KINASE CONTROL OF THE TRICELLULAR JUNCTION PROTEIN GLIOTACTIN,
AND GLIOTACTIN-INDUCED PHENOTYPES IN EPITHELIA

by

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Abstract

This thesis investigates the kinase-mediated regulation of the tricellular junction protein, Gliotactin and signaling pathways involved in Gliotactin overexpression-induced detrimental phenotypes. Tricellular junctions (TCJ) are uniquely placed permeability barriers formed in polarized epithelia where tight junctions in vertebrates or septate junctions in invertebrates from three cells converge. Misregulation of TCJ specific proteins is detrimental to life. However, mechanisms of their localization, maintenance, and potential signaling are largely unknown. Gliotactin is a transmembrane protein unique to TCJ in Drosophila and is essential for the maturation and maintenance of both bicellular and tricellular septate junctions. However, overexpression of Gliotactin leads to the spread of Gliotactin away from the TCJ and disrupts epithelial architecture by signaling for overproliferation, delamination, migration and apoptosis. One mechanism to control Gliotactin is phosphorylation of two highly conserved tyrosine residues and subsequent endocytosis. However, Gliotactin tyrosine phosphorylation also elicits detrimental phenotypes when dysregulated. The kinases involved in Gliotactin phosphorylation had not been broadly investigated prior to this work. We carried out an RNAi screen for phospho-regulators (kinases and some kinase-associated proteins) to determine which could modify the detrimental phenotypes triggered by Gliotactin overexpression. Four suppressors, four partial suppressors, and 53 enhancers were identified by screening 275 RNAi lines covering 164 genes. We determined that Gliotactin overexpression phenotypes involved TNF-JNK, PI3K-Akt signaling pathways and Btk29A. C-terminal Src kinase (Csk), Ret, PI4KIII-α, Skittles and Pkaap were also identified as candidates for further studies. We focused our analysis on Csk and determined Csk is a regulator of Gliotactin endocytosis and plays a role in the regulation of Gliotactin at the TCJ. Although Csk is known as a negative regulator of Src kinases, we identified that the effect of Csk on Gliotactin
is independent of Src, and likely occur through an AJ-associated complex. Taken together, this thesis provides novel insights on the function of Csk and identifies other candidate kinases that have the potential to regulate localization and/or signaling events associated with TCJ formation and function.
Lay Summary

Epithelia are a specialized group of cells that cover or line organs to prevent the entrance of germs and stop the loss of fluid. These cells are tightly packed and joined together with proteins that form junctions, like making a wall with bricks and cement. These junctions or the cement which occur in all animals, hold cells together and seal the spaces. Interestingly, when three cells meet, there is a unique group of proteins/cement to seal the corners of these cells. The spread of corner proteins to other locations is harmful and can even cause cancer. This study was done on one such specific protein to understand how this corner protein is limited only to these contacts and how it leads to cancer-like conditions when it spreads to other places. Using genetic approaches, we identified key proteins that regulate the localization and the signaling associated with the corner protein.
Preface

Chapter 2: “The tricellular junction protein Gliotactin overexpression induces Grindelwald to activate the TNF pathway.”

Work in this chapter has produced a manuscript in preparation as: G. D. N. Gayathri Samarasekera, M. P. Barmchi and Vanessa Jane Auld. The tricellular junction protein Gliotactin overexpression induces Grindelwald to activate the TNF pathway.

For this publication, I conducted all the experiments and analysis except the PTEN-RNAi co-expression in Gli-WT which was done by M.P. Barmchi. Both myself and Vanessa Auld contributed to all the other aspects of the manuscript including study conception and design, data interpretation, writing and editing of the manuscript.

Chapter 3: “C-terminal Src kinase (Csk) regulates the tricellular junction protein Gliotactin independent of Src.”

Work in this chapter has produced a manuscript which was submitted to the Molecular Biology of the Cell on April 20, 2017, as G. D. N. Gayathri Samarasekera and Vanessa Jane Auld. C-terminal Src kinase (Csk) regulates the tricellular junction protein Gliotactin independent of Src.

For this publication, I conducted all the experiments and analysis. Both myself and Vanessa Auld contributed to all the other aspects of the manuscript including study conception and design, data interpretation, writing and editing of the manuscript.
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<tbody>
<tr>
<td>AiP</td>
<td>Apoptosis induce proliferation</td>
</tr>
<tr>
<td>AJ</td>
<td>Adherens junction</td>
</tr>
<tr>
<td>ap</td>
<td>Apterous</td>
</tr>
<tr>
<td>ASPP</td>
<td>Ankyrin-repeat, SH3-domain, and proline-rich-region containing protein</td>
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<tr>
<td>BDSC</td>
<td>Bloomington drosophila stock center</td>
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<td>BL</td>
<td>Bloomington</td>
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<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<td>BSK</td>
<td>Basket</td>
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<td>Btk</td>
<td>Bruton’s tyrosine kinase at 29A</td>
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<td>Cas3</td>
<td>Caspase3</td>
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<td>CBP</td>
<td>Csk binding protein</td>
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<td>Csk homologous Kinase</td>
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<td>Contactin</td>
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<td>Cora</td>
<td>Coracle</td>
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<td>Csk</td>
<td>C-terminal Src kinase</td>
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<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<tr>
<td>Dlg</td>
<td>Discs large</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant negative</td>
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<tr>
<td>DSHB</td>
<td>Developmental studies hybridoma bank</td>
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<tr>
<td>Ecad</td>
<td>E-cadherin</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<td>EM</td>
<td>Electron micrographs</td>
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<tr>
<td>FADD</td>
<td>Fas-associated protein with death domain</td>
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<td>FAK</td>
<td>Focal adhesion kinase</td>
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<tr>
<td>FERM</td>
<td>Four.1, Ezrin, Radixin, Moesin</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>Gli</td>
<td>Gliotactin</td>
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<tr>
<td>GliDD</td>
<td>Phospho-mimic form of Gliotactin</td>
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<td>GliFF</td>
<td>Phosphorylation blocked form of Gliotactin</td>
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<tr>
<td>Gli-WT</td>
<td>Gliotactin -wild type</td>
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<tr>
<td>ILDR</td>
<td>Immunoglobulin-like domain-containing receptor</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
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<tr>
<td>JAK/STAT</td>
<td>Janus kinase/Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>JAM</td>
<td>Junctional adhesion molecule</td>
</tr>
<tr>
<td>JNK</td>
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</tr>
<tr>
<td>JNKKK</td>
<td>Jun kinase kinase kinase</td>
</tr>
<tr>
<td>LSR</td>
<td>Lipolysis stimulated lipoprotein receptor</td>
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<tr>
<td>MAGUK</td>
<td>Membrane-associated guanylate kinases</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MAPKKK</td>
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<td>MARVEL</td>
<td>MAL and related proteins for vesicle trafficking and membrane linking</td>
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<td>MMP1</td>
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<tr>
<td>MVB</td>
<td>Multivesicular bodies</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical aperture</td>
</tr>
<tr>
<td>ND</td>
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</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation signals</td>
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<tr>
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<td>Nrv2</td>
<td>Nervana2</td>
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<tr>
<td>NS</td>
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<td>PAG</td>
<td>Phosphoprotein associated with glycosphingolipid-enriched microdomains</td>
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<td>PDZ</td>
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<tr>
<td>PH</td>
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<td>Phospho-histone3</td>
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<td>PKA</td>
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<tr>
<td>POSH</td>
<td>Plenty of SH3s</td>
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<tr>
<td>PSF</td>
<td>Point-spread function</td>
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<tr>
<td>pSJ</td>
<td>Pleated Septate Junction</td>
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<tr>
<td>PTEN</td>
<td>Phosphatase and tension homolog</td>
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<td>Abbreviation</td>
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<td>--------------------------------------</td>
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<tr>
<td>SD</td>
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<td>Septate junction</td>
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<td>Sub-perineurial glia</td>
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<td>sSJ</td>
<td>Smooth Septate Junction</td>
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<td>Transforming growth factor beta-activated kinase</td>
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<td>TJ-associated Marvel domain protein</td>
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<td>Tricellular junction</td>
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<td>Transforming growth factor beta</td>
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<td>Tumor necrosis factor</td>
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<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
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<td>TNF-receptor-associated factor</td>
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<td>Tricellulin</td>
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<td>UAS</td>
<td>Upstream activating sequence</td>
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<td>Vienna Drosophila RNAi Center</td>
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<td>WT</td>
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A very big thank to all of those who supported me and I am very sorry if I have missed someone here.
Dedication

To the other name of my success, Mark

for keeping aside all your dreams,

Know that; you are free to live in your dreams

To my stress sponges, Binithi and Kovidha

for being so lovely and patient,

Know that; dedicate your life to what you love

To my late Dad who did not see any of my achievements

for being an invisible strength,

Know that; there is a drop of tears in my heart forever
Chapter 1: Introduction

Epithelial tissues are one of the primary tissues in our body and originated early in the animal lineage such as in diploblastic cnidarians, i.e., Hydra (Tyler 2003). An epithelium consists of tightly packed cells on a basement membrane. Therefore, epithelia have sheet-like arrangements maintained by the cell to extracellular matrix (ECM) contacts and diverse forms of cell to cell junctions such as adherens junctions (AJs), septate junctions (SJs) and tight junctions (TJs) along the apical side of the lateral cell membrane. Due to the sheet-like arrangement, epithelial cells make bicellular and tricellular contacts. In many tissues, permeability barriers are created to control paracellular flow through the lateral space that is created at these contacts. In invertebrate epithelia, permeability barriers are formed by the septate junctions (SJs) at bicellular contacts and tricellular septate junctions (TCJs) at tricellular contacts. The invertebrate junctions are functionally homologous to vertebrate bicellular tight junctions (TJs) and vertebrate tricellular tight junctions, respectively. These bicellular and tricellular junctions are considered interdependent protein complexes for their formation, localization of components, maturation and maintenance of barrier function (Schulte et al., 2003; Ikenouchi et al., 2005). The focus of this thesis is the transmembrane protein Gliotactin (Gli), which is restricted to tricellular septate junctions in invertebrate epithelia (Schulte et al., 2003). The downregulation of Gliotactin disrupts barrier function at both tricellular and bicellular contacts leading to the loss of permeability barriers and the death of the organism (Schulte et al., 2003). In contrast, overexpression of Gliotactin disrupts the entire epithelial architecture by signaling for cell delamination, migration, death via apoptosis and cell overproliferation (Padash-Barmchi et al., 2010). These phenotypes are dependent on the phosphorylation of Gliotactin as well as the septate junction associated protein Discs large (Dlg) (Padash-Barmchi et al., 2010; Padash-
Therefore, phosphorylation plays a key role in the level and/or the localization of Gliotactin, which is strictly controlled for the normal development of epithelial tissues. When Gliotactin is overexpressed, tyrosine phosphorylation of Gliotactin plays a role in endocytosis of Gliotactin but can also elicit deleterious phenotypes. However, the mechanism is not known (Padash-Barmchi et al., 2010). Gliotactin contains highly conserved tyrosine and serine/threonine residues, yet the kinases that control Gliotactin phosphorylation are not known. The overall goals of this thesis were to determine:

i) what kinase(s)/pathway(s) are activated leading to cell delamination, cell migration, apoptosis and cell overproliferation when Gliotactin is overexpressed

ii) what kinase(s) are involved in controlling the localization of Gliotactin at tricellular junctions.

1.1 Bicellular and tricellular SJs/TJs in epithelia form permeability barriers and perform multiple functions

The primary selective advantage of the evolution of epithelia is thought to be compartmentalization. This function is mainly achieved by the septate junctions (SJs) in invertebrate epithelia and tight junctions (TJs) in vertebrate epithelia (Tyler 2003). Hence, epithelial tissues by means of SJs/TJs form physical barriers that regulate paracellular flow between cells (Auld et al., 1995; Knust and Bossinger, 2002; Genova and Fehon, 2003; Paul et al., 2003). Barrier function is vital to maintain different molecular/ion composition for organ function and to prevent the entrance of microorganisms including pathogens into the body or organs. For instance, these junctions in the epithelial lining of the respiratory tract make a barrier that separates the air filled respiratory tract from the inner aqueous environment (Rao 2013; Wittekindt, 2017). This prevents the entrance of microorganisms, toxins and allergens from the
outside environment into the body as well as prevents leakage of components in an aqueous
environment into the respiratory airways (Rao 2013; Wittekindt, 2017). In *Drosophila*, it has
been shown that the disruption of permeability barriers can result in paralysis and death (Auld et
al., 1995; Baumgartner et al., 1996). In addition to the paracellular barrier function, these
junctions are also required to maintain cell polarity in epithelial tissues by preventing the
movement of proteins/molecules along the apicobasal axis or by anchoring polarity domains
within cells (Tepass et al., 2001, Nakajima et al., 2013). It has also been shown that the epithelial
architecture, the layer-like arrangement of the tissue, is highly dependent on SJs/TJs (Nakajima
et al., 2013). These junctions also regulate the movement of epithelial sheets during the dorsal
closure of embryos (Fehon et al., 1994). Further, recent evidence shows that SJs may play a
signaling role in important cellular events such as mitosis (Gibson et al., 2006; Bergstralh et al.,
2013; Bosveld et al., 2016). Therefore, deregulation of these junctions allows pathogens to enter,
the exchange of fluids across the tissue, along with disruption of tissue homeostasis, and cancer.
Indeed, the majority of cancers (about 90%) in human are of epithelial origin (Christiansen and
Rajasekaran, 2006; McCaffrey and Macara, 2011) and some of these are directly due to
misregulation of tight junction proteins (Zhong et al., 1994; Quan and Lu, 2003; Christiansen
and Rajasekaran, 2006; Martin, 2014). Therefore, in humans, the disruption of epithelial barrier
junctions is correlated with a variety of diseases (Rao 2013; Wittekindt, 2017). In particular,
some cancers, respiratory disorders’ like asthma and inflammatory bowel diseases are linked to
malfunction of epithelial barriers.

Permeability barriers are created not only between two cell surfaces but also at the
corners where three cells meet at junctions called the tricellular junctions (TCJs). The disruption
of TCJs disrupts the barrier function of epithelia in both invertebrates and vertebrates (Schulte et
al., 2003; Tsukita et al., 2008). Further, the downregulation of TCJ proteins in vertebrates has been shown to linked with cancers however, the mechanisms are not known (Kondoh et al., 2011; Patonai et al., 2011). The upregulation and/or mislocalization of tricellular junction components is also deleterious in both systems (Padash-Barmchi et al., 2010; Korompay et al., 2012; Somorácz et al., 2014; Takasawa et al., 2016). In addition to the permeability barrier function, TCJ proteins are involved in controlling other cellular events, and that will be discussed in detail later in section 1.6 and 4.3. Overall, for the above reasons, it is important to understand how these junction domains are formed and regulated as well as the potential signaling pathways associated with regulation of these junctions (Rao, 2013).

### 1.2 Permeability barriers (SJ/TJs and TCJs) are also found outside the epithelia

SJ/TJs and TCJs are found in all types of epithelial cells including glia, specifically in the sub-perineurial glia (SPG) in *Drosophila* and the myelinating glia of vertebrates. In the vertebrate nervous system, the paranodal junctions present between myelinating glia (oligodendrocytes and Schwann cells) and axons are homologous to septate junctions in invertebrates (Tepass, 2001; Hortsch and Margolis 2003). These junctions are critical in both invertebrates and vertebrates to protect the central and peripheral nervous systems. In insects, septate junctions (SJ) found in subperineurial glia (SPG) ensheathing the brain and peripheral nervous system form barriers separating the nervous system from the circulating hemolymph (blood). The SJs are critical for the function of nervous system and mutants that disrupt the SJ are paralyzed and embryonic lethal (Schulte et al., 2003).

Paranodal junctions of vertebrates also play a role in nerve insulation. Paranodal junctions have been shown to act as barriers between axonal surface proteins and to mediate
signals between myelinating glia and axons (Banerjee et al., 2006a; O’Brien et al., 2010). The molecular and structural analysis shows that the components of invertebrate SJs are more related to vertebrate paranodal junction components than to vertebrate TJs and it is possible that paranodal junctions are evolved from invertebrates SJs (Hortsch and Margolis 2003). Although this thesis details work done only on the epithelial tissues in Drosophila, the findings will have implications on equivalent junctions found between some glia cells, vertebrate tricellular and bicellular tight junctions, paranodal junctions and provide a better understanding of the evolutionary relationships among these tissues.

1.3 Structure and proteins of permeability barriers at bicellular contacts in vertebrate epithelia

In vertebrate epithelia, the tight junctions (TJs) and tricellular tight junctions seal the bicellular and tricellular contacts, respectively (Fig. 1.1A; Fig. 1.2A). Tight junction proteins make strands that encircle the apical side of the epithelial cells. At cell-cell contacts, the tight junction strands from opposing cells meet each other only at certain points along the lateral cell membranes making fusion points known as kissing points (Tsukita et al., 2001). Therefore, in electron micrographs, tight junctions appear as a series of kissing points along the space between the apical side of the lateral cell membranes of nearby cells (Fig. 1.1C).

More than 30 proteins including transmembrane proteins and cytosolic scaffolding proteins are known to involve in making tight junctions. Claudins (Clds), MAL and related proteins for vesicle trafficking and membrane link (MARVEL) such as Occludins, Junctional adhesion molecules (JAMS), and membrane-associated guanylate kinases (MAGUK) such as Zonular Occludins, are the main protein families involved in making tight junctions (Fig. 1.1B).
Among these, Claudins are the core TJ proteins required for making TJ strands. Claudins make homophilic interaction with Claudins in opposing cells (Piontek et al., 2008; Furuse, 2010). There are over 25 Claudin proteins in vertebrates, and the composition of Claudin proteins are different depending on the organ and the permeability of TJs (Günzel and Yu, 2013). Occludins are required for TJ formation and stability, and the precise role of Occludin is not clear (Blasig et al., 2011). The TJ-associated proteins like ZO-1, link Claudin and Occludin molecules to the actin cytoskeleton (Hartsock and Nelson, 2008a). Junctional adhesion molecules (JAMs) are another group of tight junction-associated proteins (Ebnet et al., 2004). These are single-pass transmembrane proteins with Ig domains in the extracellular side and with various protein-protein binding domains in the cytosolic tail. JAMs mainly act as scaffolding proteins at TJs interacting with ZO-1 (Nomme et al., 2011) and polarity proteins like Par3 (Itoh et al., 2001).

1.4 Structure and proteins of permeability barriers at bicellular contacts in invertebrate epithelia

The physiological role and some of the components involved junction formation are common between vertebrate TJs and invertebrate SJs. One of the main differences between SJ and TJ is their subcellular localization. TJs are found apical to the adherens junctions (AJ), septate junctions are found basal to the adherens junction (Fig. 1.1A). While both junctions have highly organized structures, the structural organization of these junctions is different. In electron micrographs, septate junctions appear as series of strands that run parallel to each other at the apical side of the bicellular contacts (Fig. 1.1C). In invertebrates, the intercellular space at the bicellular contacts is about 15-20 nm wide (Jonusaite et al., 2016) and the septa spanning this space give a ladder-like appearance to the septate junction. These strands, called septa,
completely seal the space between cells and are formed by the septate junction co-components made from opposing cells. With the exception of the Claudin-like proteins, SJ components are different from those involved in TJ formation in vertebrates. Despite the structural and molecular level differences, SJs and TJs show many functional similarities as both make permeability barriers, maintain apicobasal polarity and regulate cell signaling.

There are two fundamentally different types of septate junctions known as pleated septate junctions (pSJs) and smooth septate junctions (sSJs) (Banerjee et al., 2006b). Pleated septate junctions are ectodermal in origin, and the septa are spaced by regular gaps. However, the smooth septate junctions have an endodermal origin, and the septa are fused to each other. In addition to the structural differences, these junctions have unique components localized to each type of junction. However, they also have components common to both junctions. The epithelial lining found in the midgut of Drosophila has sSJs (Yanagihashi et al., 2012). The pSJs are the common type of septate junctions found in invertebrates and are the focus of this thesis. Therefore, pSJ are hereafter referred to as septate junctions (SJs) in this thesis.

Over the years, many core and associated components of septate junctions have been identified. The total number of genes that have been annotated as a component of SJ or a component required for the assembly of SJ is 48 (Resnik-Docampo et al., 2016). However, exact mechanisms of formation, maturation and maintenance of those junctions remain poorly understood. SJ proteins can be categorized into two groups (Fig. 1.1B). The group one proteins are the core septate junction proteins that are required for the formation of septa/stands along the bicellular contacts of cells. In Drosophila, examples for core septate junction proteins are Neurexin IV (NrxIV), Coracle (Cor), Na/K ATPase (both α subunit (ATPα) and beta subunits (Nrv2), Neuroglian (Nrg) Contactin (Cont) (Fig. 1.1B). Mutations in any of these core proteins
disrupt SJ formation and hence barrier function (Baumgartner et al., 1996; Genova and Fehon 2003; Schulte et al., 2003; Faivre-Sarrailh et al., 2004; Schulte et al., 2006; Oshima and Fehon, 2011). This group of proteins is considered co-dependent in that a loss of any one protein leads to the disruption of the entire SJ complex. Among these proteins, Neurexin IV (NrxIV), a member of Neurexin family, is a transmembrane protein with a cytoplasmic domain homologous to glycophorin C (Baumgartner et al., 1996). Coracle (Cora) is a cytoplasmic protein (from the Band 4.1 family) and interacts with the glycophorin C domain of NrxIV (Baumgartner et al., 1996). Neuroglian (Nrg) is also a transmembrane protein with multiple Ig domains, and Contactin is an Ig domain GPI-anchored protein (Genova and Fehon 2003; Faivre-Sarrailh et al., 2004). The above four core proteins (NrxIV, Cora, Nrg, Cont) presumably make one complex along the bicellular contacts of the plasma membrane (Genova and Fehon 2003; Hortsch and Margolis, 2003; Ganot et al., 2015). In addition, both the α subunit (ATPα) and beta (Nrv2) subunits of Na/K ATPase are necessary for the formation of SJ (Genova and Fehon 2003; Paul et al., 2003) and are linked to the core complex in a manner that has not been determined.

Macroglobulin Complement-related protein (Mcr) is a transmembrane protein, homologous to vertebrate Mcr and interacts with Neuroglian (Bätz et al., 2014; Hall et al., 2014). Mcr is part of a subcomplex at the SJ such that while Nrg and Mcr are interdependent for localization and function at the SJ (Bätz et al., 2014; Hall et al., 2014).

