and IP. If Gliotactin PDZ binding motif is necessary for the formation of the TCJ complex, loss of interaction between Gli/Scrib and Gli/Dlg is expected.

4.4 Regulation of Scrib complex through microRNA control

In chapter three of the thesis, we show that there is a Scrib complex at the TCJ. The formation of this complex is important to assemble and maintain the integrity of the TCJ. Since the Scrib complex regulates the TCJ function, the expression level of each component should be tightly regulated. Whether similar to Gliotactin, TCJ complex components Scrib, Dlg, and Bark are regulated through microRNAs needs to be investigated. Previous studies show that miRNAs play fundamental role to regulate the Scrib polarity complex. miR-296 is one of the main repressors of tumorigenesis and is inhibited in variety of cancers including breast, prostate, and colorectal cancers (Vaira et al., 2012; Wei et al., 2010). miR-296 regulates cell motility in epithelial cells via direct inhibition of Scrib expression. Loss of miR-296 leads to Scrib upregulation and mislocalization at the junctions in human cancer cells and results in excessive tumor cell migration and invasion (Savi et al., 2014; Vaira et al., 2012). Re-expression of miR-296 in tumor
cells rescues the random cell migration and reduces the progression of tumor growth (Savi et al., 2014; Vaira et al., 2012). Thus, miR-296 plays important role to maintain cell polarity through regulation of Scrib in epithelial cells. In vitro microRNA screening studies show that Dlg, a member of Scrib complex, is potentially be repressed by miR-19, miR-21, miR-23, and miR-29, also miR-338-3p and miR-451 functions to regulate epithelial cell polarity and epithelial cell differentiation. However, the target proteins and molecular mechanism of this regulation still remain unknown (Tsuchiya et al., 2009). In Drosophila, the miR-310-313 cluster regulates Dlg accumulation at the neuromuscular junction (NMJ). Loss of miR-310-313 leads to the induction of kinesin family protein Khc-73, changes in Dlg distribution at presynaptic region, and enhancement of neurotransmitter release. Therefore, miR310-313 negatively controls Dlg and synaptic transmission at the NMJ (Tsurudome et al., 2010). However, the role of microRNAs in the regulation of Scrib complex has been poorly investigated in Drosophila. Using the TargetScanFly algorithm (Ruby et al., 2007), a series of conserved miRNA target sequences were identified for junctional proteins including Scrib, Dlg and Gliotactin. To test the involvement of these miRNAs in the regulation of Scrib TCJ complex, the function of each miRNA needs to be repressed through the use of miR-sponge screening. Then, the effect on the formation of TCJ complex tested. Next, the function of selected miRNAs will be investigated further by changing the expression level of potential targets and assessing the effect on TCJ formation. Furthermore, TargetScanFly predictions indicate that Scrib, Dlg, and Guk-Holder are potential targets of miR-277. It is worth testing whether miR-277 plays a role to control Scrib/Guk-holder/Dlg interaction at the septate junction. To test this idea, the function of miR-277 needs to be repressed through using the miR-277 sponge, and its effect on localization and interaction between Scrib, Dlg, and Guk-holder will be investigated. Then, the level of miR-277
needs to be measured by qRT-PCR when Scrib, Dlg, and Guk-holder are downregulated or overexpressed. Changes in the level of miR-277 suggest the involvement of this microRNA in Scrib and Dlg regulation. It is expected that overexpression of these proteins results in the induction of miR-277 and as a consequence leads to the downregulation of Scrib and Dlg. It is likely that miR-277 through regulation of Scrib and Dlg functions as a regulator of TCJ complex. Overall, the formation of Scrib TCJ complex is critical to control the function of septate and tricellular junctions, therefore, the level of Scrib complex components needs to be tightly regulated. We suggest that miRNAs are potential mediators for this regulation.