There is another group of proteins (Group 2), which are the SJ-associated proteins that indirectly regulate SJs in numerous ways. Some of the key regulators include Discs large (Dlg) (Woods et al., 1996), Scribble (Scrib) ((Bilder et al., 2000) and Lethal giant larvae (Lgl) (Bilder et al., 2000) (Fig. 1.1B). These three proteins function to establish basolateral polarity, which is required for the formation of SJs (Tepass et al., 2001). They are also thought to act as scaffolding
proteins at the SJ. Other associated proteins including a group involved in SJ protein trafficking. Specifically, the Ly6 superfamily proteins such as Boudin, crooked, coiled and crimped are involved in trafficking of some SJ proteins (i.e., NrxIV) to the membrane (Hijazi et al., 2009; Nilton et al., 2010). The one group of proteins with the most similarity to tight junction components is the Claudin-like proteins including Megatrachea (Behr et al., 2003), Sinuous (Wu et al., 2004), and Kune-kune ((Nelson et al., 2010). These proteins are necessary for SJ permeability barrier formation (though are not part of the core complex) and for tracheal development. However, their role in SJs formation is largely unknown (Behr et al., 2003; (Wu et al., 2004; Nelson et al., 2010). In addition, Pasiflora 1 and Pasiflora 2 are recently identified tetraspanin membrane proteins that are required for the SJ formation (Deligiannaki et al., 2015). Overall, many core and associated components of septate junctions have been identified. However, their relative roles and the exact mechanism of formation, maintenance of these junctions are largely unknown.
Figure 1.1: Position, components and structure of bicellular barrier junctions in vertebrates and invertebrates.
A: Schematics showing cellular localization of vertebrate TJs and invertebrate SJs. TJs and SJs form paracellular barriers at the bicellular cell contacts in epithelial tissues. In vertebrates, TJs are localized apical to the adherens junctions (AJs), in invertebrates SJs are localized basal to the AJs.

B: Components of TJs and SJs. Claudin and Occludin, the two main tight junction proteins in vertebrates, make homodimers between cells. Proteins like ZO-1 link Claudin and Occludin molecules to the actin cytoskeleton. NrxIV, Coracle, Contactin are the main proteins involved in making SJ in invertebrates. Septate junction associated proteins, Dlg and Scribble are shown. Adapted from (Browne, 2009).

C: Using TEM analysis, TJs show ‘kissing points’(arrow) like appearance and invertebrate SJs consist with ladder-like structures called septa (arrow). (Furuse and Tsukita, 2006) (Limmer et al., 2014) http://pubmedcentralcanada.ca/pmcc/articles/PMC4231875.

1.5 Structure and proteins of tricellular Junctions (TCJs) in vertebrate and invertebrate epithelia

Barriers must be created not only at bicellular contacts but also at tricellular contacts. Tricellular junctions (TCJs) form barriers at the convergence of three cell contacts. Similar to bicellular junctions, the tricellular junctions in vertebrates and those in invertebrates are functionally homologous. However, the structural organization and the components involved in the formation of these junctions are different. At bicellular contacts, the SJ or TJ strands run perpendicular to the long axis of the cells (Fig. 1.2C). At the tricellular contacts, these strands make a 90 degree turn towards the basal side of the cells and run parallel to the long axis (Fig. 1.2C). In vertebrates, the descending TJ strands from each cell fused along the long axis making a central tube of about 10 nm diameter at tricellular contacts (Fig. 1.2A) (Staehelin 1973; Krug et al., 2009; Furuse et al., 2014).

In contrast, electron micrographs show that in invertebrates, at the corners where three cells meet, a series of electron dense plug-like structures or diaphragms are formed giving a different structural organization compared to the TCJs in vertebrates (Fig. 1.2C). In invertebrates, the descending SJ strands at cell corners (called limiting septa) are in close contact.
with the diaphragms that seal the tricellular space of about 25-30 nm (Byri et al., 2015). The limiting septa may hold diaphragms in the correct place (Fristrom, 1982; Noirot-Timothée et al., 1982). The core septate junction proteins and septate junction-associated proteins in the limiting septa may be in close contact with the proteins involved in making diaphragms.

In both vertebrates and invertebrates, TCJ specific proteins are localized to tricellular junctions. However, the components are different (Fig. 1.2, B and D). In vertebrates, Tricellulin is the most studied TCJ protein. Tricellulin is a four-pass transmembrane protein similar to Occludins. Due to the presence of the tetraspanin MARVEL (MAL and related proteins for vesicle trafficking and membrane link) domain, this is also known as a MARVEL family member (MARVELD2). In addition to TRIC, two other MARVELD family proteins, MARVELD1 and MARVELD3 are known to localize to TJ in vertebrates (Raleigh et al., 2010; Mariano et al., 2011). An Angulin family member, Lipolysis stimulated lipoprotein receptor (LSR) is another TCJ specific protein found in vertebrates (Fig. 1.2B). LSR is a type 1 transmembrane protein and has an Ig domain. LSR regulates the localization of Tricellulin to the TCJ (Masuda et al., 2011) and the mechanism is largely unknown. In addition to LSR, two other LSR related Angulin family members: immunoglobulin-like domain-containing receptor (ILDR) 1 and ILDR2 have been identified as TCJ proteins in vertebrates (Hauge et al., 2004; Dokmanovic-Chouinard et al., 2008). However, they are not found in all the epithelial tissues, and their relative roles in TCJ formation or function is largely unknown (Furuse et al., 2014).

In invertebrates, Gliotactin was the first identified TCJ protein, and recently Bark/Anakonda (Aka) was identified (Byri et al., 2015; Hildebrandt et al., 2015). All septate junction core components converge at the tricellular corners as septa run along the long axis, and some septate junction proteins such as Dlg are enriched at tricellular junctions. These bicellular
junction proteins may play a role in the formation and/or function of TCJs, none of these, however, are specific to tricellular junctions like Gliotactin or Bark in invertebrates or Tricellulin and LSR in vertebrates (Fig. 1.2, B and D). However, Gliotactin and Bark are structurally different from TRIC and LSR. Overall, the structure and the components identified so far in invertebrate TCJs are different from those in vertebrates. However, these junctions are functionally homologous.
Figure 1.2: Structure and components of tricellular junctions (TCJs).

A and C: Tricellular junctions (TCJs) are formed at tricellular contacts. At tricellular corners, TJs/SJs converge and start to run parallel to the longitudinal axis of epithelial cells (A and C). In invertebrates, at TCJs, characteristic plug or diaphragms like structures are formed along this long axis (C). (Arrows and arrowheads mark TCJs or TJ stands). (Noirot-Timothe and Noirot, 1982), (Furuse et al., 2014) (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4117683), (Masuda et al., 2011) (http://jcs.biologists.org/content/124/4/548), (Ikenouchi et al., 2005)
B and D: In vertebrates, Tricellulin (TRIC) and in insects, Gliotactin (Gli) specifically localized to TCJs. Lipolysis-stimulated lipoprotein receptor (LSR) (Angulin family protein) is another TCJ specific protein in vertebrates (B). Bicellular junctional proteins, Occludin in vertebrates and Dlg in insects show a highly overlapping localization with TCJ proteins. (Arrows and arrowheads mark TCJs and SJ strands). (Schulte et al., 2006) http://jcs.biologists.org/content/119/21/4391.long

1.6 Formation, maintenance and functions of tricellular junctions

Tricellular junctions are highly specialized junctions that are formed at three cell meeting points. How this highly organized tricellular complex is assembled and maintained at this restricted localization is largely unknown in both vertebrates and invertebrates (Byri et al., 2015). Disruption of TCJs leads to the disruption of the bicellular junctions, suggesting that TCJs are important for the formation, maturation, maintenance or the function of both TJs and SJs. However, details about the interaction between components in TCJs and bicellular junctions are also largely unknown. Like bicellular junctions, TCJs also have barrier independent functions. For instance, Tricellulin is thought to control the organization of the entire cell through regulating the cell shape and the F-actin network (Oda et al., 2014).

If overexpressed or dysregulated, the TCJ proteins Gliotactin or Tricellulin spread to ectopic locations (Ikenouchi et al., 2008; Takasawa et al., 2013; Byri et al., 2015). Overexpression studies demonstrate that TCJ proteins cause deleterious effects such as cell delamination, cell migration, apoptosis and cell overproliferation when they spread to ectopic locations (Padash-Barmchi et al., 2010; Somorácz et al., 2014; Takasawa et al., 2016). Therefore, there are likely to be inhibitory mechanisms to downregulate the level of expression and restrict these proteins to the TCJ. However, how the TCJ-specific localization is exactly achieved and maintained and what potential pathways are activated by TCJ proteins at ectopic locations are largely unknown. Kinases are strong candidates in regulating TCJ protein levels, localization,
and function in both vertebrates and invertebrates. Therefore, an overview of kinases and some of their known functions in controlling junctional proteins is given below.

1.7 Kinases and kinase control of permeability barriers

1.7.1 Kinases

Kinases are enzymes that regulate their target substrates by adding a phosphate group from adenosine triphosphate (ATP) onto a hydroxyl group of target substrate. The addition of phosphate group can lead to the activation, inactivation, changes in the localization, or inter-intra molecular interactions of the target substrate, changing the downstream activity of the target substrate (Lahiry et al., 2010). The target substrate can be either a protein, lipid, carbohydrate or a nucleic acid. Thereby, kinases mediate and/or coordinate almost all the fundamental as well as complex biological events (Lahiry et al., 2010). Kinases usually act in cascades (signaling pathways), sensing and transducing external and internal signals to a cell or between cells leading to changes in gene transcription and cell responses. Kinases act as molecular switches and they themselves can be in active (on) or inactive (off) state. Receptor kinases can be activated by ligand binding or other stimulus initiating the kinase cascade. Non-receptor kinases are activated either by autophosphorylation or by transferring of a phosphate group by another kinase onto the phosphorylation site at the activation loop. Binding of allosteric effectors, dissociation from a inhibitory protein or changing subcellular localization (such as recruitment to the membrane) can also switch on or off a kinase (Shchemelinin et al., 2006; Pereira et al., 2011). A signaling pathway can be misregulated directly due to a mutation in a kinase or indirectly due to a mutation in a negative regulator or downstream signaling regulator such as a phosphatase (Lahiry et al., 2010). Mutations in kinase pathways are the main cause of
malignancy reported so far in humans. In addition, a wide variety of diseases including inflammatory diseases, diabetes, infectious diseases, cardiovascular diseases are due to aberrant kinase function (Blume-jensen and Hunter, 2001; Fabbro et al., 2015). Kinases also play a key role in the regulation of junctional proteins.

Among kinases, the protein kinase family represents one of the largest protein families in eukaryotes. Eukaryotic protein kinases fall into three types based on the phosphorylation site: tyrosine, serine/threonine or both tyrosine and serine/threonine (dual-specificity protein kinases) (Douville et al., 1994; Fabbro et al., 2015). Tyrosine kinases have, a conserved tyrosine residue in the catalytic group and once activated tyrosine kinases phosphorylate tyrosine residues in the target proteins. While serine/threonine kinases have either serine or threonine in the catalytic domain and theses kinases phosphorylate serine/threonine residues in the target proteins. Tyrosine kinases are further divided into two types as receptor or non-receptor tyrosine kinases. Non-receptor tyrosine kinases are found in the cytoplasm or in the nucleus. Ser/threonine kinases are also present in the cytosol or in the nucleus. Some of the serine/threonine kinases can be in the membrane bound form, however, they usually do not contain a transmembrane domain (Douville et al., 1994; Lahiry et al., 2010). Based on the sequence and the structure of the catalytic domain there are seven sub-groups of human kinases (Lahiry et al., 2010). Those include, tyrosine kinases (TK), tyrosine kinase-like (TKL), homologous of the yeast sterile 11 and sterile 20 kinases (STE), Casein kinase 1 (CK1), family of protein kinase A, G and C (AGC), Calcium/calmodulin-dependent protein kinases (CAMKs), and CMGC subgroup which contains kinases such as cyclin-dependent-kinases (CDKs) and Mitogen activated protein kinases (MAPKs) (Shchemelinin et al., 2006; Lahiry et al., 2010).
About 250 kinases have been reported in the fly genome (Morrison et al., 2000) with about 40 tyrosine kinases and the rest serine/threonine kinases. At least 538 kinase genes have been identified in humans with the majority represented by serine/threonine kinases (Fabbro et al., 2015). Drosophila does not appear to have any fly specific kinase family, and every Drosophila kinase has a human ortholog, showing the highly conserved nature of the Drosophila and human kinomes (Read et al., 2013).

In addition to protein kinases, lipid kinases also control a wide range of cellular processes. Phosphatidylinositol (PI) kinases and related protein kinases in particular play important roles in maintaining plasma membrane lipid composition, actin organization, membrane trafficking and cell signaling by modifying phosphoinositides (Odorizzi et al., 2000; Shewan et al., 2011). Phosphatidylinositol (PI) kinases act on phosphatidylinositol lipids (PI lipids) making PI phosphates (PIPs or phosphoinositides). The composition of the PIPs gives a unique membrane identity for the plasma membrane and for each organelle within a cell. PIPs act as substrates in key signaling pathways including those involved in tissue formation and patterning during development (Tan and Brill, 2014; Tan et al., 2014).

Phosphatidylinositol lipids (PI lipids) have three potential phosphate addition sites in the cytosolic inositol ring. Production of PI4-phosphate (PI4P) by a PI4K is the first step in the PIP pathway, and this makes the precursor for PI(4,5)bisphosphate (PIP₂) and then PI (3,4,5)-trisphosphate (PIP₃). PI4P is the most abundant PIP in the cell membrane, and PIPs can be interconverted by lipid kinases changing the membrane PIP lipid composition and thereby the biochemical properties of the membrane (Giepmans and van Ijzendoorn, 2009; Shewan et al., 2011). These changes lead to the changes in the composition of the proteins associated with the cell membrane. When PIP₂ acts as a substrate for phospholipase C (PLC), IP₃ and diacylglycerol
(DAG) are produced. Subsequently, this leads to signaling for protein kinase c (PKC). The production of PIP3 by PI3-kinase leads to signaling for the mTOR pathway through the activation of Akt. The mTOR pathway acts as a master growth regulator by receiving growth related signals (both nutritional and environmental) and controlling anabolic and catabolic processes such as gene transcription, protein translation and autophagy (Sarkar, 2013; Zarogoulidis et al., 2014). Recent evidence shows that not only PIP2 and PIP3 but also PI4P also has an important signaling role in addition to its function as a precursor molecule. In particular, PI4P is known to be involved in regulation of the cytoskeleton and membrane trafficking.

1.7.2 Role of kinases and phosphatases in regulation of permeability barriers

As outlined below in section 1.8, phosphorylation followed by endocytosis is a part of the mechanism to control level and localization of the TCJ protein Gliotactin (Padash-Barmchi et al., 2010). When overexpressed, Gliotactin phosphorylation is also known to activate signaling pathway(s) leading to deleterious phenotypes, such as cell delamination, cell migration, cell death and tissue overproliferation (Padash-Barmchi et al., 2010). However, the kinases that regulate Gliotactin level and/or localization or the kinases that are involved in Gliotactin signaling are not known. There are numerous possible kinases that may mediate these events.

The assembly, dissociation and functions of cell-cell or cell-matrix junctional complexes in epithelial tissues are regulated by various signaling pathways through various mechanisms. i.e., by increasing the transcriptional expression of target proteins, by phosphorylating the proteins leading to changes in localization or protein-protein interactions (Takahashi et al., 2005; Wang et al., 2006; Bertocchi et al., 2012; Takasawa et al., 2013). These events can be regulated by phosphorylation of junctional proteins themselves or other proteins that interact...
with scaffolding proteins in the junctions. In addition, these junctions also act as signaling hubs that receive or transmit various inter- and intra- cellular signals (Matter and Balda, 2003). For instance, TJs regulate events such as cell proliferation, cytoskeletal organization and differentiation by interacting with various signaling pathways (Matter and Balda, 2003; Leech et al., 2015).

Multiple studies have shows that TJ proteins interact with kinases. For instance, TJs, Occludin and Zonular Occludins interact with Hippo pathway component LATS (warts) and Yap (Yorkie) to regulate cell proliferation (Meng et al., 2016; Zihni et al., 2016). The Hippo pathway functions to inhibit the growth by phosphorylating the downstream target Yap (Yorkie) via LATS (warts). The phosphorylated Yap (Yorkie) remains in the cytoplasm or associated with tight junctions and does not enter the nucleus to activate the transcription of genes that regulate growth (Nishio et al., 2013; Meng et al., 2016; Zihni et al., 2016). TJ proteins Occludin and MARVELD3 regulate mitogen-activated protein kinase (MAPK) signaling (Steed et al., 2014). In particular, MARVELD3 has an inhibitory effect on MEKK1 (MAP3K)-JNK signaling. The removal of MARVELD3 from tight junctions through endocytosis leads to the activation of MEKK1(MAP3K1)-JNK pathway signaling. Overall, the permeability junction proteins have the ability to modify signaling pathways through interactions with kinases.

In contrast, some kinases control tight junction proteins and thereby the permeability. The kinase control of tight junctions has been studied to some extent in vertebrates, and it is known that their formation, function and dissociation rely on various kinases. For instances, Epidermal growth factor receptor (EGFR) regulates the expression and localization of the tight junction proteins Occludins and Zonular-Occludins (ZO-1) (Wang et al., 2006b). Some cell signaling pathways regulate tight junctions through transcriptional control of TJ proteins or by changing
the localization of tight junction proteins by phosphorylation. For instance, in lung epithelia, TNF-alpha signaling downregulates the transcription of some tight junction proteins (cldn2, cldn5 and ZO-1) and localization of some other TJ proteins (Wittekindt, 2017). Transforming growth factor-β (TGFβ) increases the expression of claudin during some bacterial infections to increase TJ resistance (Howe et al., 2005). In contrast, the interaction between the TJ protein Occludin and TGFβ leading to junction dissociation has also been shown (Barrios-Rodiles et al., 2005). Alternatively, kinase control leads to direct phosphorylation of tight junction proteins to control function and downregulation through endocytosis (González-Mariscal et al., 2008). For instance, TNF-alpha can control protein turnover at TJs through Src kinase activity (Amasheh et al., 2010). The TJs in human keratinocytes and colonic epithelial cells are disassembled by JNK activity (Lee et al., 2009; Naydenov et al., 2009; Kojima and Sawada, 2012). Further, the phosphorylation of Occludin at serine/threonine residues changes its function and localization. Occludin is phosphorylated by numerous kinases including protein kinase C (PKC), protein kinase A (PKA), MAPK kinases, phosphatidylinositol 3-kinase (PI3K), casein kinase 1 (CK1) and casein kinase 2 (CK2) and the outcome of these phosphorylation events depend on the tissue type and the signaling components involved (González-Mariscal et al., 2008; Dörfel et al., 2009). In general, phosphorylation of Occludin at conserved serine/threonine residues leads to localization of Occludin to the TJ domain and increased barrier function (Rao, 2009). However, the tyrosine phosphorylation of Occludin generally leads to loss of interaction with other TJ proteins and junction disassembly (Rao, 2009).

In addition to kinases, phosphatases play key roles in the regulation of cell-cell junctions. The involvement of protein phosphatase 2A (PP2A), protein phosphatase 1 (PP1) and density enhanced phosphatase-1 (DEP-1) in the regulation of Occludin has been shown. PP2A and PP1
act on the phosphorylated serine/threonine sites in the C-terminal end of Occludin, while DEP-1 acts on the phosphorylated tyrosine sites (Seth et al., 2007; Hartsock and Nelson, 2008). These phosphatases may also interact with other TJ proteins such as Claudins and ZO-1, to control TJs, and the PP2A and PP1 activity generally lead to junction disassembly (Rao, 2009). Further, PTEN (phosphatase and tension homolog deleted on chromosome ten) controls the expression level of several TJ proteins including Occludin, ZO-1 and Claudin (Langlois et al., 2010). This suggests that phosphatases act not only to counteract kinase activity but also have other functions. However, this thesis study did not investigate the function of phosphatases in relation to junctional proteins.

While some kinases that regulate TJ proteins are well studied, very little is known about kinase control of TCJ proteins in vertebrates. The vertebrate TCJ protein Tricellulin has multiple threonine phosphorylation sites (Ikenouchi et al., 2008; Takasawa et al., 2013). It has been shown that the degree of Tricellulin phosphorylation changes in relation to the disruption or formation of tight junctions (Takasawa et al., 2013). However, the kinases associated with those phosphorylation sites and the consequences of those phosphorylation events have yet to be elucidated (Mariano et al., 2011). Phosphorylated Tricellulin is found in the bicellular contacts and in the cytoplasm suggesting that Tricellulin localization is regulated by phosphorylation (Ikenouchi et al., 2008; Takasawa et al., 2013). Tricellulin seems to localize to ectopic domains such as bicellular contacts, and apical and basolateral cell membranes if overexpressed or dysregulated (Ikenouchi et al., 2008). An elevated level of Tricellulin was reported in some cancerous cells in pancreatic ductal carcinoma where JNK activity may upregulate the expression of Tricellulin (Korompay et al., 2012; Kojima et al., 2013; Takasawa et al., 2016). However, most studies on the phosphorylation of Tricellulin were done in cell culture studies,
and the mechanisms, roles and potential signaling pathways associated with Tricellulin phosphorylation *in vivo* are not known yet.

Similar to vertebrate TCJs, the kinase control of permeability barriers in invertebrates is largely unknown for both bicellular septate and tricellular junctions.

1.8 **Gliotactin is a main TCJ component, and its localization must be strictly controlled**

Gliotactin is a core TCJ protein, and the localization of Gliotactin to the TCJ is regulated by phosphorylation leading to endocytosis to ensure the correction localization to the TCJ (Padash-Barmchi et al., 2010) (The background studies behind this statement are explained in detail below in section 1.8). However, the kinases involved in Gliotactin phosphorylation are not known. Gliotactin is a single pass transmembrane protein from the Neuroligin family and the first protein identified to have a unique and concentrated localization at tricellular junctions in invertebrates (Schulte et al., 2003). Stage 13 embryos of *Drosophila* shows that Gliotactin is first localized along the lateral cell membrane and then restricted to the TCJ domain by stage 16. It has been suggested that Gliotactin is also localized to bicellular junctions by default and later it is restricted to the tricellular corners (Byri et al., 2015). Hence, Gliotactin may not have the ability to localize specifically to the TCJs, and other proteins and mechanisms are involved in restricting localization to the TCJ.

One mechanism thought to control the localization of Gliotactin to the TCJ is through the recently identified TCJ protein Bark beetle/Anaconda (Byri et al., 2015; Hildebrandt et al., 2015). Bark contains a large extracellular domain with an unusual triple-repeat. The large extracellular domain of Bark/Aka is important for the initiation of tricellular junction assembly and the recruitment of Gliotactin to TCJs (Byri et al., 2015). In Bark/Aka mutant embryos,
Gliotactin is not localized to the TCJs. However, the septate junction core proteins or associated proteins are not affected, showing that bicellular SJ assembly is independent of Bark/Aka (Byri et al., 2015). Similarly, mutants of core septate junction proteins still show Bark/Aka protein localization at the TCJs, showing Bark/Aka localization at TCJs is independent of the bicellular junctions. Byri et al., (2015) showed that expression of Bark/Aka is sufficient to initiate Gliotactin localization to TCJs. Interestingly, when overexpressed, Bark/Aka is localized not only to TCJs but also to the bicellular septate junctions. However, Gliotactin is not recruited to the bicellular junctions (Byri et al., 2015). This shows that Bark/Aka is capable of recruiting Gliotactin only to the tricellular junctions and that other protein(s)/mechanisms are required for regulating Gliotactin localization.

_Gliotactin_ null mutants and overexpression of Gliotactin both give rise to detrimental phenotypes, showing the importance of having Gliotactin at and restricted to the TCJs. _Gliotactin_ null mutant animals are paralyzed due to the disruption of permeability barriers (Fig. 1.3A). In particular, the blood-brain and blood-nerve barriers are disrupted, and they die in late embryogenesis (Schulte et al., 2003). Electron micrographs showed that while a few septa are formed, the components are loosely packed, core SJ proteins spread basally, and barrier function is compromised in the _Gli_ null mutant (Fig. 1.3B) (Schulte et al., 2003). Conversely, Gliotactin is mislocalized in loss of function mutants in SJ genes (Schulte et al., 2003). This suggests that unlike for Bark/Aka, SJ components are directly or indirectly required for the Gliotactin localization (Schulte et al., 2003). While Gliotactin is important to maintain barrier function and for the maintenance of the SJ domain, _Gli_ null clones are cytotoxic and do not divide (Schulte et al., 2006). Loss of Gliotactin triggers epithelial cells to undergo apoptosis and delamination.
(Schulte et al., 2006). Notably, Gli null cells do not lose apical-basal polarity (Padash-Barmchi et al., 2013), suggesting that Gliotactin might have other roles in addition to barrier function.

![Figure 1.3](image)

**Figure 1.3: Presence of Gliotactin at the TCJs is critical for barrier function and structure.**
A: Disruption of barrier function in Gli null mutants. Penetration of dextran dye into the lumen of salivary glands in Gli null mutants is shown.
B: Electron micrographs (EM) showing tightly packed septate junction strands in WT and less number of loosely packed septa in Gli null mutants (some septa are missing in the mutant). (Schulte et al., 2003).

Localization and restriction of Gliotactin to the tricellular corners seem to be equally important. When overexpressed in the columnar epithelia of the wing imaginal disc using the ap>GAL4 driver, Gliotactin spreads around the cell and along the baso-lateral membrane in the cells in the apterous side of the wing disc (Fig. 1.4). (a brief introduction on the wing imaginal disc of *Drosophila* and the Gal4-UAS binary expression system is given below in section 1.9). When overexpressed, the ectopic localization of Gliotactin leads to cell delamination, cell migration to the non-apterous side of the wing disc and apoptosis (Fig. 1.4, B’, C and E) (Padash-Barmchi et al., 2010). Further, ectopic folds are formed due to overproliferation (star in Fig. 1.4, C). Overexpressed Gliotactin is also found in large internal vesicles (Fig. 1.4D). In Gliotactin overexpressing backgrounds, the detrimental phenotypes are due to an interaction
between ectopic Gliotactin and Dlg (Padash-Barmchi et al., 2013). Further, these phenotypes seem to be generated through the activation of Jun kinase (JNK) as the knockdown of JNK leads to suppression of phenotypes (Padash-Barmchi et al., 2013). However, the details of the pathway(s) involved in and the mechanisms that underlie the activation of JNK through Gliotactin and/or Dlg are not known (Padash-Barmchi et al., 2010; Padash-Barmchi et al., 2013).

**Figure 1.4: Gliotactin overexpression phenotypes and localization of overexpressed Gliotactin.**

In all panels, the boundary between dorsal/apterous (ap) and ventral (non-apterous/wild type) compartments is marked by the yellow dotted line.

A: Low magnification image (20x) of 3rd instar wing imaginal discs expressing apterous-Gal4 driven UAS-NLS-GFP in the dorsal half. Ectopic folds were not visible. The arrow and the inset in the upper right corner show no spreading of cells from the apterous side to non-apterous side.
In addition to the activation of the JNK pathway, whether some other signaling pathways are also activated when Gliotactin is overexpressed is not known.

Overall, Gli null and overexpression experiments clearly show that Gliotactin levels and localization must be strictly controlled. However, the signaling pathways or mechanisms involved in regulating Gliotactin localization have not yet been fully identified. Beyond the role of Bark/Aka in initiating Gliotactin recruitment to TCJ and the interaction with Dlg, direct interactors with Gliotactin that are involved in its localization or function have not been identified.

### 1.8.1 Molecular structure and regulation of Gliotactin

Although Gliotactin is functionally homologous to Tricellulin in vertebrates, its closest vertebrate homolog is Neuroligin 3 (NLGN3) (Gilbert et al., 2001; Gilbert and Auld, 2005).
NLGN3 is found in many classes of Glia cells and it is a synaptic protein. Clustering of NLGN3 at the synapses induces the clustering of Neurexin and this is mainly act as an adhesive protein in the nervous system (Ichtchenko et al., 1996; Krueger et al., 2012). Further, the downregulation of NLGN3 affect the vesicular endocytosis. Dlg is another protein that is known to interact with Neuroligins (Ichtchenko et al., 1996). A cleaved and secreted form of NLGN3 (extracellular domain) activates PI3K-mTOR pathway promoting the glioma cell proliferation and invasion (Venkatesh et al., 2015). Like Neuroligins, Gliotactin is a single-pass transmembrane protein consisting of an extracellular non-enzymatic serine esterase domain, a transmembrane domain and a cytoplasmic domain (Fig. 1.5A). The extracellular domain is thought to be involved in protein-protein interactions, and Gliotactin may form dimers or oligomers through this domain (Venema et al., 2004; Gilbert and Auld, 2005). Similarly, the vertebrate homologue to Gliotactin, Neuroligin 3 (NLGN3) also form dimers or oligomers (Gilbert and Auld, 2005). The cytosolic domain of Gliotactin contains a range of highly conserved domains: two highly conserved tyrosine residues at amino acids 760 and 799 (Gilbert and Auld, 2005), a conserved PDZ binding domain, a protein kinase C (PKC) phosphoserine motif and predicted phosphatase target sites. One or both phosphotyrosine sites are involved in endocytosis and downregulation of Gliotactin (Fig. 1.5 B-D) (Padash-Barmchi et al., 2013). When Gliotactin is overexpressed, the tyrosine residues are phosphorylated, and Gliotactin is endocytosed into endosomes and lysosomes, and then degraded (Padash-Barmchi et al., 2010). Gliotactin phosphorylation in the Gliotactin overexpressing background, has been confirmed in-vitro by phosphotyrosines antibody labeling in western blots (Padash-Barmchi et al., 2010). Overexpression of a phosphomimetic form of Gliotactin (GliDD) does not generate the deleterious phenotypes generated by overexpression of Gli-WT (wild type Gliotactin) (Fig. 1.5C), but triggers the formation of large endocytotic
vesicles (Fig. 1.5C’). In contrast, overexpression of phosphorylation-blocked form of Gliotactin (GliFF) leads to enhanced phenotypes (in particular, cell migration and overproliferation phenotypes are enhanced) and production of only small vesicles (Fig. 1.5 D and D’). The mechanism behind enhancement of phenotypes by GliFF is described later in this section. The change of tyrosine to aspartic acid or glutamic aside is a common practice in phosphomimetic studies for kinases, however this might not exactly work for other proteins. In particular, this might not work if the phosphorylation has to be followed by a recognition by another protein (i.e., recognition of phosphotyrosines by SH2 domain containing proteins like Cbl, Grb2 for subsequent endocytosis (Jiang et al., 2003). However, somehow when overexpressed, GliDD increased the endocytosis, mimicking the tyrosine phosphorylation.

The identity of large vesicles formed in Gli-WT and GliDD has been determined (Padash-Barmchi et al., 2010). The large internal vesicles formed in both the Gli-WT and GliDD backgrounds colocalized with endosome, lysosome and ubiquitin markers indicating that they were in a degradation pathway (Padash-Barmchi et al., 2010). When assayed by Western analysis Gli-WT showed degraded products whereas Gli-FF did not (Padash-Barmchi et al., 2010). Further, we would expect Gli-WT and GliDD proteins to be colocalized with ER markers if Gli-WT or GliDD activate ER stress due to an unfolded protein response. However, they did not colocalize with Golgi or ER markers showing that these constructs were secreted out as normal proteins (Padash-Barmchi et al., 2010). Further, all three constructs, Gli-WT, GliDD and GliFF rescued the embryonic lethal Gli null mutant to the adult stage (Padash-Barmchi et al., 2010) confirming that they could restore the normal function of Gliotactin. Overall, when overexpressed Gliotactin is endocytosed and sent for degradation, and the deleterious phenotypes seen in this background are not due to a stress response caused by unfolded proteins.
In addition, when endocytosis is blocked using a Rab5DN construct in otherwise wild type epithelia, Gliotactin spreads away from the TCJ (Fig. 1.5F) (Padash-Barmchi et al., 2013). These data suggest that phosphorylation and endocytosis is a mechanism to restrict Gliotactin to the TCJ in wild type tissues (Padash-Barmchi et al., 2013). However, the kinase(s) involved in this important phosphorylation event(s) are not known. It has been shown that Src can phosphorylate the intracellular C-terminal domain of Gliotactin in vitro (Padash-Barmchi et al., 2010). However, Src is a kinase with a wide range of substrates (Ferrando et al., 2012) and whether Src can phosphorylate Gliotactin in vivo is not known. In addition, four predicted serine phosphorylation sites (Fig. 1.5A) are conserved in all Gliotactin homologues. However, their role(s) are not known. Therefore, not only tyrosine kinases but also serine/threonine kinases may also have a role in controlling Gliotactin localization and/or function, and these have not been investigated yet.
Figure 1.5: Gliotactin localization and level is controlled through tyrosine phosphorylation and endocytosis.

A: Schematic showing Gliotactin protein and tyrosine phosphorylation sites on the cytosolic side. Two conserved tyrosine residues (760, 799) are indicated. Blue stripes represent conserved serine phosphorylation sites.

B-D: Gliotactin overexpression phenotypes change with the phosphorylation state of Gliotactin. Third instar wing imaginal discs, expressing different Gliotactin constructs driven with the apterous-Gal4 driver in the dorsal half. Yellow arrows pointing to vesicles like structures positive for Gliotactin
B: *ap>Gli-WT*. Overexpression of wild type Gliotactin (Gli-WT) leads to cell migration (B). The Overexpressed Gliotactin is found around the cell membrane and in large vesicle-like structures (B’).

C: *ap>GliDD*. Overexpression of phosphomimic form of Gliotactin (GliDD) does not lead to cell migration to the ventral compartment (C) and shows many cells with large vesicles (C’).

D: *ap>GliFF*. Overexpression of phosphorylation blocked form of Gliotactin (GliFF) enhances the migration and ectopic fold (overgrowth) phenotypes seen in A and shows only a small number of vesicles. The vesicle size also was smaller (D’).

E: Puncta like restricted localization of Gliotactin in a wild type epithelium.

F: *ap>RAb5-DN*. Blocking endocytosis by expressing a dominant negative Rab5 leads to mislocalization of Gliotactin. Endogenous Gliotactin spreads away from the TCJs (blue arrows) when endocytosis is blocked. (E and F panels are from the original paper and hence are at different magnifications)

All the images except the schematic A were from (Padash-Barmchi et al., 2010) (http://jcs.biologists.org/content/123/23/4052.long),
Figure 1.6: Endogenous Gliotactin interacts with overexpressed Gliotactin constructs leading to changes in the degree of endocytosis and deleterious phenotypes.

A-C: Schematic diagrams showing changes to endocytosis and signaling with changes to Gliotactin phosphorylation state as overexpressed Gliotactin forms oligomers or dimers with the endogenous Gliotactin. (A) Overexpressed Gliotactin phosphorylates and leads to both endocytosis and deleterious signaling. (B) A phosphomimetic form of Gliotactin (GliDD) is rapidly removed out from the membrane through endocytosis, giving less time for the deleterious signaling. (C) A phosphorylation blocked form of Gliotactin (GliFF) has decreased endocytosis. When GliFF is dimerized with endogenous Gliotactin this slows endocytosis and increases deleterious signaling as this stay longer on the membrane.

The overexpression of Gliotactin in otherwise wild type imaginal disc points to Gliotactin endocytosis as a clear mechanism that operates to control Gliotactin levels. However, in this background, Gliotactin phosphorylation plays a role not only in the control of protein levels but
also in triggering the deleterious phenotypes. The overexpression of GliDD construct leads to an increased endocytosis, however, does not trigger the deleterious phenotypes (Fig. 1.5C; Fig. 1.6B). Gliotactin is known to function as dimer or oligomer (Venema et al., 2004). Of interest, when the Gliotactin wild type construct (Gli-WT) is expressed in a Gli null background this also lead to the spread of Gliotactin around the cell membrane and generated the deleterious phenotypes. But overexpression of the tyrosine-phosphorylation blocked form of Gliotactin (GliFF) in a Gli null background does not lead to Gliotactin endocytosis or deleterious phenotypes even though the GliFF spreads around the membrane (Padash-Barmchi et al., 2010). This shows that phosphorylation at conserved tyrosine sites is required for both Gliotactin endocytosis and deleterious phenotypes. The lack of Gliotactin overexpression phenotypes when GliFF is overexpressed in Gli null background is in contrast to what occurs when GliFF is expressed in the presence of endogenous Gliotactin, where Gliotactin phenotypes were enhanced (Fig. 1.5D; Fig. 1.6C). This has led to a model where GliFF forms a complex with endogenous Gliotactin and the reduced endocytosis of GliFF keeps the phosphorylated Gliotactin (endogenous) longer at the membrane to signal for deleterious phenotypes (Padash-Barmchi et al., 2010) (Fig. 1.6C). Overall, these data suggest that tyrosine phosphorylation is required for both endocytosis and activation of deleterious signaling pathways in Gliotactin overexpression background. However, the kinase(s) that phosphorylate Gliotactin are not known. Further, the overexpression of mCD8::GFP, NrxIV or Dlg do not lead to formation of large vesicles or deleterious phenotypes. It is the specific overexpression of Gliotactin that leads to these phenotypes. However, the signaling pathways involved in generating the Gliotactin overexpression phenotypes are not known. As discussed in section 1.8.3, Gliotactin overexpression also somehow trigger the phosphorylation of Dlg at 797S (serine) residues.
coexpression of phosphorylation blocked form of Dlg with Gliotactin suppresses the Gliotactin overexpression phenotypes, however, the coexpression of phosphomimetic form of Dlg enhances the phenotypes. Since Gliotactin or Dlg are not kinases, the activation of signaling pathways is likely due to ectopic interactions and/or interfering with inhibitors of some signaling pathways when Gliotactin spreads away from TCJs.

1.8.2 Gliotactin function beyond the permeability barrier

The barrier function of Gliotactin is well known and has been shown both in epithelia and in the nervous system of Drosophila (Auld et al., 1995; Schulte et al., 2003). TCJ proteins apparently coordinate other cellular events in addition to the barrier function (i.e., in controlling polarity, cell division and differentiation). For instance, Venema et al (2004) showed that Gliotactin together with Cora function to form distally oriented, parallel arrays of prehairs in pupal wings independent of the frizzled pathway, suggesting a role of Gliotactin in the establishment of planar cell polarity. In addition, a possible role for Gliotactin in controlling the orientation of cell division was recently discovered (Bosveld et al., 2016). The Dynein-associated protein, Mud2 is involved in generating mechanical pulling force to control the spindle orientation, and the speed of centrosome recoil during cell division (Bosveld et al., 2016). Mud2 is localized to TCJs in a Gliotactin-dependent manner. Further, Bosveld et al (2016) suggested that Gliotactin together with Mud2 provide spatial landmarks for TCJs in rounded mitotic cells during cell division. Resnik-Docampo et al (2016) showed that in the midgut epithelia of adult flies, Gliotactin is lost at the TCJs in differentiated epithelial cells as the flies get older. This leads to a cell non-autonomous effect on the undifferentiated stem cell population in the midgut epithelia and increases their cell proliferation in a JNK-dependent
manner (Resnik-Docampo et al., 2016). In imaginal disc columnar epithelia, the plane of cell division localized to the level of SJ and TCJ domain and further, at least one centrosome is localized to TCJ (Charish, 2011). Expression of Gliotactin RNAi or Gli null clones in imaginal disc epithelia is cell lethal, suggesting Gliotactin is required for cell survival and perhaps for cell division (Charish, 2011).

When overexpressed, Gliotactin indirectly or directly affects the cell cycle and induces extra cell proliferation giving extra folds and overgrowth of the wing disc. A large amount of pyknotic nuclei and increased Caspase 3 (Cas3) activity showed that the overexpression of Gliotactin leads to apoptosis. Therefore, cell overproliferation might be largely due to apoptosis induced proliferation (AiP) (Mollereau et al., 2013) or through activation of the JNK pathway (Padash-Barmchi et al., 2010). Overall, the mechanism(s) behind restricting Gliotactin level and localization, details on barrier independent functions, kinases that directly phosphorylate Gliotactin and potential signaling pathways that are activated by Gliotactin are areas that are not yet fully understood.

1.8.3 Kinase-mediated functional interaction between Gliotactin and Dlg

Like Gliotactin, Discs large (Dlg) is also expressed both in epithelia and glia cells. In epithelia, Dlg is found at SJs and concentrated at TCJs, showing an overlapping localization with Gliotactin at TCJs (Schulte et al., 2006). Dlg is a SJ associated protein involved in many cellular processes such as polarity, tumor suppression, scaffolding, and cell signaling (Humbert et al., 2003). Various studies have indicated potential genetic, functional and biochemical interactions between Gliotactin and Dlg (Padash-Barmchi et al., 2013). When Dlg is knocked-down, Gliotactin is lost from the TCJs suggesting that Dlg recruits Gliotactin to the TCJ (Padash-
Barmchi et al., 2013). However, direct binding between Gliotactin and Dlg has not been proven (Schulte et al., 2006). Although Gliotactin also has multiple predicted protein-protein interacting domains (Zeev-Ben-Mordehai et al., 2003), so far Dlg is the only component that has been found in a protein complex with Gliotactin. Padash-Barmchi et al (2013) further showed a functional interaction between Gliotactin and Dlg such that when Gliotactin was overexpressed, Dlg is downregulated. In addition, overexpression of Dlg enhances the overexpression phenotypes of Gliotactin suggesting an ectopic interaction between these two proteins (Padash-Barmchi et al., 2013). Moreover, phosphorylation of Dlg at S797, a PAR1 phosphorylation site, plays a critical role in this interaction. Expression of Gliotactin with DlgS797A-GFP blocks all the deleterious effects of Gliotactin overexpression (Padash-Barmchi et al., 2013). Those results indicate that Dlg phosphorylation is also a critical event in generating the phenotypes seen when Gliotactin is overexpressed. Blocking Basket (Bsk; Drosophila JNK) function when either Gliotactin is overexpressed or when Gliotactin and Dlg are co-expressed suppresses all the Gliotactin overexpression phenotypes (Padash-Barmchi et al., 2013). JNK phosphorylation of Dlg has been reported in other genetic contexts (Massimi et al., 2006). However, in a Gliotactin overexpression background, JNK activation is downstream of Gliotactin and Dlg phosphorylation (Padash-Barmchi et al., 2013). Therefore, what kinase mediates the functional interaction between Gliotactin and Dlg is not known.

1.9 The model organism, the tissue and the main genetic tools used in the study

The research presented in this thesis utilized the columnar epithelia of the Drosophila wing imaginal disc as the model tissue (Fig. 1.4 A and B). Wing imaginal discs are present in the larval stage, and these are developed into adult wings during metamorphosis. In larval
stages, these discs consist of two layers of epithelial stem cells: a tall columnar epithelial layer and a squamous parapodial cells (Fig 1.4 B and B’). The tall columnar epithelial cell layer allows us to visualize polarized cellular phenotypes during differentiation and development of the adult wing. Genetic manipulations in wing discs are not lethal as wings are not essential for viability. Further, mutants and RNAi lines for most of Drosophila genes have been generated, and many genetic tools are available for this model organism. Signaling pathways are often very complicated and complex to understand. However, Drosophila often has only one copy of each kinase making it a good model to understand in vivo roles and interactions of signaling pathways.

Since many inter and intra-cellular signaling pathways are conserved throughout evaluation, the findings of this study can contribute to the understanding of vertebrate tricellular junction protein regulation and potential signaling events associated with these junctions.

Among the genetic tools available for research in Drosophila, the GAL4/UAS system is a powerful system for studying tissue specific gene functions. GAL4 is a yeast regulatory protein that activates gene expression downstream of the GAL4 Upstream Activating Sequence (UAS) (Brand et al., 1993). Using this system, target genes can be expressed in a temporal and tissue specific manner by fusing the GAL4 gene to various promoters and the UAS with genes of interest (Brand et al., 1993), RNAi or reporter sequences to express target genes in a time- and tissue-specific manner. GAL4 expression can be further refined in a temperature dependent manner by using a temperature sensitive form of the GAL4 regulator GAL80 (GAL80ts) (McGuire et al., 2004). GAL4 expression increases from a low-level at 18°C to maximum activity at 29°C. In contrast, at 18°C, GAL80ts functions normally repress GAL4 expression, thereby preventing the activation of the UAS and subsequent target gene expression. At the restrictive
temperature 29⁰C, GAL80ts is inactivated, permitting GAL4 expression and subsequently target and subsequent gene expression.

In all the experiments done for this thesis, the GAL4 fused to the apterous promoter was used to drive expression of various target gene constructs. Apterous is expressed only in the dorsal compartment of the wing imaginal disc enabling us to use the ventral compartment as an internal wild type control (Fig 1.4B).

1.10 Thesis questions and hypotheses

The work presented in this thesis addressed the following research questions.

1. What pathways and mechanisms are activated when the tricellular junction protein Gliotactin is overexpressed?

   What components upstream of JNK are activated when Gliotactin is overexpressed?

   What other kinase pathways are activated when Gliotactin is overexpressed and what other kinases can modify the Gliotactin overexpression phenotypes?

2. How is Gliotactin restricted to the TCJs and its protein levels controlled?

   What kinase(s) are responsible for the regulation of endogenous Gliotactin?