4.5 Potential roles for Scrib at the TCJ

TCJ proteins besides their functions as regulators of the barrier have non-permeability barrier roles at the TCJ. In vertebrate, Tricellulin controls actomyosin organization and regulates the epithelial junction architecture. Tricellulin recruits F-actin and MyosinII to the junction and activates Cdc-42 through direct interaction with Cdc-42 GEF Tuba. Loss of Tricellulin disrupts the organization of F-actin at the TCJ and impairs the polygonal shape of the cells (Oda et al., 2014). Tricellulin is also regulated by epithelial-specific cell adhesion molecule (EpCAM) (Salomon et al., 2017). EpCAM is a regulator of epithelial cell configuration. Loss of EpCAM leads to displacement of actomyosin and Tricellulin from the lateral membrane, generation of greater cell contractility (Guillemot et al., 2001; Salomon et al., 2017), and disruption of permeability barrier (Kozan et al., 2015; Lei et al., 2012). Thus, EpCAM modulates cortical tension homeostasis, actomyosin patterning, and barrier integrity at the TCJ of the epithelial cells (Salomon et al., 2017).
It has been shown that Scrib regulates actin remodeling, epithelial morphogenesis, and astrocyte orientation through direct interaction with β-PIX (guanine nucleotide exchange factor for the small GTPases Rac and Cdc-42) and PAK (p21-activated kinase) (Audebert et al., 2004; Eastburn et al., 2012; Nola et al., 2008; Osmani et al., 2006). Scrib/β-PIX complex regulates Cdc-42 localization and activation in polarized astrocytes (Osmani et al., 2006), and β-PIX works as a mediator between the Scrib PDZ domains and Cdc-42. In mouse, loss of Scrib disorganizes microtubule structures and the actin network and impairs Cdc-42 and Dlg recruitment to the leading edge of the astrocytes (Osmani et al., 2006). These findings suggest that Scrib acts to stabilize cell polarization and orientation through regulation of Cdc-42. Scrib also interacts with cytoskeletal proteins in bronchial epithelial cells. β-spectrin directly binds to the Scrib C-terminus to recruit and stabilize Scrib at the membrane. Mutations interfering with Scrib interaction with β-spectrin leads to disruptions of epithelial cell polarization and orientation (Boeda and Etienne-Manneville, 2015). Scrib plays an important role in maintaining lung epithelial cell organization where loss of Scrib impairs cytoskeletal organization. In cells lacking Scrib, F-actin has multiple patches pattern, myosinII and Vangle2 (a regulator of cytoskeleton dynamics) are mislocalized, and epithelial cell association and alignments are disrupted (Yates et al., 2013). It has become clear that Gliotactin is also involved in non-permeability roles at the TCJ. Gliotactin is colocalized with Mud, a Dynein-associated protein, at the TCJ during the cell division. Loss of Gliotactin reduces the level of Mud from the TCJ. It is suggested that Gliotactin along with Mud controls microtubule pulling force and spindle orientation in mitosis (Bosveld et al., 2016).

Similar to Tricellulin and Giotactin, Scrib may function at the TCJ to reorganize actin filaments and regulate the TCJ positioning at the tricellular contacts of the epithelial cells. Scrib
regulates epithelial cell organization and alignment through interaction with actomyosin regulators. Whether Scrib at the TCJ modulates F-actin patterning, epithelial cell tension, and polygonal shape of the cells is still unknown. To test this idea, the interaction between Scrib and cytoskeletal regulators, and their effects on the TCJ positioning needs to be tested. The level of Scrib at the TCJ will be changed, and the localization of F-actin filament, activation of cytoskeletal regulators like Cdc-42, orientation of microtubule proteins, and distribution of myosin and zipper will be checked. If changes in Scrib level disrupts the organization of actomyosin network, thus, it is likely that Scrib at the TCJ plays role to regulate epithelial cell configuration. For further investigation, Scrib constructs with deletions in different PDZ domains used to find out which Scrib domains are involved in this regulation. Finally, It is also important to understand whether Scrib plays role to regulate Gliotactin-Mud interaction at the TCJ of dividing cells. To test this idea, the level of Scrib will be changed and its effect on Mud localization at the TCJ and spindle orientation in mitotic cells investigated. Overall, we propose that Scrib besides its polarity role may function at the TCJ to regulate actin organization, microtubule structure, and epithelial cell architecture.

In summary, the aim of this thesis was to understand how tricellular junction is regulated. We show that the TCJ is regulated at two different levels through signaling and scaffolding. First, Gli expression at the corner of cells is tightly regulated at the mRNA level through BMP-dependent induction of miR-184. Secondly, scaffolding proteins Scrib and Dlg play important role to regulate the TCJ assembly and recruitment of the TCJ proteins Bark and Gli to the membrane. While many questions remain unanswered we have provided a foundation from which we can investigate the TCJ regulation further.
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