Gliotactin levels and the localization must be tightly controlled for normal development of epithelial tissues. Gliotactin levels are in part controlled by tyrosine kinase phosphorylation, endocytosis and degradation (Padash-Barmchi et al., 2010). However, the kinase(s) required for this phosphorylation and/or subsequent degradation has not been identified yet. We hypothesize that knock-down of a kinase required for Gliotactin downregulation will enhance Gliotactin overexpression phenotypes. When overexpressed Gliotactin spreads away from the TCJ causing
deleterious effects such as delamination paired with migration, apoptotic cell death, and extracellular proliferation leading to tissue overgrowth and ectopic folds (Padash-Barmchi et al., 2010). These phenotypes suggest that if Gliotactin is not tightly regulated, it has the potential to activate signaling pathway(s) that lead to these phenotypes. We hypothesize that the loss of pathway components that are activated in Gliotactin overexpressing background will suppress the Gliotactin overexpression phenotypes. As blocking endocytosis using a dominant negative form of Rab5 causes endogenous Gliotactin to spread away from the TCJs (Padash-Barmchi et al., 2010), we also hypothesize that blocking of kinases responsible for Gliotactin phosphorylation and/or endocytosis will lead to the spread of endogenous Gliotactin away from the TCJs.
Chapter 2: The tricellular junction protein Gliotactin overexpression induces Grindelwald to activate the TNF pathway

2.1 Synopsis

The formation and control of occluding junctions are critical to control solute and pathogen movement across a wide range of epithelia in both invertebrates and vertebrates. A key point in the formation of these barriers is the presence of the tricellular junction (TCJ) formed at the convergence of the bicellular junctions of three epithelial cells. TCJ proteins are uniquely localized at the corners of epithelial cells, yet little is known about the mechanisms that regulate this. Gliotactin is a vital component of the Drosophila TCJ and is tightly regulated by tyrosine phosphorylation paired with endocytosis to ensure the correct localization to the TCJ. When overexpressed, Gliotactin spreads away from the TCJ, and this spread triggers a number of deleterious phenotypes including overproliferation, apoptosis and migration of delaminated epithelial cells. These effects are also dependent on tyrosine phosphorylation. However, little is known about which kinases are involved in the control of Gliotactin or other TCJ proteins and to address this question; we carried out an RNAi screen of the Drosophila kinome and some kinase-related genes. From our screen, we identified many components of the TNF-alpha receptor/JNK pathway, including the TNF receptor Grindelwald (Grnd). Our results also identified components of the lipid kinase pathways, including PI4IIIK-alpha, PI3K and the downstream effectors Akt and Btk29A. This study provides the groundwork for further investigation of kinases that can control the regulation and potential signaling of Gliotactin and other potential tricellular junction proteins.
2.2 Introduction

Septate junctions (SJs) in invertebrate epithelia and tight junctions (TJs) in vertebrate epithelia are occluding junctions that form physical barriers to regulate paracellular flow (Auld et al., 1995; Knust and Bossinger, 2002; Genova and Fehon, 2003; Paul et al., 2003; Tyler, 2003). These junctions are also responsible for maintenance of cell polarity (Tepass et al., 2001, Nakajima et al., 2013) and epithelial architecture (Nakajima et al., 2013). In additions to these canonical functions, these junctions were later identified as signaling hubs that regulate the junctions themselves as well as various other inter- and intracellular events (Matter and Balda, 2003; Papagiannouli and Mechler, 2010; Fairchild et al., 2016; Zihni et al., 2016). Tricellular junctions (TCJs) are subdomains of occluding junctions, and these are formed at the convergence of bicellular occluding junctions from three neighboring cells. Gliotactin (Schulte et al., 2003) and Bark-beetle (Bark) /Anakonda (Byri et al., 2015; Hildebrandt et al., 2015) are transmembrane proteins that are unique to tricellular junctions in Drosophila. Similarly, Tricellulin, a tetramembrane-spanning protein similar to Occludin and MarvelD3, and the Angulin family members, lipolysis-stimulated lipoprotein receptor (LSR), immunoglobulin-like domain-containing receptor (ILDR1) and ILDR2 are tricellular TJ specific proteins (Ikenouchi et al., 2005; Masuda et al., 2011; Higashi et al., 2013). Beyond the formation of an occluding barrier, evidence points to important cellular roles for TCJ proteins. Gliotactin is required for the correct orientation of cell division of epithelial tissues (Bosveld et al., 2016) and the loss of Gliotactin from the differentiated midgut epithelia leads to activation of a JNK pathway increasing the proliferation of stem cells (Resnik-Docampo et al., 2016). However, very little is known about the regulation and restriction of these proteins to the tricellular corners or about the signaling associated with the TCJ.
Restricted localization of TCJ proteins seems to be critically important in both invertebrates and vertebrates. For instance, Tricellulin is upregulated in adenocarcinoma in human pancreatic ducts (Kojima and Sawada, 2012). Tricellulin localization and levels show distinct changes in relation to the differentiation levels in pancreatic cancer where Tricellulin interacts with MAPK and PKC pathways, changing the degree of cancer progression and metastasis (Takasawa et al., 2016). Tricellulin is also linked to the development of some forms of hepatocellular carcinoma (Somorácz et al., 2014). Furthermore, misregulation of the other vertebrate TCJ protein LSR (lipolysis stimulated lipoprotein receptor) also results in endometrial carcinoma (Shimada et al., 2016). In Drosophila, overexpression of Gliotactin in epithelia triggers deleterious phenotypes including apoptosis, overproliferation, and migration of delaminated cells (Padash-Barmchi et al., 2010). Gliotactin levels are tightly controlled within epithelia both at the level of mRNA control by microRNA (SharifKhodaei et al., 2016) and protein endocytosis and degradation (Padash-Barmchi et al., 2010). Phosphorylation of two highly conserved tyrosine residues in the cytosolic domain of Gliotactin leads to endocytosis and degradation of Gliotactin. Blocking endocytosis leads to the spread of Gliotactin away from the TCJ (Padash-Barmchi et al., 2010). However, tyrosine phosphorylation itself is responsible for the activation of signaling pathway(s) that generate the deleterious phenotypes observed with excess Gliotactin. This has led to a model where tyrosine phosphorylation by one kinase controls endocytosis and phosphorylation triggering the deleterious phenotypes is controlled by a different kinase (Padash-Barmchi et al., 2013). Similarly, serine phosphorylation of Discs large (Dlg), a MAGUK, PDZ protein that localizes with Gliotactin at the TCJ, is also key to the deleterious phenotypes (Padash-Barmchi et al., 2013). Yet the identity of these kinases(s) and those that control TCJ proteins in general are unknown.
To identify the kinases involved in phosphorylation of Gliotactin, we carried out a modifier screen using RNAi lines to the *Drosophila* kinome and tested for the effect on both overexpressed Gliotactin and endogenous Gliotactin at normal physiological levels. We identified multiple components of the TNF/JNK pathway, including *Drosophila* Tak1, Tab2 Traf6 and the TNF receptor, Grindelwald (Grnd), that when downregulated completely suppressed the overexpression phenotypes of Gliotactin. A lipid kinase pathway was also identified, including PI3K and the downstream effectors Bruton’s tyrosine kinase at 29A (Btk29A) and Akt. Finally, the receptor tyrosine kinase, Ret, was identified and we observed that activated forms of two *Drosophila* Ret constructs, analogous to human Ret^{MEN2A} and Ret^{MEN2B}, have differential effects. Given that more than 90% of human cancers are epithelial in origin and some of those are directly due to misregulation of occluding junction proteins (McCaffrey and Macara, 2011; Martin, 2014; Leech et al., 2015; Shimada et al., 2016) our results demonstrate the usefulness of Gliotactin overexpression as a model for understanding the key kinases that regulate TCJ protein distribution and signaling within epithelia. The implications of this study are discussed in detail in Chapter 4.

2.3 Materials and methods

2.3.1 Fly stocks

*Drosophila* fly Stocks were either obtained from the Bloomington *Drosophila* Stock Center (BL/BDSC), the Vienna *Drosophila* RNAi Center (VDRC), Kyoto stock Center (K/DGRC) or from researchers who made them. *w^{1118}* (BDSC) was the control. *apterous–GAL4* (BDSC) was the GAL4 driver. The UAS transgenes used in this study include: Gli-WT (Padash-Barmchi et al., 2010), NLS-GFP (a gift from Dr. Douglas Allan), Bsk-DN (Weber et al., 2000),
Tak1-DN (kinase dead) (Takatsu et al., 2000), Tak1-WT (Tak1-WT#3) (Takatsu et al., 2000), activatedPI3K (25908 BDSC), Btk29A (109095 DGRC) (Tsikala et al., 2014), Hipk-DN (kinase dead) (Choi et al., 2005), RetMEN2A and RetMEN2B (Dar et al., 2012). UAS driven RNAi lines used in the primary screen from VDRC and BDSC are listed in the Appendix 1. Other RNAi, transgenes and/or mutants screened to search for upstream of Tak1 are given in the Table 2.2.

All crosses except experiments that involved Taki-WT overexpression were carried out at 29°C under standard conditions using virgin females from the parental stock: ap-GAL4, GAL80ts; UAS-Gli-WT#5/SM-TM6, Tb. Since the Tak1-WT overexpression was lethal, the crosses were kept at 18°C and transferred to 29°C for 24 hours at the third instar larval stage.

2.3.2 Immunofluorescence labeling

Third instar wing imaginal discs were dissected and immunolabeled using standard protocols: larvae were dissected in 1XPBS and fixed with 4% paraformaldehyde for 20 mins. All subsequent washes were in PBS plus 0.1% Triton X-100 (PBST) and discs were blocked in PBST with BSA (0.5%) prior to primary antibody incubation overnight at 4°C, secondary antibodies were incubated for 2 hours at room temperature. Discs were cleared in 70% glycerol overnight and mounted with Vectashield (Invitrogen). Primary antibodies were used at following dilutions: mouse anti-Gli IF6.3 at 1:100 (Auld et al., 1995), rabbit anti-Gli at 1:600 (the Gliotactin polyclonal antibody was generated by immunizing rabbits with a peptide corresponding to the amino acid sequence (CQPAAQPRRTHLVEGVPQTS) and subsequent affinity purification, both by YenZym Antibodies, LLC), mouse anti-Dlg 4F3 at 1:100 (DSHB) (Parnas et al., 2001), rat anti-DE-Cadherin at 3:100 (DSHB), rabbit anti-Nrv2 (1/300), rabbit anti-pRet (1/25), rabbit anti-phosphoHistone3 (1/600) (Novus Biologicals, Ca). DAPI was used at
Secondary antibodies were used at 1:300: goat anti rabbit Alexa488, goat anti-rabbit Alexa568, goat anti rabbit Alexa647, goat anti mouse Alexa488, goat anti-mouse Alexa568, goat anti-mouse Alexa647, goat anti-rat Alexa568, goat anti-rat Alexa647, goat anti-guinea pig Alexa488 (Invitrogen).

### 2.3.3 Imaging

For higher magnification images, z-series stacks were collected with 0.2 μm steps using DeltaVision Spectris microscope (Applied Precision, GE) with a 60x (1.4 NA) oil immersion lens and CoolSnap HQ digital camera. Lower magnification images were collected with a 20X air lens. Deconvolution was done with SoftWorx (Applied Precision) software with 6-10 iterations using a point-spread function (PSF) calculated with 0.2 μm beads conjugated with Alexa Fluor 568 (Molecular Probes) mounted in Vectashield. Image processing and side projections were done using SoftWorx. Figures were made using Adobe Photoshop 4 or CC 2017. Zeiss Axioskop (at 20x objective) and Northern Eclipse software were used for images of whole wing discs.

### 2.3.4 Statistical analysis

The statistical analysis and graphing were done using Prism 6.0. One Way ANOVA was done to perform pairwise comparisons among multiple genotypes. Tukey post-hoc was done to determine significance. Students T-test was done when only two genotypes were to be compared. Cell migration: Using Image J (http://imagej.nih.gov/ij/), the migrated distance of Gliotactin positive cells from the apterous boundary to the wild type side was measured for each disc and expressed as a ratio to the total distance from the apterous boundary to the tip of the wild type
side of the wing disc. To measure each distance, the line tool and the Analyze/Measure option in ImageJ were used. For all panels, the dorsal/ventral apterous side was identified based on immunolabeling of the overexpressed Gliotactin at the level of the septate junction domain as the epithelium/boundary is intact at this level.

Immunolabeling intensity: Using ImageJ, intensities of immunolabeling (mean gray values) were measured in two constant size boxes drawn on the apterous and wild type side of the pouch area of wing disc and the ratios of the apterous/wild type were calculated.

Cell proliferation: In each disc, the number of phosphohistone3 (pH3) positive cells were counted manually in constant size boxes drawn on the dorsal and ventral compartments of the wing pouch area.

2.4 Results

2.4.1 Screen to identify enhancers and suppressors of the Gliotactin phenotypes

In the intracellular domain, Gliotactin contains two highly conserved tyrosines, for which a clear function in endocytosis and signaling has been identified, and four highly conserved serine residues for which less is understood. The kinases involved in phosphorylation of Gliotactin in vivo are not known and neither is the signaling pathway(s) involved in generating the detrimental phenotypes associated with Gliotactin overexpression. Therefore, we carried out a modifier screen using RNAi mediated knock-down of the Drosophila kinome to determine the effect of each kinase on overexpressed Gliotactin. To screen for those kinases involved, we used the wing imaginal disc and the apterous-GAL4 driver to overexpress Gliotactin (UAS-Gli-WT) in the dorsal side of the disc (Fig. 2.1B; Fig. 2.2 A and A”). The migration of Gliotactin overexpressing cells can be visualized as a cloud of cells that crosses from the apterous side into
the wild type side along the basal side of the disc columnar epithelia (Fig. 2.1B; Fig. 2.2 A, A”). In this context, the wing disc epithelium contains extra folds due to the overproliferation of cells in the Gliotactin overexpressing/apterous side (star, Fig. 2.1B). The migration distance can be used to quantify the severity of the phenotype, as the migration distance is proportional to the degree of regulation of Gliotactin protein levels, including endocytosis and phosphorylation (Padash Barmchi et al., 2010; Padash Barmchi et al., 2013; SharifKhodaei et al., 2016). For each RNAi line, we tested for enhancement or suppression of the three main phenotypes: migration of Gliotactin expressing cells into the wild type side, for the degree of extra tissue folds or tumor-like growth, and overall size of the wing imaginal disc. The Drosophila kinome has about 32 tyrosine kinases and over 250 serine/threonine kinases (Morrison et al., 2000; Read et al., 2013; Swarup et al., 2015). We screened 275 RNAi lines that covered 164 kinases and kinase-associated proteins in Drosophila (Appendix 1). Overall, we screened RNAi lines for all the known tyrosine kinases and approximately half of the serine/threonine kinases. Amongst these 100 genes were tested with at least two independent RNAi lines (Appendix 1). From our initial screen, four suppressors, four partial suppressors and 53 enhancers of the Gliotactin phenotypes were identified (Table 2.1). Of those lines that suppressed, we observed RNAi lines that completely suppressed all the phenotypes (Table 2.1) including RNAi to Tak1 (TGF-β activated kinase 1) (Fig. 2.1C), Akt and Btk29A. Some lines were partial suppressors as they did not suppress all the phenotypes (Table 2.1) including the RNAi to Hipk (Homeodomain interacting protein kinase) (Fig. 2.1D), which suppressed the cell migration phenotype but not the overproliferation. When a dominant negative form of Hipk (Hipk-DN) was co-expressed with Gli-WT the cell migration phenotype was further suppressed. However, the extra folds were still observed (Fig. 2.2B). RNAi lines that enhanced the Gliotactin phenotypes were more common
(Table 2.1) and included Csk (C-terminal Src kinase) (Fig. 2.1H), Ret (REarranged during Transformation) (Fig. 2.1E), rolled (Drosophila ERK) (Fig. 2.1G) and PI4KIII-α (Fig. 2.1F). In particular, for those RNAi lines that enhanced the Gliotactin phenotypes, the phenotypes could be due to a direct effect on Gliotactin or due to additive indirect effects (i.e., deleterious Gliotactin phenotypes coupled with deleterious effect caused by knockdown of a protein). Therefore, a subset of suppressors and enhancers were further studied through a secondary screen to confirm these results and tested whether each kinase plays a direct role in the regulation of endogenous Gliotactin. Only four enhancers (Csk-RNAi, PI4KIII-α-RNAi, Skittle-RNAi and Pkaap-RNAi) out of the twenty lines tested in the secondary screen showed effects on endogenous Gliotactin suggesting most of the enhancers were more likely due to additive effects (Table 2.1). From our screen, we then tested for the effects of a subset of suppressors and enhancers including Ret, and components of the TNF-pathway and the lipid kinase pathways.
Table 2.1: Modifiers of the Gliotactin overexpression phenotypes identified from the primary screen and the effect of selected lines on endogenous Gliotactin.

<table>
<thead>
<tr>
<th>Gene name (abbreviation)</th>
<th>Full Gene name</th>
<th>stock number(s) or reference</th>
<th>Phenotype in Gli-WT overexpressing background</th>
<th>Phenotypic class*</th>
<th>Effect on endogenous Gliotactin</th>
</tr>
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<tbody>
<tr>
<td><strong>Non-Receptor tyrosine kinases</strong></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Ack</td>
<td>Activated Cdc42 kinase-like</td>
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<td>Btk29A</td>
<td>Btk family kinase at 29A</td>
<td>VDRC: 22675/106962</td>
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<td>Csk</td>
<td>C-terminal Src Kinase</td>
<td>VDRC: 32877/109813 BL: 35174</td>
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<td>6</td>
<td>spread away from TCJ</td>
</tr>
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<td>Fps85D</td>
<td>Fps oncogene analog</td>
<td>VDRC: 36053/107266</td>
<td>enhanced</td>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td>Wee</td>
<td>Wee 1 kinase</td>
<td>VDRC: 26543/106329</td>
<td>enhanced</td>
<td>6</td>
<td>not affected</td>
</tr>
<tr>
<td><strong>Receptor tyrosine kinases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cad96Ca</td>
<td>Cadherin 96Ca</td>
<td>VDRC: 1091</td>
<td>enhanced</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td>dnt</td>
<td>doughnut on 2</td>
<td>VDRC: 27057/106056</td>
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<td>3</td>
<td>not affected</td>
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<td>Egfr</td>
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<td>Eph receptor tyrosine kinase</td>
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<td>Pvr</td>
<td>PDGF- and VEGF-receptor related</td>
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<td>Ret</td>
<td>Ret oncogene</td>
<td>VDRC: 30832/107648</td>
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<td>not affected</td>
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<td>Gene name (abbreviation)</td>
<td>Full Gene name</td>
<td>stock number(s) or reference</td>
<td>Phenotype in Gli-WT overexpressing background</td>
<td>Phenotypic class*</td>
<td>Effect on endogenous Gliotactin</td>
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<tr>
<td>Ror</td>
<td>Ror</td>
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<td>ND</td>
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<tr>
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<td>sax</td>
<td>Saxophone</td>
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<tr>
<td>Tak1</td>
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<td>VDRC: 101357/Bl: 35180/33404</td>
<td>suppressed</td>
<td>1</td>
<td>not affected</td>
</tr>
<tr>
<td>tor</td>
<td>torso</td>
<td>BL: 33627</td>
<td>enhanced</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td>Wnd</td>
<td>wallenda</td>
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<td>ND</td>
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<td>Akt</td>
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<td>ND</td>
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<td>Cdc2c</td>
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<td>enhanced</td>
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<td>cert</td>
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<tr>
<td>Doa</td>
<td>Darkener of apricot</td>
<td>VDRC: 20120/102520</td>
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<td>Erk 7</td>
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<td>fu</td>
<td>Fused</td>
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<tr>
<td>Gene name (abbreviation)</td>
<td>Full Gene name</td>
<td>stock number(s) or reference</td>
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<td>Phenotypic class*</td>
<td>Effect on endogenous Gliotactin</td>
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<td>GCKiii</td>
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<td>mos</td>
<td>Mos oncogene</td>
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<td>Protein kinase N</td>
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<td>ninaC</td>
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<td>VDRC: 110702</td>
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<td>enhanced</td>
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<td>VDRC: 37570</td>
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<td>Full Gene name</td>
<td>stock number(s) or reference</td>
<td>Phenotype in Gli-WT overexpressing background</td>
<td>Phenotypic class*</td>
<td>Effect on endogenous Gliotactin</td>
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<td>----------------</td>
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<td>-----------------------------------------------</td>
<td>-------------------</td>
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<tr>
<td>PI3K21B</td>
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<td>VDRC: 33556</td>
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<td>ND</td>
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<tr>
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<td>PI4KIlla</td>
<td>VDRC: 105614</td>
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<td>7</td>
<td>spread away and baso-laterally</td>
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<tr>
<td>Sktl</td>
<td>skittles</td>
<td>VDRC: 6229</td>
<td>enhanced</td>
<td>7</td>
<td>spread away from TCJ</td>
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</table>

*Characteristics of each phenotypic class:  
1- all the Gliotactin phenotypes were suppressed,  
2- cell migration was suppressed, but overproliferation or smaller discs were observed,  
3- discs with tumor-like growths and enhanced cell migration,  
4- the periphery of the wing pouch area overproliferated relative to the center,  
5- many extra folds were prominent compared to other Gliotactin phenotypes,  
6- smaller discs with enhanced cell migration,  
7- enhanced proliferation giving extra parts on wing discs.  
Stock numbers are given for the RNAi lines from the VDRC or Bloomington (BL) stock centers. References are given when the dominant negative or mutants were used.  
*The Pek phenotype were not consistent across individual RNAi lines. The effect on endogenous Gliotactin was tested only with some of the modifiers. ND stands for not determined.
Figure 2.1: Screen to identify enhancers and suppressors of the Gliotactin phenotypes.
Third-instar wing imaginal discs with Gliotactin-WT and kinome RNAi lines driven by apterous-GAL4 (ap>), immunolabeled for Gliotactin (Gli) and labeled for DAPI. The dashed lines mark the apterous boundary between the dorsal and ventral compartments with the apterous side (dorsal compartment) on the top. Arrows indicate the leading edge of the migratory cells. Scale bars: 30 µm. For each genotype n=10 discs.

A: Morphology of a control wing disc (w1118).

B: Gliotactin overexpression (ap>Gli-WT) led to distinct phenotypes including migration of Gliotactin overexpressing cells into the wild type side of the disc (arrow, B’) and extra folds (star, B) due to increased proliferation on the apterous side.

C: Suppression of Gliotactin overexpression phenotypes by co-expression of TakK1-RNAi. Cell migration to the wild type side was suppressed (C’) and no extra folds were formed (C and C’).

D: Partial suppression of Gliotactin overexpression phenotypes by co-expression of Hipk-RNAi. Cell migration to the wild type side was suppressed (D’), extra folds were still formed (D and |D’).

E: Enhancement of Gliotactin overexpression phenotypes by co-expression of Ret-RNAi. Cell migration was enhanced (arrow) and the apterous side showed tumor-like growth (star) (E’).

F: Enhancement of Gliotactin overexpression phenotypes by co-expression of PI4KIIIα-RNAi. The apterous side showed tumor-like growth (star), extra folds and extra tissue (blue arrows) (F and F’). Cell migration was present but was not prominent (yellow arrow) (F’).

G: Enhancement of Gliotactin overexpression phenotypes by co-expression of rolled-RNAi. Cell migration was enhanced (arrow) and the apterous side showed overproliferation mostly at the periphery of the pouch area (stars) relative to the center (G’).

H: Enhancement of Gliotactin overexpression phenotypes by co-expression of Csk-RNAi. Cell migration was enhanced (arrow) and the apterous side was smaller (H’).
Figure 2.2: Apterous expression domain, migration of Gliotactin overexpressing cells and suppression of cell migration by Hipk-DN.

A: A schematic of *Drosophila* wing imaginal disc. The apterous expression area is shown in green and the boundary between dorsal (apterous) and ventral (wild type) compartments is marked by the yellow line. The side-view below shows the apical squamous cell layer and basal columnar epithelial cell layer of the wing disc.

A’: A schematic showing the migration of Gliotactin overexpressing cells to the wild type side. Migration distances were measured for cells from the apterous boundary into the wild type/ventral compartment (red arrow) compared to the total distance across the ventral compartment from the apterous boundary to the distal edge of the disc (black arrow).

B: further suppression of migration of Gliotactin overexpressing cells by co-expression of Hipk-DN. Cell migration to the wild type side was suppressed, extra folds were still formed.
2.4.2 Overexpression of Ret mutants differentially affect the migration of Gliotactin overexpressing cells

Ret is a receptor tyrosine kinase with proto-oncogenic properties (Phay and Shah, 2010). Overexpression, rearrangement or point mutations of Ret are known to activate oncogenic signaling in both human and flies (Abrescia et al., 2005; Read et al., 2005). Drosophila has one Ret gene, which produces two splice variants. Knock-down of Ret kinase (Ret-RNAi) (V107648) when Gliotactin is overexpressed (ap>Gli-WT, Ret-RNAi) enhanced the deleterious phenotypes caused by excess Gliotactin (Fig. 2.1E,2A). Specifically, the cell migration and overproliferation were enhanced leading to tumor-like growth in the wing disc (Fig. 2.1E; 2.3A). Specificity was confirmed with a second independent Ret-RNAi line (V30832) (Table 2.1). As the downregulation of Ret was detrimental, we next determined whether the co-expression of Ret can suppress the Gliotactin phenotypes. We used transgenes expressing two Ret isoforms, RetMEN2A and RetMEN2B, and each was independently coexpressed with Gliotactin (Gli-WT) (ap>Gli-WT, RetMEN2A; ap>Gli-WT, RetMEN2B) (Fig. 2.3B,D). These Ret transgenes are analogous to two Ret point mutations found in multiple endocrine neoplasia type 2A (MEN2A) and 2B (MEN2B) in human (Read et al., 2005). RetMEN2A is a mutation in the extracellular domain that leads to a constitutively dimerized (activated) form and RetMEN2B is a mutation in the kinase domain that leads to constitutive kinase activity (Read et al., 2005). We found that RetMEN2A but not RetMEN2B completely blocked the cell migration phenotype of Gliotactin overexpressing cells (arrow, Fig. 2.3B’). None of the transgenes were capable of suppressing the overproliferation phenotypes (Fig. 2.3B, D). This was not unexpected given that Ret is an oncogene (Sonoshita and Cagan, 2016) and as each transgene was capable of stimulating cell overproliferation when expressed alone (Fig. 2.3C, E”). Suppression of the Gliotactin cell
migration phenotype by only one transgene suggests that Ret\textsuperscript{MEN2A} and Ret\textsuperscript{MEN2B} may interact with different pathways. Immunostaining with antibodies to phosphorylated Ret identified an accumulation of pRet at the level of adherens junction with strong localization at the tricellular corners (Fig. 2.4A”, B”). We next tested the effect of Ret-RNAi on endogenous Gliotactin (\(ap > Ret\)-RNAi) and found that knock-down of Ret had no effect on the localization of Gliotactin to the TCJ or within the apical/basal domain (Fig. 2.4D, E). While Ret-RNAi expression with Gli-WT enhanced the Gliotactin phenotypes (\(ap > Gli\)-WT, Ret-RNAi), alone Ret-RNAi did not lead to cell migration or tumor-like growth (Fig. 2.4C). Overall, our results suggest that Ret may negatively regulate a signaling pathway involved in generating the Gliotactin overexpression phenotypes.
Figure 2.3: Ret mutants differentially affect the migration of Gliotactin overexpressing cells.

Third-instar wing imaginal discs with transgenes or RNAi lines expressed by the apterous-GAL4 (ap>) driver and immunolabeled for Gliotactin, Dlg and DAPI. The dashed lines marked the
apterous boundary between the dorsal and ventral compartments with the apterous side (dorsal compartment) on the top or on the left. Arrows indicate the leading edge of the migratory cells. Scale bars: 30µm. Each genotype n≥8 discs.

A: Enhancement of Gliotactin overexpression phenotypes by co-expression of Ret-RNAi. Cell migration was enhanced (arrow) (A’) and the apterous side had tumor-like growths (star)(A’’’)

B: Partial suppression of Gliotactin overexpression phenotypes by co-expression of Ret\textsuperscript{MEN2A}. Cell migration to the wild type side was completely blocked (arrow) (B’), extra folds were still formed (stars) (B-B’’’).

C: Overexpression of Ret\textsuperscript{MEN2A} (ap > Ret\textsuperscript{MEN2A}) alone led to extra folds (stars) on the apterous side (C- C’’’).

D: Co-expression of Ret\textsuperscript{MEN2B} did not suppress the cell migration phenotype induced by overexpressed Gliotactin (arrow) (D’), extra folds were still formed (stars) (D’-D’’’).

E: Overexpression of Ret\textsuperscript{MEN2B} (ap > Ret\textsuperscript{MEN2B}) alone did not lead to cell migration, however extra folds were observed (stars) on the apterous side (E” and E’’’).
Figure 2.4: Phospho-Ret distribution and lack of an effect on endogenous Gliotactin by Ret knock-down.
A-B: High-magnification images (60X) from the wing pouch from wild type discs from 3\textsuperscript{rd} instar larvae, immunolabeled for Ecad (Green), pRet (red) and DAPI or Gliotactin (blue). \textit{En face} panel represents a single Z slice taken at the adherens junction level. Scale bars: 15µm. n=10 discs.

A: Wild type disc with Ecad and pRet labeling all around the cell membrane (A’ and A’’). Ecad and pRet immunolabeling colocalize and concentrated at tricellular corners (A).

B: Corresponding side projections. pRet labeling was found throughout from basal to apical with the highest concentration at the adherens junctions (B’ and B’’). Gliotactin (Blue in B) was found mostly below the Ecad labeling and no colocalization with pRet.

C-E: Third-instar wing imaginal discs with NLS-GFP and Ret-RNAi expressed by the apterous-GAL4 (ap>) driver and immunolabeled for Gliotactin (Gli, green), Dlg (red) and NLS-GFP (blue). Apterous boundary is indicated with a dashed line with the apterous side on the top or left. Each panel represents a single Z slice at the septate junction level. Scale bars: (C 30µm) and (D-E 15µm). n=10 discs.

C: \textit{ap>NLS-GFP, Ret-RNAi} at low magnification (20x). There was no cell migration (arrow, C’’) or overproliferation in the Ret knocked-down apterous side compared to the non-apterous side.

D: \textit{ap>NLS-GFP, Ret-RNAi} at high magnification (60x). Gliotactin localization (D’) or the Dlg localization (D’’) was not affected.

E: Corresponding side projections.

\textbf{2.4.3 Tak1 and the TNF pathway regulates the Gliotactin overexpression phenotypes}

Based on our prior work, the simple spreading of Gliotactin around the cell membrane itself is not sufficient to trigger deleterious phenotypes; instead, the phosphorylation of Gliotactin at one or both conserved tyrosine residues is necessary to trigger these phenotypes (Padash-Barmchi et al., 2010). Further, we found that these phenotypes require the phosphorylation of Dlg at a conserved serine residue (Ser797) and that JNK is a key component of both the cell migration and overproliferation phenotypes (Padash-Barmchi et al., 2010; Padash-Barmchi et al., 2013). Tak1 (TGF-β activated kinase 1) RNAi was identified from the kinome screen as a strong suppressor of the Gliotactin-overexpression phenotypes (Fig. 2.1C). Tak1 is a known regulator of the JNK signaling pathway (Igaki and Miura, 2014) and thus we concentrated on Tak1 and other components in this pathway. Two independent RNAi lines to Tak1 gave the same suppressed phenotypes when expressed with Gliotactin (\textit{ap>Gli-WT, Tak1-RNAi}) confirming the specificity of the RNAi knock-down (Fig. 2.6A, B). We hypothesized if
the Tak1 was a component in the signaling pathway that was activated by Gli overexpression, then the co-expression of Tak1 with Gliotactin would enhance the phenotypes. To test this hypothesis, we coexpressed Tak1 (Tak1-WT) with Gliotactin (ap>Gli-WT, Tak1-WT). Since this combination was lethal, we drove coexpression by shifting expression to 29°C only for 24 hours at the third instar larval stage. Even with that short expression frame, we found that the phenotypes including cell migration and overproliferation were enhanced (Fig. 2.5C). We also observed overproliferation and pyknotic nuclei indicating cell proliferation and death in Tak1-WT overexpression alone (ap>Tak1-WT), but overexpression of Tak1-WT did not lead to cell migration (Fig. 2.5D). If Tak1 is required for the regulation of Gliotactin at the TCJ, the downregulation of Tak1 in otherwise wild type background should affect the normal distribution of Gliotactin at the TCJ. However, Tak1 knockdown (ap>Tak1-RNAi) did not have an effect on endogenous Gliotactin protein localization (Fig. 2.6E) suggesting that Tak1 is a component of the signaling pathway triggered by overexpression of Gliotactin.
Figure 2.5: Tak1 regulates the Gliotactin overexpression phenotypes.
Third-instar wing imaginal discs with transgenes or RNAi lines expressed by the apterous-GAL4 (ap>) driver and immunolabeled for Gliotactin, and Dlg with DAPI labeling. The dashed lines marked the apterous boundary between the dorsal and ventral compartments with the apterous side (dorsal compartment) on the left. Arrows indicate the leading edge of the migratory cells. Scale bars: 30µm. Each genotype n≥8 discs.
A: Gliotactin overexpression (ap>Gli-WT) led to migration of Gliotactin overexpressing cells into the wild type side of the disc (A’), and formation of extra folds (stars) in the apterous side (A-A’’’).
B: Suppression of Gliotactin overexpression phenotypes by co-expression of Tak1-DN. Cell migration to the wild type side was suppressed (B’), and no extra folds were formed (B-B’’’).
C: Enhancement of Gliotactin overexpression phenotypes by co-expression of TAK-WT. Cell migration was enhanced (arrow) and the apterous side showed many extra folds (star) (C-C”).

D: Overexpression of Tak1-WT (ap>Tak1-WT) itself led to some extra folds in the non-apterous side (stars) (D-D”).

Figure 2.6: Specificity, suppression of cell overproliferation, and lack of an effect on endogenous Gliotactin by loss of Tak1.

Third-instar wing imaginal discs with transgenes and RNAi lines driven by apterous-GAL4 (ap>). The dashed lines mark the apterous boundary between the dorsal and ventral compartments with the apterous side (dorsal compartment) on the top. Arrows indicate the leading edge of the migratory cells. Scale bars: 30µm in C and 15um in E For each genotype n=10 discs.
A-B: Suppression of Gliotactin overexpression phenotypes by co-expression of independent Tak1-RNAi lines. Immunolabeled for Gliotactin (Gli) and labeled for DAPI. Cell migration to the wild type side was suppressed (A’ and B’) and no extra folds were formed.

C: Suppression of Gliotactin overproliferation phenotype by co-expression of Tak1-DN. Immunolabeled for Gliotactin (Gli), pH3 (Red) and labeled for DAPI. The number of pH3 positive cells in the apterous side was not different from wild type side.

D: Statistical analysis of the cell proliferation in ap>Gli-WT, Tak1-DN. The mean number of pH3 positive cells in the apterous side and WT side are shown. n=10 discs. The difference was not significant. Error bars represent the standard deviation (SD).

E: ap>NLS-GFP, Tak1-DN at high magnification (60x). Gliotactin localization (E’) or the Dlg localization (E’”) was not affected.

Tak1 and its signaling partners have been well characterized in other genetic contexts (Fig. 2.7F) (Shim et al., 2005). We hypothesized that the loss of different components of this pathway should also suppress the overexpressed Gliotactin phenotypes. To identify and confirm the different components that function upstream of Tak1, we carried out a secondary RNAi screen by crossing a range of RNAi lines with Gli-WT (Table 2.2). Knock-down of either Tab2 (Tak1-associated binding protein 2) or Traf6 (TNF-receptor-associated factor 6) led to the same suppressed phenotypes as knock-down of Tak1 (Fig. 2.7B, D). Multiple pathways can activate Tak1 through either or both Tab2 and Traf6 (Fig. 2.7F) (Takaesu et al., 2000; Takaesu et al., 2001; Shim et al., 2005; Landström, 2010; Pérez-Garijo et al., 2013) and we tested a range of potential pathways.

Knock-down of the following proteins did not suppress the Gliotactin phenotypes: Baboon (TGF-beta Type II receptor), Tkv [Bone morphogenic protein (BMP) , Type II receptor], Toll, POSH (Plenty of SH3s), and ben (Bendless), an E2 ubiquitin-conjugating enzyme, which links the TNF pathway with TAK-JNK (Ma et al., 2014) (Table 2.2). Activation of Traf6, Tak1 and JNK pointed to the involvement of the TNF pathway (Igaki and Miura, 2014), thus we tested RNAi lines for Eiger and Wengen, the ligand and a receptor of the
## Table 2.2: Screen of candidates upstream of Tak1 kinase.

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<th>Gene name (abbreviation)</th>
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<td>BL: 9152/ (Kanda et al., 2002)</td>
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<tr>
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<td>Eiger</td>
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<td>not affected</td>
</tr>
<tr>
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<td>enhanced (5)</td>
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<td>Baboon</td>
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<tr>
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<tr>
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<td>BL: 43677 grnd[M105292]/ VDRC: 104538</td>
<td>suppressed</td>
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</table>

Enhanced 5: many extra folds were prominent over other Gliotactin-overexpression phenotypes.
Enhanced 6: smaller discs with enhanced cell migration.
Stock numbers are given for the RNAi lines obtained from VDRC or Bloomington (BL) stock centers, References (and stock numbers were appropriate) are given when the dominant negative or mutants were used.
Drosophila TNF pathway respectively (Kanda et al., 2002; Kauppila et al., 2003). Surprisingly, the knock-down of either (using multiple RNAis known to effectively knock-down each) did not suppress the phenotypes (Table 2.2). However, when we tested an RNAi line to a newly identified Drosophila TNF receptor, Grindelwald (Grnd) (Andersen et al., 2015), knock-down of Grnd suppressed the Gliotactin phenotypes (ap>Gli-WT, Grnd-RNAi) (Fig. 2.7A). Similarly, a grnd heterozygous mutant (grnd[MI05292]) was able to suppress (ap>Gli-WT; grnd[MI05292/+]) (Table 2.2; Fig. 2.8C, I, J). This suggests that when overexpressed, Gliotactin activates the TNF-JNK pathway via Grnd but not through Wengen or Eiger. However, the localization of endogenous Gliotactin was not affected by either Tak1 or Grnd knock-down suggesting that the TNF pathway is not involved in regulation of Gliotactin protein level or localization to the TCJ (Fig. 2.6E). Overall, our results suggest that if Gliotactin spreads beyond the TCJ, the deleterious effects involve Grnd and TNF-alpha signaling pathway which leads to JNK activation through Tak1 kinase, resulting in cell delamination, migration, cell death via apoptosis and, directly or indirectly overproliferation.
Figure 2.7: TNF pathway regulates the Gliotactin overexpression phenotypes.

Third-instar wing imaginal discs with transgenes or RNAi lines expressed by the apterous-GAL4 (ap>) driver and immunolabeled for Gliotactin and label for DAPI. The dashed lines marked the apterous boundary between the dorsal and ventral compartments with the apterous side (dorsal compartment) on the top. Arrows indicate the leading edge of the migratory cells. Each genotype n≥10 discs.

A-E: Downregulation of TNF pathway components in Gliotactin overexpression background suppressed the Gliotactin-induced cell migration and cell overproliferation. Suppression by co-expression of grnd-RNAi (A), TRAF6-RNAi (B), Tak1-DN (C), TAB2-RNAi (D), Bsk-DN (E).
F: Schematic of some pathways activated upstream of TAK that may lead to JNK induced apoptosis. The downregulation of components boxed in red led to suppression of Gliotactin overexpressing phenotypes.

2.4.4 Gliotactin overexpression phenotypes are sensitive to Tak1 and Grnd levels

To understand the mechanism involved in activation of the TNF-alpha pathway, we further investigated and quantified the cellular changes to Gliotactin and Dlg when TNF-JNK signaling is reduced. We used multiple approaches to reduce Tak1 signaling (Fig. 2.8B) including RNAi knock-down, Tak1 heterozygous null mutants (Tak1[179], Tak1[2]) (Vidal et al., 2001; West et al., 2015); a dominant negative form of Tak1 (UAS-Tak1-DN) (Takatsu et al., 2000) and a Tak1 transgene lacking the kinase domain (UAS-Tak1-CT-HA) (Stronach et al., 2014) (Fig. 2.8A). In all cases, a reduction or loss of Tak1 suppressed the Gliotactin overexpression phenotypes (Fig. 2.8B, G, H). In all discs examined for all the different modes of Tak1 loss of function, cell migration was completely blocked. The distance the Gliotactin expressing cells migrate from the apterous boundary into the wild type side of the disc was measured and compared to the distance from the apterous boundary to the furthest edge of the wing discs (Fig. 2.8B). This ratio is normally 0.5 (50% of the ventral half) for Gli-WT alone (Fig. 2.8B). The overproliferation phenotype was also suppressed by loss of Tak1 and this was quantified using immunolabeling for phospho-Histone 3 to measure mitosis on the apterous side versus the wild type side of the wing disc (Fig. 2.6C, D). These results suggest that the Gliotactin phenotypes are highly sensitive to changes to Tak1.

Gliotactin overexpression is also accompanied by an increase in large intracellular vesicles previously identified as multi-vesicular bodies and lysosomes (Padash Barmchi et al., 2010) (Fig. 2.8E’, E”). At higher magnification, we observed that large vesicles positive for Gliotactin were
increased in prevalence when Tak1 was downregulated (ap>Gli-WT, Tak1-DN) (Fig. 2.8G’, G’’’) compared to the Gli-WT overexpression alone (ap>Gli-WT) (Fig. 2.8E’, E’’’). These results further suggest that Tak1 is not a part of the phosphorylation pathway necessary for Gliotactin endocytosis as the loss of Tak1 would then lead to a decrease in endocytosis. Previously we found that changes in the levels and the phosphorylation state of Dlg, when Gliotactin is overexpressed, have profound effects on the Gliotactin overexpression phenotypes and normally Gliotactin overexpression leads to a reduction of Dlg at the membrane (Padash Barmchi et al. 2013). When we tested the level of Dlg downregulation, we found that loss of Tak1 along with Gliotactin (ap>Gli-WT, Tak1-RNAi) overexpression had the same degree of Dlg reduction as that observed with Gli-WT overexpression alone (Fig. 2.8D). Therefore, the suppression of the Gliotactin phenotypes by Tak1 knock-down was not due to changes to Dlg. Overall, our data suggest that Tak1 is unlikely to directly phosphorylate Gliotactin or Dlg to affect protein distribution and trafficking, and it is more likely a signaling component activated downstream of Gliotactin and Dlg phosphorylation events.

Knock-down of the TNF-alpha receptor Grnd or the loss of function mutant, grnd[MI05292]/+, suppressed the Gliotactin overexpression phenotypes including a decrease in the cell migration ratio (Fig. 2.7A; Fig. 2.8C, I, J). Of note, the reduction of Grnd did not lead to a complete suppression of cell migration unlike the loss of Tak1 showing that Gliotactin phenotypes were less sensitive to Grnd protein levels or that the Grnd mutant is weaker. However, similar to Tak1 knock-down, the large Gliotactin positive vesicles were prevalent (Fig. 2.8I, I’’’). There was no difference in Dlg downregulation in ap>Gli-WT; grnd[MI05292]/+ compared to ap>Gli-WT alone (Fig. 2.8D). These data indicate that Grnd is not required for the endocytosis of Gliotactin and the suppression may not due to changes to Dlg. Overall, loss or
block of Grud, Tak1 or JNK [Drosophila Bsket (Bsk)] when Gliotactin is overexpressed leads to suppression of Gliotactin phenotypes with no change in the Dlg downregulation observed with Gli-WT overexpression (Fig. 2.8D; Fig. 2.9). These results suggest that the TNF-JNK pathway likely represents signaling components activated downstream of Gliotactin and Dlg. Interestingly, loss of function of any of these components leads to an increased prevalence of large Gliotactin positive vesicles compared to those in Gli-WT expression alone (Fig. 2.8E-J; Fig. 2.7A; Fig. 2.9A). These data suggest that the suppression of the Gliotactin-triggered signaling pathway somehow leads to an increase in endocytosis of Gliotactin.
Figure 2.8: Gliotactin overexpressing phenotypes are sensitive to changes in Tak1 and Grmd levels.
A: Schematic of the Tak1 protein showing the locations of mutations and size and nature of Tak1 transgenes used (Stronach et al., 2014).

B-D: Statistical analysis of Gliotactin phenotypes.

B: Quantification of the cell migration ratios with Gliotactin overexpressed alone (ap>Gli-WT), co-expressed with Tak1-RNAi, Tak1-DN, Tak1-CT or overexpression in Tak1[179], or Tak1[2] heterozygous mutants. The mean cell migration ratio in ap>Gli-WT was 0.5 and in all Tak1-downregulated backgrounds, the mean cell migration ratio was 0.0, a significant reduction (**** P<0.0001). (n=10 discs for each, except n=15 in ap>Gli-WT). Bars represent the standard deviation (SD).

C: Quantification of the cell migration ratios with Gliotactin overexpressed alone (ap>Gli-WT), overexpressed in a grnd[MI05292]/+ heterozygous mutant or co-expressed with grnd-RNAi. All showed a significant difference in the cell migration ratios (**** P<0.0001) compared to ap>Gli-WT. The difference in cell migration ratios between (ap>Gli-WT, grnd[MI05292]/+) and (ap>Gli-WT, grnd-RNAi) was not significant (NS). (n=10 discs for each, except n=15 in ap>Gli-WT). Bars represent the standard deviation (SD).

D: Quantification of changes to Dlg immunolabeling. For each genotype, the mean ratios of Dlg intensities in the apterous side versus WT side are shown. n=10 discs. In all genotypes, the differences were not statistically significant (NS) from each other. Error bars represent the standard deviation (SD).

E-J: High-magnification images (60X) from the pouch region of third-instar wing imaginal discs with transgenes expressed using the apterous-GAL4 (ap>) driver. Discs were immunolabeled for Gliotactin (Gli, green), Dlg (red) and DAPI (blue). The dashed lines represent the apterous boundary with the dorsal (apterous) compartment on the left. Boxed regions were digitally magnified (28X) and shown on the right. Yellow arrows point to the internal vesicles. Each en face panel represents a single Z slice taken at the septate junction level. Scale bars: 15µm, except 5µm in digitally magnified panels. n=10 discs in each genotype.

E: ap>Gli-WT. On the apterous side, Gliotactin is found around the membrane and in internal vesicles. Not all the cells contained large vesicles (E’ and E’’). Dlg immunolabeling is shown in E’”.

F: Corresponding side projections showing basal migration of Gliotactin overexpressing cells to the non-apterous side (F’) and downregulation of Dlg immunolabeling in the apterous side (blue arrows, F’”).

G-J: Co-expression of Gli-WT with Tak1-DN (ap>Gli-WT, Tak1-DN) or grnd heterozygous mutant (ap>Gli-WT, grnd[MI05292]/+) suppressed Gliotactin phenotypes and showed that many cells were positive for large vesicles (G’, G’”, I’ and I’”). Dlg immunolabeling is shown in G” and I”. In each background, corresponding side projections showed suppression of cell migration of Gliotactin overexpressing cells to the non-apterous side (H’ and J’) and downregulation of Dlg immunolabeling in the apterous side (blue arrows, H’” and J’”).

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Figure 2.9: Suppression of Gliotactin overexpression phenotypes by Bsk-DN.

A-B: High-magnification images (60X) from the pouch region of third-instar wing imaginal discs with transgenes expressed using the aperous-GAL4 (ap>) driver. Discs were immunolabeled for Gliotactin (Gli, green), Dlg (red) and DAPI (blue). The dashed lines represent the aperous boundary with the dorsal (aperous) compartment on the left. Boxed regions were digitally magnified (28X) and shown on the right. Each en face panel represents a single Z slice taken at the septate junction level. Scale bars: 15µm, except 5µm in digitally magnified panels. n=10 discs in each genotype.

A: ap>Gli-WT, Bsk-DN. Co-expression of Bsk-DN suppressed Gliotactin phenotypes leading to an increase in cells with large vesicles (A’ and A”). Yellow arrows point to the internal vesicles. Dlg was downregulated in the aperous side (A”).

B: Corresponding side projections showing suppression of cell migration of Gliotactin overexpressing cells to the non-aperous side (B’) and downregulation of Dlg immunolabeling in the aperous side(B”). Blue arrows point to the delaminated cells enriched with overexpressed Gliotactin (B’), Dlg (B”) and DAPI (B”’) labeling.

2.4.5 Downregulation of JNK (Bsk) but not other TNF pathway components completely blocks apoptosis

To further characterize the changes in the Gliotactin-induced phenotypes when Grnd, Tak1 or JNK (Drosophila Bsk) was blocked or knocked-down, we compared the amount of apoptosis in these three backgrounds (Fig. 2.10). An analysis of the basal side of the wing imaginal disc identified many pyknotic nuclei in both the aperous and wild type sides of wing discs with Gliotactin overexpression (ap>Gli-WT) (Fig. 2.10A’). When either Tak1 (ap>Gli-WT, Tak1-RNAi) or Grnd (ap>Gli-WT, Grnd-RNAi) were knocked-down fewer pyknotic nuclei were
present, and these were limited to the apterous side (Fig. 2.10B’, C’). While cell migration is
suppressed in these two backgrounds compared to Gli-WT alone, there was still some degree of
apoptosis observed. In contrast, blocking JNK using a dominant negative form of *Drosophila*
JNK (Bsk-DN) generated very few or no pyknotic nuclei when Gliotactin was overexpressed
(*ap > Gli-WT, BskDN*) (Fig. 2.10D’) similar to the level observed in wild type wing imaginal
discs (Delanoue et al., 2004). Overall it appears that the Gliotactin-induced cell death is
completely blocked when JNK/Bsk function is blocked. The lower but consistent amount of cell
death when Tak1 or Grnd were knocked-down suggests that another pathway or pathways,
independent of Tak1 or Grnd, are induced by Gliotactin and signal through JNK. This suggests
that when Gliotactin is overexpressed JNK can be activated by multiple pathways.
Figure 2.10: Downregulation of JNK (Bsk) but no other TNF pathway components completely blocks apoptosis.
A-D: High-magnification images (60X) from the basal side of pouch region of third-instar wing imaginal discs with transgens expressed using the apterous-GAL4 (ap>) driver. Discs were immunolabeled for Gliotactin (Gli, green), Dlg (red) and DAPI (blue). The dashed lines represent the apterous boundary with the dorsal (apterous) compartment on the top or left. Yellow arrows point to the leading edge of cell migration and blue arrows to the pyknotic nuclei. Each en face panel represents a single Z slice taken at the basal side. Scale bars: 15µm. n=10 discs in each genotype.

A: Gliotactin overexpression (ap>Gli-WT) showed many pyknotic nuclei at the basal side of both apterous and wild type (ventral) compartments (A’). Dlg immunolabeling at the basal side (A’’).

B: Gliotactin overexpression in a grnd mutant (ap>Gli-WT, grnd[MI05292]/+) showed fewer numbers of pyknotic nuclei and only on the apterous side (B’). Dlg immunolabeling at the basal side (B’’).

C: Gliotactin co-expression with Tak1-DN (ap>Gli-WT, Tak1-DN) also showed fewer numbers of pyknotic nuclei and only on the apterous side (C’). Dlg immunolabeling at the basal side (C’’).

D: Gliotactin co-expression with Bsk-DN (ap>Gli-WT, Bsk-DN) occasionally had very few pyknotic nuclei and only at the apterous side (D’). Dlg immunolabeling at the basal side (D’’).

2.4.6 Components of the lipid kinase pathway have opposite effects on Gliotactin overexpression

Our screen identified that knock-down of PI3K, Akt or Btk29A in the Gliotactin overexpressing background can lead to suppression of the Gliotactin-induced phenotypes (Fig. 2.11C, D; Fig. 2.13A). In contrast, the knock-down of two upstream components in the PI3K pathway; PI4KIII- α or Skittles enhanced the phenotypes (Fig. 2.11A, B). We first focused on the suppression of phenotypes by downregulation of PI3K or Akt. A dominant negative transgene of PI3K (PI3K-DN) was effective at suppressing the Gliotactin induced cell migration (Fig. 2.12A). However, the discs were smaller than with the RNAi knock-down, a phenotype observed by others and linked to the role of PI3K in cell growth (Britton et al., 2002). Since the downregulation of PI3K suppressed the phenotypes, we upregulated PI3K levels by coexpressing activated PI3K with Gliotactin (ap>Gli-WT, activated-PI3K) and found that phenotypes were enhanced (Fig. 2.12B). Given that PI3K regulates phosphoinositol(3,4,5)P3 (PIP3) levels in the
membrane, the loss of PI3K would lead to an increase in PIP2 and a decrease in PIP3. If loss of PIP3 levels can suppress the Gliotactin overexpression, we hypothesized that loss of the PIP3 phosphatase PTEN would lead to increased levels of PIP3 and enhance the Gliotactin phenotypes. This is what we observed when PTEN-RNAi was expressed with Gli-WT (ap>Gli-WT, PTEN-RNAi) (Fig. 2.11E).

Akt is well known as a downstream component activated by PIP3 downstream of PI3K. Akt promotes cell proliferation and migration by directly or indirectly activating mTOR kinase (LoPiccolo et al., 2007). Therefore, suppression of Gliotactin phenotypes by both PI3K and Akt is consistent. However, the suppression of the apoptosis phenotypes triggered by Gliotactin, when Akt was knocked-down is not consistent as Akt is well known as an inhibitor of apoptosis (Lynch et al., 2016). Further, Akt is not known to activate JNK/Bsk directly (Lynch et al., 2016) suggesting that the PI3K and Akt signaling may function through another kinase. Overall, our data suggest that PI3K and Akt play a role in the Gliotactin overexpression phenotypes, parallel to the TNF-alpha pathway and the relationship between these two pathways in this context, remains to be identified.
Figure 2.11: Components of the lipid kinase pathway have opposite effects on Gliotactin overexpression.
Third-instar wing imaginal discs with co-expression of Gliotactin transgene (Gli-WT) with RNAi lines by the apterous-GAL4 (ap>) driver and immunolabeled for Gliotactin and labeled for DAPI. The dashed lines marked the apterous boundary between the dorsal and ventral compartments with the apterous side (dorsal compartment) on the top. Arrows indicate the leading edge of the migratory cells. For each genotype n≥10 discs).

A-B: Downregulation of some components in lipid kinase pathway in Gliotactin overexpression background enhanced Gliotactin induced cell overproliferation. Enhancement by co-expression of PI4KIIα-RNAi (A) and Sktl-RNAi (B). The apterous boundary was not clear enough to identify and mark.

C-D: Downregulation of some components in lipid kinase pathway altered the Gliotactin-induced phenotypes. Suppression by co-expression of PI3K-RNAi (C) and Akt-RNAi (D). Enhancement by co-expression of PTEN-RNAi (E).

F: Schematic showing some of the components involved in lipid metabolism and downstream lipid kinase pathway signaling. Phosphatases except PTEN are not shown (marked with orange arrow). A side pathway generated by PLC activity on PIP2 is shown with pink arrows.

**Figure 2.12: Effect of changing lipid kinases levels on Gliotactin overexpressing and wild type discs.**

A: Third-instar wing imaginal discs with transgenes and RNAi expressed by the apterous-GAL4 (ap>) driver and immunolabeled for Gliotactin (Gli, green), Dlg (red) and labeled for DAPI (blue). Apterous boundary is indicated with a dashed line with the apterous side on the top or left. Each panel represents a single Z slice. Scale bars: (A-B 30µm) and (C 15µm). n=10 discs.

A: ap>Gli-WT, PI3K-DN at low magnification (20x). Wing disc was small, did not produce extra folds and cell migration was suppressed (yellow arrow, A’).

B: ap>Gli-WT, PI3K-activated at low magnification (20x). large extra folds were formed (stars, B’) and cell migration was enhanced (yellow arrow, B’).
C: ap>NLS-GFP, PI4KIII-α-RNAi at high magnification (60x). Z stack was taken at the basal side. Blue arrows point to the delaminated cells with pyknotic nuclei (C’’’).

2.4.7 Knock-down of Btk29A suppresses Gliotactin overexpression phenotypes

The results of our screen implicated two signaling pathways, TNF/JNK and the PI3K/Akt pathway and thus the identification of Btk29A-RNAi as a strong suppressor of the Gliotactin phenotypes was of interest. For instance, PI3K kinase, through increased PIP3 levels, is known to act on Btk29A, which in turn can activate JNK (Kawakami et al., 1998; Tomlinson et al., 2001). Btk29A (Bruton’s tyrosine kinase at 29A) is a member of the Tec family of tyrosine kinases, which are implicated in multiple signaling pathways including the JNK and phospholipid PIP3 pathways (Qiu and Kung, 2000; Lindvall and Islam, 2002). Knock-down of Btk29A suppressed the migration of the Gliotactin expressing cells and the cell overproliferation phenotype (Fig. 2.13A, E). The co-expression of a Btk29A transgene (ap>Gli-WT, Btk29A-WT) enhanced the phenotypes suggesting that Btk29A is part of a signaling pathway that mediates Gliotactin phenotypes (Fig. 2.13B). To determine the potential interactions and mechanisms that link Btk29A and overexpressed Gliotactin, we made use of a line with Btk29A endogenously tagged with GFP (Btk29A::GFP). We observed in wild type wing discs, Btk29A is uniformly distributed throughout the disc (Fig. 2.13D) and localized mainly to the basal side (Fig. 2.14A, B). Interestingly, there was an upregulation of Btk29A in the Gliotactin overexpression background where Btk29A levels were elevated uniformly throughout the apterous side. Btk29A::GFP was also concentrated in the Gliotactin overexpressing delaminated and migrated cells in the ventral side of the disc (Fig. 2.13C’’; blue arrows, Fig. 2.14C’’). This was different compared to Btk29A localization in wild type discs (yellow arrows, Fig. 2.14A’’’, C’’). Further, cells strongly positive for Btk29A::GFP were found throughout the epithelia from the basal to the
apical side (blue arrows, Fig. 2.14D”). In wild type discs, the Btk29A::GFP did not show such a localization and found mainly towards the basal side (yellow arrows, Fig. 2.14B”). Our data suggest that increased levels of Gliotactin also lead to an upregulation or stabilization of Btk29A and that Btk29A plays a role in the Gliotactin-triggered signaling pathways.

**Figure 2.13: Knock-down of Btk29A suppresses the Gliotactin overexpression phenotypes.**

A-D: Third-instar wing imaginal discs with transgenes or RNAi lines expressed by the apterous-GAL4 (ap>) driver and labeled for DAPI and immunolabeled for Gliotactin and Dlg or expressing GFP. The dashed lines marked the apterous boundary between the dorsal and ventral compartments with the apterous side (dorsal compartment) on the top or left. Arrows indicate the leading edge of the migratory cells. Scale bars: 30µm. Each genotype n≥10 discs.
A: Co-expression of Gliotactin (Gli-WT) with Btk29A-RNAi led to suppression of migration of Gli overexpressing cells into the wild type side of the disc (A’) and downregulation of Dlg immunolabeling (A”).

B: Co-expression of Gliotactin (Gli-WT) with Btk29A-WT led to enhancement of migration of Gliotactin overexpressing cells into the wild type side of the disc (B’). The apterous side was smaller (B”). Downregulation of Dlg immunolabeling (B”).

C: Overexpression of Gliotactin (Gli-WT) in with Btk29A endogenously tagged with GFP (Btk29A::GFP). Migration of Gliotactin overexpressing cells to wild type side (C’). Btk29A::GFP showed an elevated level of fluorescence in the apterous side and in wild type side where migrated Gli overexpressing cells present (C”). GFP also showed an elevation in the wild type side immediately after the leading edge of cell migration (star) (C”).

D: Wild type wing disc showing Btk29A::GFP expression. Gliotactin immunolabeling (D’). Btk29A::GFP showed a uniform level of fluorescent intensity throughout the wing disc.

E: Quantification of the cell migration ratios with Gli overexpressed alone (ap>Gli-WT) and co-expressed with Btk29A-RNAi. In co-expression, the cell migration ratio was significantly reduced (**** P<0.0001) compared to ap>Gli-WT. (n=15 for ap>Gli-WT, n=10 for ap>Gli-WT; Btk29A•RNAi). Bars represent the standard deviation (SD).

Figure 2.14: Changes to Btk29A::GFP in Gliotactin overexpression.
High-magnification images (60X) from the pouch region of third-instar wing imaginal discs with Btk29A::GFP in wild type or in Gli-WT overexpression with the apterous-GAL4 (ap>) driver. Discs were immunolabeled for Gliotactin (Gli, green), and DAPI (blue). Yellow arrows point to the basal localization and normal pattern of Btk29A::GFP. Blue arrows point to the accumulated distribution of Btk29A::GFP. Each en face panel represents a single Z stack. Scale bars: 15µm. n=10 discs in each genotype.

A: Wild type disc with Btk29A::GFP. Puncta like Gliotactin labeling (A’) and presence of GFP throughout the cytoplasm. GFP was found highly concentrated at the basal side (A’’').

B: Corresponding side projections with Gliotactin at the septate junction (B’). GFP was found mostly in the basal side (B’’').

C: ap>Gli-WT, Btk29A::GFP. The single Z stack was taken from the basal side. Gliotactin overexpression led to accumulation of Btk29A::GFP in the cytoplasm (blue arrows) changing the wild type distribution pattern (yellow arrow) of the protein (C’’'). Migration of Gliotactin overexpressing cells (C’).

D: Corresponding side projections with overexpressed Gliotactin (D’). Btk29A::GFP was found throughout from basal to apical instead of basal accumulation (D’’'). Some cells were more concentrated with GFP compared to other cells (blue arrows).

2.4.8 Downregulation of PI4KIII-α affects overexpressed and endogenous Gliotactin

The downregulation of PI3K and Akt suppressed the Gliotactin overexpressing phenotypes indicating that phospholipids might have a signaling role in generating these phenotypes. However, RNAi to two upstream components, PI4KIII-α or Skittles (Phosphatidylinositol-4-phosphate 5-kinase) had the opposite results. Knock-down of either PI4KIII-α or Skittles enhanced the Gliotactin overexpression phenotypes (Fig. 2.1F; Fig. 2.11A, B; Fig. 2.15A). In particular, the cell overproliferation phenotype was strongly enhanced by knock-down of PI4KIII-α or Skittles. Overexpression of Gliotactin in a PI4KIII-α [3]/+ heterozygote mutant background (ap>Gli-WT, PI4KIII-α [3]/+) also enhanced the phenotypes confirming the specificity of the RNAi (Fig. 2.15B). To test the effect of downregulation of PI4KIII-α in otherwise wild type background, we expressed PI4KIII-α-RNAi alone (ap> PI4KIII-α-RNAi). Interestingly the knock-down of PI4KIII-α in otherwise wild type background did not trigger overproliferation to the same extent that expression with Gli-WT did (Fig. 2.15C, D). Only a few
extra folds were visible and only in some discs (n=2 out of 10 discs) (Fig. 2.15D). However, in every discs a large amount of cell death was evident in the apterous side where PI4KIII-α was knocked-down (Fig. 2.12C’’’).

The massive proliferation observed with PI4KIII-α knock-down in the presence of Gliotactin overexpression suggests that there is a genetic interaction between Gliotactin and PI4KIII-α. Therefore, we tested for changes to endogenous Gliotactin protein localization when PI4KIII-α was knocked-down in an otherwise wild type wing disc (ap > PI4KIII-α-RNAi).

Endogenous Gliotactin was upregulated and spread beyond the tricellular junction in many cells in the apterous side (Fig. 2.15E’, I) compared to non-apterous side (Fig. 2.15E’, I’). The side projections indicated that Gliotactin was mislocalized and spread along the basolateral cell membrane in the cells on the apterous side (arrow, Fig. 2.15F’). To determine if the effect was specific to Gliotactin, we immunolabeled other SJ and AJ proteins. Immunolabelling for Dlg and Nervana2 (Nrv2) indicated that those proteins also spread baso-laterally when PI4KIII-α was knockeddown (Fig. 2.15E”’), while Nrv-2 was reduced from the membrane (Fig. 2.15H’), suggesting a misregulation of the SJ domain. Immunolabeling for the adherens junction marker, Ecad (E-cadherin) determined that the adherens junctions were present (Fig. 2.15, G” and H”’ and suggested that only the SJ proteins were affected. Since the tricellular junctions and bicellular junctions are interdependent for their localization and functions (Schulte et al., 2003; Schulte et al., 2006; Padash-Barmchi et al., 2013), it is not clear whether the changes to Gliotactin were the reason for mislocalization of other septate junction proteins or vice versa. Overall, our results suggest that PI4KIII-α has a role in controlling the localization of tricellular junctional protein Gliotactin either directly or indirectly through changes to other SJ proteins.
Figure 2.15: Downregulation of PI4KIII-α affects both overexpressed and endogenous Gliotactin.

A-D: Third-instar wing imaginal discs with transgenes or RNAi lines expressed by the apterous-GAL4 (ap>) driver and immunolabeled for Gliotactin and labeled for DAPI. The dashed lines
marked the apterous boundary between the dorsal and ventral compartments with the apterous side (dorsal compartment) on the top. Arrows indicate the leading edge of the migratory cells. For each genotype n≥10 discs.

A: Enhancement of Gliotactin overexpression phenotypes by co-expression of PI4KIII-α-RNAi. The apterous side showed many extra folds and tumor-like growth.

B: Enhancement of Gliotactin overexpression phenotypes by overexpression Gli-WT in a PI4KIII-α [3]/+ mutant background. The apterous side showed many extra folds and tumor-like growth.

C-D: Expression of PI4KIII-α-RNAi in otherwise wild type background did not generate extra folds or tumor-like growth (C). Occasionally (2 out of 10) discs showed a few extra folds (C”).

E-H: High-magnification images (60X) from the pouch region of third-instar wing imaginal discs with ap>NLS-GFP, PI4KIII-α-RNAi expression. Discs were labeled for DAPI and immunolabeled for Gliotactin or Nrv2 (green), and Dlg or Ecad (red). The dashed lines represent the apterous boundary with the dorsal (apterous) compartment on the left. Boxed regions were digitally magnified (28X) and shown in I. Each en face panel represents a single Z slice taken at the septate junction level. Arrows in the side projections point to the basolateral spreading of proteins in the apterous side. Scale bars: 15µm, except 5µm in digitally magnified panels. n=10 discs in each genotype.

E: ap>NLS-GFP, PI4KIII-α-RNAi. On the wild type side (right) Gliotactin immunolabeling appears as tight puncta (E’). On the apterous side (left), Gliotactin spread beyond tricellular corners (E’). Dlg immunolabeling was downregulated in the apterous side (E”).

F: Corresponding side projections showing basolateral spreading of Gliotactin (F’) and Dlg (F”) in the PI4KIII-α-RNAi expressing apterous side.

G: ap>NLS-GFP, PI4KIII-α-RNAi. Nrv2 immunolabeling was downregulated in the apterous side (G’). Ecad level was slightly downregulated in the apterous side (G”).

H: Corresponding side projections showing basolateral spreading of Nrv2 (F’). The Ecad did not spread along the basolateral membrane (H”) in the PI4KIII-α-RNAi expressing apterous side.

I and I’: Digital magnification (28X) of areas marked by boxes in E’ of apterous (left) and wild type (right), respectively. Note that I and I’ are the same magnification (scale bar =5µm).

2.5 Discussion

2.5.1 Kinases associated with permeability barriers have not been well studied

Disruption of TCJs disrupts the barrier function of epithelia in both invertebrates and vertebrates, and the disruption of epithelial TJs is correlated with a variety of diseases including cancer (Rao 2013). The overexpression of Gliotactin signals for aberrant phenotypes including overproliferation, cell delamination and apoptosis and previously JNK was found to play a key role in this process (Padash-Barnchi et al., 2010). However, the identity of the kinase pathways and mechanisms that control permeability barriers or the signaling events associated with
permeability barriers was unknown. From our screen of the *Drosophila* kinome, we found that the generation of the Gliotactin overexpression phenotypes involves at least two main signaling pathways: TNF-JNK pathway through Grnd, and the phospholipid pathway through the involvement of PI3K, Akt and possibly Btk29A.

### 2.5.2 Gliotactin and the TNF-JNK pathway

Knock-down of one of the *Drosophila* TNF receptors Grnd suppressed the Gliotactin phenotypes but the knock-down of the other TNF receptor, Wengen, and the ligand, Eiger, did not. While the function of Eiger is dependent on the receptor Grnd or Wengen, Grnd does not always depend on its ligand Eiger (Andersen et al., 2015). For instance, the overexpression of the intracellular domain of Grnd is sufficient to activate ectopic activation of Wg, JNK and apoptosis suggesting that Eiger is not always required for the Grnd activity (Andersen et al., 2015). Furthermore, Andersen et al., (2015) showed that the neoplastic growth and tumors caused by Syntaxin knock-down were not sensitive to changes in Eiger levels but to changes in Grnd. In vertebrates, receptor trimerization and subsequent signaling of the TNF pathway can occur in the presence or absence of TNF ligand (Locksley et al., 2001). Overall, this has led to the suggestion that Grnd can activate JNK signaling and apoptosis in a ligand (Eiger) independent manner (Andersen et al., 2015; Fogarty et al., 2016).

Interestingly only the loss of Grnd but not Wengen suppressed the Gliotactin phenotypes. Eiger-induced apoptotic cell death can occur through Wengen (Kanda et al., 2002) but also through Grnd (Andersen et al., 2015), and Grnd and Wengen functions are not redundant (Andersen et al., 2015). While Wengen does not possess the conserved TNF receptor death domain or TRAF binding domains (Kanda et al., 2002; Kauppila et al., 2003), Wengen can bind
the *Drosophila* TRAF2 homolog, dTRAF2/TRAF6 (Kauppila et al., 2003). In contrast, Grnd has a conserved dTRAF2/TRAF6 binding motif similar to many TNFR receptors and Grnd physically binds with dTRAF2/TRAF6 (Andersen et al., 2015). We determined that dTRAF2/TRAF6 is key to the Gliotactin phenotypes and while it remains to be determined whether Gliotactin overexpression phenotypes are Eiger independent, it is certain that those phenotypes are Grnd dependent and Wengen independent.

If Gliotactin phenotypes involve an Eiger independent function of Grnd, this is likely through the action of intermediate proteins. Grnd is expressed in the most apical region of the epithelia and colocalized with Crumbs and when overexpressed Gliotactin can spread into this region. However, it is unlikely that Gliotactin can interact Grnd directly. Alternatively, Eiger can signaling in various tissues through both cell autonomous and cell non-autonomous activities (Andersen et al., 2015). Similar to mammalian TNF, Eiger is cleaved and released as a soluble ligand to act in distant locations (Wang et al., 2006a). Therefore, we cannot exclude the possibility that the wing discs are responding to a non-autonomous source of Eiger such as from the other compartment of the wing (where the RNAi was not expressed) or other nearby sources such as the fat bodies or hemocytes. Therefore, the exact mechanism of Grnd activation by overexpressed Gliotactin is not certain and remains to be further investigated.

### 2.5.3 Gliotactin and the PI3K pathway

The knock-down of either PI3K or Akt led to suppression of Gliotactin overexpression phenotypes indicating PI3K-Akt is likely another pathway involved when Gliotactin is overexpressed. However, how excess Gliotactin is linked to PI3K activity is not known. PI3K is known to bind with proteins with YEY motifs (Schulze et al., 2005), and Gliotactin has a YEY
motif at Y799 and Y801. In the future, it will be interesting to find out whether PI3K directly binds with the phosphorylated YEY motif of Gliotactin leading to activation of the PI3K-Akt pathway. If the Gliotactin and PI3K interaction is not direct, this might be through changes to either PIP2/PIP3 balance or changes to either the level or the localization of PTEN (phosphatase and tension homolog deleted on chromosome ten). Gliotactin overexpression could disrupt the distribution of PTEN leading to increased levels of PIP3 and thus increased PI3K, as PI3K is activated when PTEN is downregulated (Kim et al., 2004). Activation of JNK may downregulate PTEN (Vivanco et al., 2007). Therefore, whether the level/localization of PTEN is changed with Gliotactin overexpression would be another test to be done to get some indications on mechanism of PI3K activation. Alternatively, PI3K activity might occur through activation of Btk29A as PI3K can be activated downstream of Btk29A as well (Hinman et al., 2007). Overall, the PI3K-Akt pathway is another signaling pathway that plays a role in the generation of the Gliotactin overexpression phenotypes, however, the mechanism by which the PI3K is involved whether directly, through changes to PTEN or Btk29A must be investigated.

2.5.4 Gliotactin overexpression-induced effects may converge at JNK

The complete block of cell death when only Bsk/JNK was knocked-down, compared to the downregulation of the other pathways, suggests that there can be multiple pathways leading to activation of Bsk/JNK in the Gliotactin overexpression background. From our screen loss of both the TNF/JNK and PIP3/Akt pathways suppressed the Gliotactin phenotypes, suggesting these converge with JNK. However, activation of Akt leading to JNK activation has not been shown before, rather Akt plays an inhibitory role at multiple stages of the JNK pathway (Widenmaier et al., 2009; Zhao et al., 2015). Alternatively, Btk29A may represent the
convergence point of the PI3K and JNK pathways as knock-down of Btk29A suppressed the Gliotactin-induced phenotypes. JNK can be activated by Btk29A downstream of the two isoforms of Src in Drosophila; Src42A, Src64B (Diego et al., 1997; Roulier et al., 1998). However, we have found that neither Src plays a role in the Gliotactin phenotypes (Chapter 3).

Btk29A can also activate JNK independent of Src. One means of Btk29A activation is through binding of the PH domain to phosphoinositides (Harlan et al., 1994; Salim et al., 1996; Fukuda et al., 1996). Btk29A translocates to the membrane through the interaction of the PH domain with PIP3 (phosphatidylinositol 3,4,5-trisphosphate), hence, PI3K indirectly recruits and activates Btk29A kinase by increasing the PIP3 level (Qiu and Kung, 2000). Regulation of Btk29A downstream of Akt has also been reported (Mohammad et al., 2013). Thus, Btk29A is a strong candidate to further investigate whether the PI3K-Akt pathway converges with the JNK pathway through Btk29A.

Our results suggest that if the level and the localization of TCJ protein Gliotactin is not tightly regulated, then multiple pathways are involved in the Gliotactin-induced phenotypes leading to cell delamination, migration, death via apoptosis and cell overproliferation. Our data show that lipid kinases are also involved in Gliotactin overexpression background. More importantly, we found that PI4K-α lipid kinase directly or indirectly controls the level and/or localization of Gliotactin.
Chapter 3: C-terminal Src kinase (Csk) regulates the tricellular junction protein Gliotactin independent of Src

3.1 Synopsis

Permeability barriers created by septate junctions (SJs) in invertebrates and tight junctions (TJs) in vertebrates are vital for animal survival. Likewise, permeability barriers are formed by tricellular junctions (TCJs) at the corners of polarized epithelia where tight junctions or septate junctions from three cells converge. Gliotactin is found at TCJs in *Drosophila* and loss of Gliotactin results in SJ and TCJ breakdown leading to permeability barrier loss, animal paralysis and death. However, when overexpressed, Gliotactin spreads away from the TCJs and disrupts epithelial architecture by triggering overproliferation, delamination, apoptosis and migration and these effects are mediated by tyrosine phosphorylation of Gliotactin. Not surprisingly the level and the localization of Gliotactin is tightly controlled, either by microRNA-mediated mRNA degradation, or protein endocytosis and degradation triggered by tyrosine phosphorylation. However, the kinase(s) responsible for Gliotactin phosphorylation are not known. We identified C-terminal Src kinase (Csk) as a tyrosine kinase responsible for regulating the endocytosis of Gliotactin. Increased Csk levels completely suppress the Gliotactin-induced overexpression phenotypes by increasing endocytosis. Csk is required for the regulation of endogenous Gliotactin such that loss of Csk causes Gliotactin to spread away from the TCJ. Although Csk is known as a negative regulator of Src kinases, the effects of Csk on Gliotactin are independent of Src, and are likely to occur through another AJ-associated kinase. Overall our data suggest that Csk controls a
key component of the tricellular junction and may be necessary to regulate the formation of permeability barriers.

### 3.2 Introduction

Permeability barriers are critical to protect the body from pathogens and to maintain the compartmentation of a body to provide a specific fluid environment for each organ (Tyler, 2003; Furuse and Tsukita, 2006). Barrier function is achieved by the septate junctions (SJs) in invertebrate epithelia and tight junctions (TJs) in vertebrate epithelia (Auld et al., 1995; Genova and Fehon, 2003; Paul et al., 2003; Tyler, 2003). Furthermore, these junctions are required to maintain cell polarity in epithelial tissues by preventing movement of proteins/molecules along the apico-basal axis or by anchoring polarity domains within cells (Tepass et al., 2001; Nakajima et al., 2013). A subdomain of the SJ or TJ necessary for the formation of epithelia barriers is the TCJ, which forms at the convergence point of three epithelial cells. At this point, the bicellular junction strands perform a turn at the corners and run parallel to the long axis of the cells, where they connect to a series of plugs in insects (Fristrom, 1982; Noirot-thimothee, 1982). Many core proteins and associated proteins of septate junctions have been identified and include: Neurexin IV (NrxIV) (Baumgartner et al., 1996), Coracle (Cora) (Fehon et al., 1994)(94), Na/K ATPase [both α subunit (ATPα) and beta subunits (Nrv2)] (Genova and Fehon, 2003; Paul et al., 2003), Neuroglian (Nrg) (Paul et al., 2003), Contactin (Cont) (Faivre-sarrailh et al., 2004) and Mcr (Bätz et al., 2014; Hall et al., 2014).

In *Drosophila* epithelia two proteins are uniquely concentrated at the TCJ. Gliotactin is a single pass transmembrane protein from the Neuroligin family (Schulte et al., 2003) and Bark-beetle (Bark)/Anakonda is a transmembrane protein with a large extracellular domain harboring
a tripartite structural motif that is proposed to form the plugs sealing the TCJ (Byri et al., 2015; Hildebrandt et al., 2015). In vertebrates, the Claudin-family member Tricellulin, and the Angulin family member LSR (lipolysis-stimulated lipoprotein receptor), are concentrated at the tricellular tight junctions (Ikenouchi et al., 2005; Masuda et al., 2011). Loss of TCJ proteins disrupts the barrier function of epithelia in both invertebrates and vertebrates (Schulte et al., 2003; Ikenouchi et al., 2005; Nayak et al., 2013). In Gli null mutant animals, barrier function is compromised; fewer septa are formed and septa are not tightly packed with regular spaces as shown in transmission election micrographs (TEMs) of SJs in wild type tissues. Further, immunolabelling in Gli null mutant shows that the core SJ proteins spread basally resulting in death by late embryogenesis (Schulte et al., 2003). Conversely, when overexpressed, Gliotactin spreads around the cell and along the baso-lateral membrane triggering deleterious effects such as delamination, migration and cell death as well as extra cell proliferation (Padash-Barmchi et al., 2010). Overall, loss-of-function and overexpression experiments show that the level and the localization of Gliotactin to the TCJ must be tightly controlled to ensure proper barrier function and normal epithelial development. Therefore, there are cellular mechanisms to ensure Gliotactin is expressed at the correct levels and remains within the TCJ. One important regulation mechanism of the Gliotactin protein level is the phosphorylation of two highly conserved tyrosine residues leading to endocytosis and lysosome- mediated degradation (Padash-Barmchi et al., 2010). In addition, the Gliotactin mRNA is tightly regulated by degradation by miR-184, which is induced in a feed-back loop controlled by BMP signaling (SharifKhodaei et al., 2016). The anomalous signaling in Gliotactin overexpressing cells is mediated through an interaction with the MAGUK protein Discs large (Dlg) (Padash-Barmchi et al., 2013). This interaction is dependent on phosphorylation of Dlg by an unidentified Ser/Thr kinase and leads to a
downregulation of Dlg (Padash-Barmchi et al., 2013). While Gliotactin is phosphorylated by Src in an *in vitro* kinase assay (Padash-Barmchi et al. 2010), the kinase(s) that regulate Gliotactin *in vivo* are not known.

To identify the kinase(s) responsible for controlling Gliotactin levels and/or Gliotactin signaling, we carried out an RNAi screen of most *Drosophila* kinases. We identified C-terminal Src kinase (Csk) as a tyrosine kinase responsible for controlling the protein levels of Gliotactin. Csk and Src are closely related kinases and Csk is well known as a negative regulator of Src family kinases (Nada et al., 1991; Okada et al., 1991; Imamoto and Soriano, 1993; Okada, 2012). We found that loss of Csk enhanced the Gliotactin overexpression phenotypes while increased Csk expression suppressed the Gliotactin phenotypes. In contrast to our expectations, the two isoforms of *Drosophila* Src (Src42A and Src64B) did not suppress the Gliotactin overexpression phenotypes, showing that Csk functions independent of Src in Gliotactin protein regulation. The degree of tyrosine phosphorylation associated with Gliotactin and Gliotactin endocytosis was increased with increased Csk expression. When Csk was downregulated in an otherwise wild type background, Gliotactin spread away from tricellular junctions showing that Csk regulates not only overexpressed Gliotactin but also endogenous Gliotactin. Overall, our study highlights a Src-independent role of Csk in regulation and localization of TCJ protein, Gliotactin.

### 3.3 Materials and methods

#### 3.3.1 Fly stocks

*Drosophila* fly Stocks were either obtained from the Bloomington *Drosophila* Stock Center (BDSC), the Vienna *Drosophila* RNAi Center (VDRC), or from researchers who made
them. $w_{118}^{118}$ (BDSC) was the control. *apterous–GAL4* (BDSC) was the GAL4 driver. The Gal4 responsive UAS transgenes used in this study include: Gli-WT and GliFF (Padash-Barmchi et al., 2010), Csk-WT (Pedraza et al., 2004), Src64B-WT (Dura, 2004), NLS-GFP (a gift from Dr. Douglas Allan). UAS driven RNAi lines include: Csk-RNAi 1 (VDRC #109813), Csk-RNAi 2 (VDRC #32877), Csk-RNAi 3 (BDSC #35174), Src64B-RNAi (VDRC #35252), Src64B-RNAi (BDSC #30517), Src42A- RNAi (VDRC #26019). Mutant alleles include: *Csk*[J1D8] (Stewart et al., 2003), *Src64B*[D404N] (Strong et al., 2011), *Src64B*[KO] (Dodson et al., 1998; O’Reilly et al., 2006), *Src42A*[myri] (Tateno et al., 2016). All crosses were carried out at 29°C under standard conditions using virgin females from the parental stock: *ap–GAL4,GAL80ts;UAS-Gli-WT#5 /SM-TM6,Tb*.

### 3.3.2 Immunofluorescence labeling

Third instar wing imaginal discs were dissected and immunolabeled using standard protocols: larvae were dissected in 1X PBS and fixed with 4% paraformaldehyde for 20 mins. All subsequent washes were in PBS plus 0.1% Triton X-100 (PBST) and discs were blocked in PBST with BSA (0.5%) prior to primary antibody incubation overnight at 4°C, secondary antibodies were incubated for 2 hours at room temperature. Discs were cleared in 70% glycerol overnight and mounted with Vectashield (Invitrogen). Primary antibodies were used at following dilutions: mouse anti-Gliotactin IF6.3 at 1:100 (Auld et al., 1995), rabbit anti-Gliotactin at 1:600 (the Gliotactin polyclonal antibody was generated by immunizing rabbits with a peptide corresponding to the amino acid sequence (CQPAAQPRTTHLVEGVPQTS) and subsequent affinity purification, both by YenZym Antibodies, LLC), mouse anti-Dlg 4F3 at 1:100 (DSHB) (Parnas et al., 2001), rat anti-DE-Cadherin at 3:100 (DSHB), mouse anti-Coracle (9C and C615-
16B cocktail) at 1:100 (DSHB) (Fehon et al., 1994), guinea pig anti-Mcr at 1:800 (Hall et al., 2014), rabbit anti-Csk at 1:1200 (Langton et al., 2007), rabbit anti-phospho-Tyr at 1:100 (Cell Signaling), rabbit anti-Rbsn 5 at 1:6000, (Tanaka and Nakamura, 2008), rabbit anti-Rab7 at 1/2000 (Tanaka and Nakamura, 2008), rabbit anti Rab11 at 1/8000 (Tanaka and Nakamura, 2008), mouse anti-Arm at 1:50 (DSHB), rabbit anti-cleaved Caspase 3 at 1:200 (Cell Signaling). DAPI was used at 1:1000 (Thermo Scientific). Secondary antibodies were used at 1:300: goat anti rabbit Alexa488, goat anti-rabbit Alexa568, goat anti rabbit Alexa647, goat anti mouse Alexa488, goat anti-mouse Alexa568, goat anti-mouse Alexa647, goat anti-rat Alexa568, goat anti-rat Alexa647, goat anti-guinea pig Alexa488.

3.3.3 Proximity ligation assay

Proximity ligation assays (PLA) were carried according to the manufacturer’s guidelines (Duolink, Sigma). Mouse anti-Gliotactin IF6.3 at 1:100 (Auld et al., 1995) and rabbit anti-phospho-Tyr at 1:100 (Cell Signaling) were used in the PLA done to detect proximity between Gliotactin and phospho-tyrosine. Mouse anti-Gliotactin IF6.3 at 1:100 (Auld et al., 1995) and rabbit anti-Csk at 1:1200 (Langton et al., 2007) were used in the PLA done to detect proximity between Gliotactin and Csk. Total volumes of the PLA reactions were 80 µl for 15 wing discs. Prior to PLA, the isolation of wing imaginal discs, fixation and incubation with primary antibodies were done as described above. Discs were incubated at 37°C and timings were: initial incubation with PLA probe solution for 1.5 hours, ligation reaction for 1 hour, amplification reaction for 2 hours. Discs were equilibrated and mounted in Vectashield.
3.3.4 Imaging

For higher magnification images, z-series stacks were collected with 0.2 µm steps using DeltaVision Spectris microscope (Applied Precision, GE) with a 60x (1.4 NA) oil immersion lens and CoolSnap HQ digital camera. Lower magnification images were collected with a 20X air lens. Deconvolution was done with SoftWorx (Applied Precision) software with 6-10 iterations using a point-spread function (PSF) calculated with 0.2 µm beads conjugated with Alexa Fluor 568 (Molecular Probes) mounted in Vectashield. Image processing and side projections were done using SoftWorx. Figures were made using Adobe Photoshop 4 or CC 2017. Zeiss Axioskop (at 20x objective) and Northern Eclipse software were used for some images of whole wing discs.

3.3.5 Statistical analysis

The statistical analysis and graphing were done using Prism 6.0. One Way ANOVA was done to perform pairwise comparisons among multiple genotypes. Tukey post-hoc was done to determine significance. Students T-test was done when only two genotypes were to be compared. Cell migration: Using image J (http://imagej.nih.gov/ij/), the migrated distance of Gliotactin positive cells from the apterous boundary to the wild type side was measured for each disc and expressed as a ratio to the total distance from the apterous boundary to the tip of the wild type side of the wing disc. To measure each distance the line tool and the Analyze/Measure option in ImageJ were used. For all panels, the dorsal/ventral apterous side was identified based on immunolabeling of the overexpressed Gliotactin at the level of the septate junction domain as the epithelium/boundary is intact at this level.
Counting of Endosomes: For each disc, three Z stacks (at the level of septate junction domain) of high magnification images were compressed using the *quick projection* function in SoftWorx. Endosome counting was done manually marking each endosome with the aid of *cell counter* tool in ImageJ. For each disc, counting was done in a constant area selected from the apterous side.

Immunolabeling intensity: Using ImageJ, intensities of immunolabeling or PLA (mean gray values) were measured in two constant size boxes drawn on the apterous and wild type side of the pouch area of wing disc and the ratios of the apterous/wild type were calculated.

**Biochemistry**

**3.3.6 Purification of His-tagged Gliotactin C-terminal constructs**

BL21DE3 pLysS competent cells carrying pET28a (Novagen) plasmids ligated to Gliotactin C-terminal constructs ([Padash-Barmchi et al., 2010](#)) were cultured in large volumes (100ml) for 2 hours prior to the induction of protein expression with 1 mM IPTG for 45 min. The cells were lysed in 40 mM imidazole, 300 mM NaCl, 50 mM Tris-HCl pH 8, 1/100 PhosStop (Roche), 1/100 Complete (Roche). Nickel NTA-agarose (Qiagen) was used for the purification of His-tagged proteins and eluted with 250 mM imidazole buffer (300 mM NaCl, 50 mM Tris-HCl pH 8) and dialyzed using Slide-A-Lyzer 7000MWCO cassettes (Pierce) into Kinase Buffer (25mM MOPS, pH 7.2, 12.5 mM glycerol-2-phosphate, 20mM MgCl₂, 25 mM MnCl₂ 5mM EGTA 2mM EDTA, 0.25 mM DTT and 0.1 mM Sodium Orthovanadate).

**3.3.7 Kinase assay**

A 20 µl Kinase Reaction [0.5 µg His-tagged GliCter in pET28 (extracted using the above outlined method), 0.5 µg GST–Csk (Cell Signaling Technologies), 1x Kinase Buffer (25mM
MOPS, pH 7.2, 12.5 mM glycerol-2-phosphate, 20mM MgCl2, 25 mM MnCl2 5mM EGTA 2mM EDTA, 0.25 mM DTT and 0.1 mM Sodium Orthovanadate), 1 mM ATP] was incubated at 30°C for 30 minutes. In the control experiment for the Csk activity, 0.5 µg of His-tagged human Src (Creative Biomart) was used.

3.3.8 Li-cor western blot analysis

Half of the kinase reaction (outlined above) was loaded on 10% SDS-PAGE followed by western blot on nitrocellulose membranes. Blots were blocked using 50% Odyssey blocking buffer (LI-COR) diluted in PBS and analyzed with LI-COR Odyssey fluorescent detection systems. The primary antibodies mouse anti-Gliotactin IF6.3 at 1/5000 and rabbit anti-phosphoTyr (Cell Signaling) at 1/5000 and secondary antibodies goat anti-rabbit IRDye 680 at 1: 20,000, goat anti-mouse IRDye 800 at 1: 20,000, were used (All IRDye antibodies from Rockland).

3.4 Results

3.4.1 Gliotactin overexpression phenotypes are changed with changes to Csk level

In a wild type background (w1118 in our experiments), Gliotactin is restricted to the tricellular corners of the columnar epithelial cell layer of the Drosophila wing imaginal disc (Fig. 3.2). When overexpressed in the wing imaginal disc using the apterous-GAL4 driver (ap-GAL4), Gliotactin spreads away from the TCJ and is found around the cell and along the lateral membrane. As Padash-Barmchi et al. (2010) showed, overexpression leads to overproliferation, cell delamination and apoptosis, resulting in migration of Gliotactin expressing cells into the wild type non-apterous side of the imaginal disc (Fig. 3.1A, F, G). Gliotactin protein levels are controlled by phosphorylation of two highly conserved tyrosines leading to endocytosis and
lysosome-mediated degradation (Padash-Barmchi et al., 2010). To identify the kinase(s) responsible for controlling Gliotactin levels and/or Gliotactin signaling, we carried out a screen using RNAi from the Vienna Drosophila RNAi Center (VDRC) collection to the majority of *Drosophila* kinases. The details of the screen were given in Chapter 2. In the course of this screen, we identified C-terminal Src kinase (Csk) as a potential kinase for controlling Gliotactin protein levels. When Csk RNAi was expressed along with Gliotactin (Gli-WT), the Gliotactin overexpression phenotypes were greatly enhanced. Two independent RNAi lines to Csk were used and results were the same for both confirming the specificity (Fig. 3.2C, D). The wing imaginal discs were smaller (Fig. 3.1C, I; Fig. 3.2C, D) than both wild type control (w^{118}i) (Fig. 3.2A) or Gliotactin overexpression alone (Fig. 3.1A).

In these discs (*ap > Gli-WT, Csk-RNAi*) there was a high degree of apoptosis as measured by immunolabeling with an antibody to cleaved Caspase 3 (Song, 1997) (Fig. 3.1I‴; Fig. 3.2E). The apterous region was greatly reduced in size with increased migration of the Gliotactin expressing cells to the non-apterous side of the disc (Fig. 3.1C‴, I‴; Fig. 3.2C, D). Expression of Csk-RNAi (*ap > Csk-RNAi*) in an otherwise wild type wing disc resulted in larger apterous areas with many extra folds. A small percentage of discs (20%) had low levels of cell migration but interpretation of these discs was often difficult due to the overgrowth of the apterous side leaving a reduced or absent non-apterous side (Fig. 3.14B). These data suggest that the reduced apterous side, enhanced cell migration and cell death in Csk-RNAi and Gli-WT coexpressing were different and not likely due to an additive effect. Similar results were observed when Gliotactin was overexpressed in a heterozygous Csk mutant background (*ap > Gli-WT, Csk[J1D8]/+*) (Fig. 3.1D). The downregulation of Csk increased the Gliotactin overexpression phenotypes suggesting that Csk regulates Gliotactin. To test this hypothesis, we co-expressed Csk (Csk-WT)
(Pedraza et al., 2004) with Gliotactin (Gli-WT) and found that the Gliotactin overexpression phenotypes were suppressed (Fig. 3.1B, H). Specifically, while Gliotactin still spread around the cell, the migration of the Gli-WT cells was significantly suppressed (Fig. 3.1B’, H’, J) and the degree of ectopic folds in the Gli-WT side of the disc was reduced suggesting the overproliferation phenotype was also suppressed. Further confirming the suppression, there was a reduction in apoptosis as measured by cleaved Cas3 immunolabeling (Fig. 3.1H”; Fig. S1E).

Dlg plays a role in mediating the deleterious consequences of Gliotactin overexpression and Dlg is normally downregulated by the spread of Gliotactin away from the TCJ (Padash-Barmchi et al., 2013) (Fig. 3.1A”, 2A”). The downregulation of Dlg when Csk was co-expressed with Gli-WT was comparable to that in Gliotactin overexpression alone (Fig. 3.1B”, 2B”; Fig. 3.2F), suggesting that the suppression of Gliotactin phenotypes were not mediated through changes to Dlg. These results suggest that Csk has a role in regulating Gliotactin and this effect is independent of Dlg.
Figure 3.1: Csk levels affect the Gliotactin overexpression phenotypes.

Third-instar wing imaginal discs with transgenes or RNAi lines expressed by the apterous-GAL4 (ap>) driver. The dashed lines marked the apterous boundary between the dorsal and ventral compartments with the apterous side (dorsal compartment) on the left. Arrows indicate the leading edge of the migratory cells. Each panel represents a single Z slice. Scale bars: 30µm.

A-D: Wing discs immunolabeled for Gliotactin (green), Dlg (red) and DAPI (blue). n=15 discs except n=8 discs in D. Arrows indicate the leading edge of the Gliotactin expressing cells.

A: Gliotactin overexpression (ap>Gli-WT) leads to distinct phenotypes including migration of Gliotactin overexpressing cells into the wild type (ventral) side of the disc (A’), extra folds due to increase proliferation on the apterous side (A-A’’, G-G’’).

B: Rescue of Gliotactin overexpression phenotypes by co-expression of Csk-WT. Cell migration to the wild type side was suppressed (B’) and fewer extra folds were formed (B-B’’, H-H’’).
C: Enhancement of Gliotactin overexpression phenotypes by co-expression of Csk- RNAi. Cell migration was enhanced (arrow) and the apterous side was smaller (C’).

D: Gliotactin overexpression in the Csk[JJ1D8] heterozygous mutant phenocopied the co-expression of Csk-RNAi (D-D’) giving an enhanced cell migration and a smaller dorsal side.

E: Schematic of a Drosophila wing imaginal disc. The region of apterous expression is marked in green and the boundary between dorsal (apterous) and ventral (wild type) compartments within the pouch region marked in yellow. The side-view below shows the squamous and columnar epithelial layers of the wing disc.

F: Schematic of the migration of Gliotactin overexpressing cells. Migration distances were measured for cells from the apterous boundary into the wild type/ventral compartment (red arrow) compared to the total distance from the apterous boundary to the distal edge of the disc (black arrow).

G-I: Wing discs immunolabeled for Gliotactin (green), activated Cas3 (Cas3) (red) and DAPI (blue). Stars indicate the leading edge of the cells positive for activated Cas3.

G: Gliotactin overexpression (ap>Gli-WT) increased levels of activated Cas3 with strong expression in migrating cells (G’’).

H: Co-expression with Csk-WT (ap>Gli-WT, Csk-WT) reduced the levels of activated Cas3 (H’’).

I: Co-expression with Csk-RNAi (ap>Gli-WT, Csk-RNAi) led to increased immunolabeling for activated Cas3 (I’’).

J: Quantification of the cell migration ratios with Gli overexpressed alone (ap>Gli-WT), co-expressed with Csk-WT (ap>Gli-WT, Csk-WT) and co-expressed with Csk-RNAi (ap>Gli-WT, Csk-RNAi) or in the Csk heterozygous mutant (ap>Gli-WT, Csk[JJ1D8]/+). All showed a significant difference in the cell migration ratios (**** P<0.0001). The difference in cell migration ratios between (ap>Gli-WT, Csk-RNAi) and (ap>Gli-WT, Csk[JJ1D8]/+) was not significant (NS). (n=15 discs for each). Bars represent the standard deviation (SD).
Figure 3.2: Gliotactin localization in wild type epithelia, specificity of Csk RNAi phenotypes and quantification of Cas3 and Dlg intensity ratios in different Csk backgrounds.
A-B: Third-instar wing imaginal discs of wild type w¹¹¹⁸ flies, immunolabeled for Gliotactin (Gli, green), Dlg (red) and DAPI (blue). Each panel represents a single Z slice at the septate junction level. Scale bars: (A 30 µm), (B 15µm). n= 10 discs in each genotype.
A: The pouch area of wing imaginal disc is shown at low-magnification (20x). There were no ectopic folds in the wild type wing disc.
B: The pouch area of wing imaginal disc at high-magnification (60x) Gliotactin was concentrated at the tricellular corners and Dlg was found all around the membrane with an overlap at the tricellular corners.
C-D: Two independent Csk-RNAi lines also enhanced the Gliotactin overexpression phenotypes. Low-magnification (20x) of wing imaginal discs (ap>Csk-RNAi, Gli-WT) immunolabeled for Gliotactin (Gli) and labeled with DAPI. Dashed lines mark the apterous boundary where the apterous side (dorsal compartment) is at the top. Arrows indicate the leading edge of the Gliotactin expressing cells. For each RNAi line, cell migration was enhanced and the apterous side was smaller (C’ and D’). Each panel represents a single Z slice. n=10 discs.
E: Statistical analysis of Cas3 immunolabeling. For each genotype, the mean ratios of Cas3 immunolabeling intensities in the apterous side versus WT side are shown. n=10 discs. (**** P<0.0001). No significant (NS) difference was observed between ap>Gli-WT and ap>Gli-WT, Csk-WT. Error bars represent the standard deviation (SD).
F: Statistical analysis of Dlg immunolabeling. For each genotype, the mean ratios of Dlg intensities in the apterous side versus WT side are shown. n=10 discs. (* P<0.01 and **** P<0.0001), NS - not statistically significant. Error bars represent standard deviation (SD).

3.4.2 Coexpression of Csk increases internalization of overexpressed Gliotactin

As the coexpression of Csk suppressed the Gliotactin phenotypes, we compared the changes to cells overexpressing Gliotactin at the cellular level (Fig. 3.3, 3.5). A consistent phenotype we observed was changes to cell size. Gliotactin overexpression led to an increase in cell size (Fig. 3.3A”, 3.9A”, 3.10A” ) on both the apterous and non-apterous sides. The suppression of Gliotactin phenotypes by Csk (ap>Gli-WT, Csk-WT) also included a suppression of the increased cell size (Fig. 3.3B”, 3.9C”, 3.10C”), whereas the knockdown of Csk (ap>Gli-WT, Csk-RNAi) appeared to further increase cell size (Fig. 3.3C”, 3.9E”). The mechanism underlying this effect is not clear as both sides of the disc were affected suggesting a change in tissue morphology.

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Each vesicle-like structure that was positive for Gliotactin immunolabeling was defined as a vesicle for our analysis. However, larger vesicle-like areas may represent multivesicular bodies (MVBs) and consist of many small vesicles that cannot be distinguished with light microscopy. MVBs are reported to have large diameters (>250 nm) and to be involved in endocytosis (Altick et al., 2009). In cells co-expressing Csk and Gli-WT, there was an elevated level of Gliotactin endocytosis and the majority of cells had large internal vesicles filled with Gliotactin (Fig. 3.3B’ B’’’). This was compared to Gli-WT expression alone where not all cells had large vesicles (Fig. 3.3A, A’’’). In contrast, when Csk was knocked-down using RNAi, fewer Gli-WT expressing cells had vesicles and the vesicle size was smaller (Fig. 3.3C, C’’’’) compared to Gli-WT alone or Gli-WT plus Csk (Fig. 3.3A’’’ B’’’'). The size of vesicles was placed into one of three separate groups based on maximum diameter (type 1: d ≥ 2.5 µm; type 2: 1 µm < d < 2.5 µm; type 3: d ≤ 1 µm) (Fig. 3.3D) and then quantified (Fig. 3.3E; Table 3.1). There was no significant difference between the total number of vesicles in Gli-WT expression vs Gli-WT, Csk-WT co-expression (Fig. 3.3E; Table 3.1). However, the numbers of larger vesicles (type 1 and 2) were significantly higher in Gli-WT and Csk-WT co-expression, indicating an increased amount of Gliotactin endocytosis (Table 3.1). In contrast, the total number of vesicles as well as the numbers in each type when Csk-RNAi was coexpressed with Gli-WT (ap > Gli-WT, Csk-RNAi) was significantly lower, indicating that the Gliotactin endocytosis was reduced (Table 3.1). As Gliotactin endocytosis depends on phosphorylation of conserved tyrosine residues (Y760, Y799) in the intracellular domain of Gliotactin (Padash-Barmchi et al., 2010), our results suggest that Csk controls the endocytosis of Gliotactin likely through increased phosphorylation and thus leads to the reduction of Gliotactin levels at the membrane. These results phenocopied
previous observations with the overexpression of the GliDD transgene, which mimics phosphorylation of Gliotactin, and results in large internal endocytic vesicles (Padash-Barmchi et al., 2010).

Table 3.1: The comparison of numbers of each type (based on size) and total number of vesicles in three different Csk backgrounds; endogenous level of Csk (ap>Gli-WT), overexpressed Csk (ap>Gli-WT, Csk-WT) and Csk downregulated (ap>Gli-WT, Csk-RNAi)

<table>
<thead>
<tr>
<th>Type of Vesicles</th>
<th>Comparison of number of vesicles</th>
<th>Mean Diff.</th>
<th>Significance</th>
<th>P value</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1 (d≥ 2.5µm)</td>
<td>Gli-WT vs Gli-WT, Csk-WT</td>
<td>-3.9</td>
<td>**</td>
<td>0.0033</td>
<td>0.2981</td>
</tr>
<tr>
<td></td>
<td>Gli-WT vs Gli-WT, Csk-RNAi</td>
<td>1</td>
<td>NS</td>
<td>0.6279</td>
<td>1.286</td>
</tr>
<tr>
<td></td>
<td>Gli-WT,Csk-WT vs Gli-WT,Csk-RNAi</td>
<td>4.9</td>
<td>***</td>
<td>0.0003</td>
<td>0</td>
</tr>
<tr>
<td>Type 2 (1&lt;d&lt;2.5 µm)</td>
<td>Gli-WT vs Gli-WT, Csk-WT</td>
<td>-18.7</td>
<td>*</td>
<td>0.0374</td>
<td>7.237</td>
</tr>
<tr>
<td></td>
<td>Gli-WT vs Gli-WT, Csk-RNAi</td>
<td>32.6</td>
<td>****</td>
<td>0.0003</td>
<td>4.9</td>
</tr>
<tr>
<td></td>
<td>Gli-WT,Csk-WT vs Gli-WT,Csk-RNAi</td>
<td>51.3</td>
<td>****</td>
<td>&lt; 0.0001</td>
<td>0.7024</td>
</tr>
<tr>
<td>Type 3 (d≤ 1µm)</td>
<td>Gli-WT vs Gli-WT, Csk-WT</td>
<td>33.1</td>
<td>****</td>
<td>0.0002</td>
<td>7.022</td>
</tr>
<tr>
<td></td>
<td>Gli-WT vs Gli-WT, Csk-RNAi</td>
<td>65.6</td>
<td>****</td>
<td>&lt; 0.0001</td>
<td>4.899</td>
</tr>
<tr>
<td></td>
<td>Gli-WT,Csk-WT vs Gli-WT,Csk-RNAi</td>
<td>32.5</td>
<td>****</td>
<td>0.0003</td>
<td>1.638</td>
</tr>
<tr>
<td>All</td>
<td>Gli-WT vs Gli-WT, Csk-WT</td>
<td>10</td>
<td>NS</td>
<td>0.6772</td>
<td>11.01</td>
</tr>
<tr>
<td></td>
<td>Gli-WT vs Gli-WT, Csk-RNAi</td>
<td>98.7</td>
<td>****</td>
<td>&lt; 0.0001</td>
<td>9.235</td>
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<tr>
<td></td>
<td>Gli-WT,Csk-WT vs Gli-WT,Csk-RNAi</td>
<td>88.7</td>
<td>****</td>
<td>&lt; 0.0001</td>
<td>1.533</td>
</tr>
</tbody>
</table>

Summary results of one-way ANOVAs with Tukey post-hoc tests. Mean Difference (Mean Diff), Significance, Adjusted P value for multiple comparisons (P value) and Standard Error of Mean (SEM) are shown. The total number of vesicles in Gliotactin overexpression was not significantly different from that in co-expression with Csk-WT. Further, the number of largest group (d ≥ 2.5 µm) vesicles was also not significantly different in Gliotactin overexpression compared to co-expression with Csk-RNAi. n=10 in each genotype.
Figure 3.3: Coexpression of Csk increases internalization of overexpressed Gliotactin.

High-magnification images (60X) from the pouch region of third-instar wing imaginal discs with transgenes or RNAi lines expressed using the apterous-GAL4 (ap>) driver. Discs were immunolabeled for Gliotactin (Gli, green), Dlg (red) and DAPI (blue). The dashed lines represent the apterous boundary with the dorsal (apterous) compartment on the left. Boxed regions were digitally magnified (28X) and shown on the right. Each en face panel represents a single Z slice.
taken at the septate junction level. Scale bars: 15µm, except 5µm in digitally magnified panels. n=10 discs in each genotype.

A: ap>Gli-WT. On the apterous side, Gliotactin is found around the membrane and in internal vesicles. Not all the cells contained large vesicles (A’’’). Dlg was downregulated in the apterous side (A’’’).

B: ap>Gli-WT, Csk-WT. Co-expression of Csk-WT led to an increase cells with large vesicles. Dlg was downregulated in the apterous side (B’’’).

C: ap>Gli-WT, Csk-RNAi. Co-expression of Csk-RNAi led to the presence of small vesicles in some cells while many cells did not contain vesicles. Dlg downregulation in the apterous side was not as extensive (C’’ compared to A’’’, B’’’).

D: A digitally magnified area representing the three types of vesicles quantified. Immunolabeling of Gliotactin marked the internal vesicles and Dlg marked the cell membrane. D’’’ is a duplication of D’ showing the outline of the vesicles in green.

E: Analysis of vesicle number and size in (ap>Gli-WT), (ap>Gli-WT, Csk-WT) and (ap>Gli-WT, Csk-RNAi) respectively. Vesicles were placed into three groups based on maximum diameter (type 1: \(d \geq 2.5\mu m\); type 2: \(1\mu m < d < 2.5\mu m\); type 3: \(d \leq 1\mu m\). In each n=10 discs.

We next tested a Gliotactin transgene where both tyrosines were converted to phenylalanine to mimic the dephosphorylated state (GliFF), which leads to a reduction in endocytosis (Padash-Barmchi et al., 2013) (Fig. 3.5A-B). It is important to note that Gliotactin forms a dimer or oligomer (Venema et al., 2004), therefore expression of GliFF in wild type background triggers the formation of a complex with endogenous Gliotactin (Fig. 3.6D) and shows a dominant negative effect. The complex of GliFF with endogenous Gliotactin has reduced levels of endocytosis but remains within the membrane and is able to continue signaling increasing the deleterious phenotypes (Padash-Barmchi et al., 2013) (Fig. 3.5A; Fig. 3.6A, D).

Coexpression of Csk-WT with GliFF was also able to suppress the cellular phenotypes triggered by expression of Gliotactin in the wing disc (Fig. 3.5B; Fig. 3.6B). Csk expression triggered the formation of large Gliotactin vesicles (Fig. 3.5B’’, B’’’’) further consistent with the idea that the increased level of endocytosis of Gliotactin likely through the increased phosphorylation of the endogenous Gliotactin in the dimer or oligomer with GliFF.
Figure 3.4: Co-expression of Csk with Gli-WT does not change the Gliotactin degradation pathway.
High-magnification images (60x) from the wing pouch with transgenes or RNAi lines expressed by the apterous-GAL4 (ap>) driver. En face views are digitally magnified by 28x and single Z slice taken at the level of SJs from the apterous side. In side projections, the dashed lines mark the apterous boundary between the dorsal and ventral compartments with the apterous side on the left. Scale bars: 5 µm except in 15µm in side projections. n=10 discs in each genotype. Arrows indicate the Gliotactin positive vesicles.

Discs were immunolabeled for Gliotactin (Gli, green), Rbsn-5 (red), Rab7 (red) or Rab11 (red) and DAPI (blue).

A, E, G: ap>Gli-WT. Overexpressed Gliotactin spread around the membrane and in internal vesicles. The majority of Gliotactin positive vesicles were positive for Rbsn-5 immunolabeling (A), Rab7 (E) but not Rab11 (G).

B: Side projections showing Rbsn-5 immunolabeling concentrated at the septate junctions with no apparent change in levels or distribution when Gliotactin is overexpressed.

C, F, H: ap>Gli-WT, Csk-WT. Co-expression of Csk-WT led to an increase in the number and size of Gliotactin positive large vesicles. These vesicles were positive for Rbsn-5 immunolabeling (C), Rab7 (F) but not Rab11 (H). The vesicles were larger (C”, F”) compared to those in ap>Gli-WT (A”, E”). The size of Rab11 positive vesicles was not affected.

D: Side projections showing Rbsn-5 labeling at the level of septate junctions with no apparent change to the level and/or localization of Rbsn-5.

We confirmed that the type of vesicles that were increased in ap>Gli-WT, Csk-WT were endosomes using immunolabeling to the Rab5 effector protein Rabenosyn-5 (Rbsn-5)(Tanaka and Nakamura, 2008) (Fig. 3.4A-D) and with the late endosome marker Rab7 (Tanaka and Nakamura, 2008; Vanlandingham and Ceresa, 2009) (Fig. 3.4E, F). In Gliotactin overexpression alone and in coexpression with Csk, the majority of the vesicles were colocalized with Rbsn-5 (Fig. 3.4A-D), which is present in early to late endosomes (Mottola et al., 2010; Lőrincz et al., 2016) and with Rab7 (Fig. 3.4E, F). The size of Rbsn-5 or Rab7 positive vesicles in ap>Gli-WT (Fig 3.4A”, E”) was smaller compared to the Gliotactin coexpression with Csk-WT (ap>Gli-WT, Csk-WT) (Fig 3.4C”, F”). Of note the non-apterous wild type side also showed a strong localization of Rbsn-5 at the level of septate junctions (Fig. 3.4B”, D”) and there was no apparent change to the level and/or the localization of Rbsn-5 in the Gliotactin overexpressing apterous side compared to the wild type side. The Gliotactin filled vesicles were not positive for
the recycling endosome marker, Rab11 (Fig. 3.4G, H). Overall, the data indicates that
coexpression of Csk leads to an increased endocytosis of Gliotactin through the
endocytic/lysosome degradation pathway previously observed for Gliotactin (Padash-Barmchi et
al., 2010).

3.4.3 Coexpression of Csk increases the tyrosine phosphorylation associated with
overexpressed Gliotactin

We next wanted to check whether the level of Gliotactin phosphorylation changes with
changes to Csk. Gliotactin is phosphorylated at two conserved tyrosine residues *in vivo* (Schulte
et al., 2006) and the phospho-mimetic form of Gliotactin, GliDD increases endocytosis when
overexpressed (Padash-Barmchi et al., 2010). Since antibodies specific to the phosphorylated
form of Gliotactin are not available, we used a proximity ligation assay (PLA) (Gajadhar and
Guha, 2010) to detect changes in tyrosine phosphorylation within 40 nm of Gliotactin. We
compared the degree of phosphotyrosine and Gliotactin association in three different
backgrounds: Gli-WT expression alone (*ap>Gli-WT*), co-expressed with Csk (*ap>Gli-WT, Csk-
WT*) or co-expressed with Csk-RNAi (*ap>Gli-WT, Csk-RNAi*) (Fig. 3.5F-H). With Gliotactin
overexpression alone, there was an elevated level of the PLA signal compared to the non-
apterous side, indicating that the overexpressed Gliotactin was in close proximity with phospho-
tyrosine residues (Fig. 3.5C, F’). When coexpressed with Csk, the PLA signal level was
increased (Fig. 3.5D, G, H). Conversely when Csk was knocked-down the PLA signal was lower
than Gli-WT alone (Fig. 3.5E). This data suggests that Csk is responsible (either directly or
indirectly) for the phosphorylation of Gliotactin, a Gliotactin associated protein or a nearby non-
associated protein.
Figure 3.5: Coexpression of Csk increases the tyrosine phosphorylation associated with overexpressed Gliotactin.

Third-instar wing imaginal discs with transgenes or RNAi lines expressed by the apterous-GAL4 (ap>) driver. The dashed lines mark the apterous boundary with the dorsal (apterous) compartment on the left. Areas marked by boxes were digitally magnified 28 times and placed at the end of
corresponding rows. Each panel represents a single Z slice at the septate junction level. Scale bars: 15 µm except in C, D and E 30 µm, and A”” and B”” 5 µm.
A-B: High-magnification images (60x) from the wing disc pouch immunolabeled for Gliotactin (Gli, green), Dlg (red) and DAPI (blue). Arrows are pointing to vesicles. n=6 in A and n=5 discs in B.
A: $ap\text{-}Gli^{FF}$. On the apterous side, Gliotactin was found around the membrane and the occasional small vesicles (A, A””).
B: $ap\text{-}Gli^{FF}$, Csk-WT. Co-expression of Csk-WT led to an increase in vesicle number and size (B’ and B””).
C-G: PLA signals showing the changes to amount of phospho-tyrosine (pY) that were within 40 nm proximity to Gliotactin. n=11 in F, n=9 in G and n= 8 discs in H.
C’ and D’: Higher resolution images (60x) from the pouch area comparing the levels of PLA in apterous side vs WT side. E’: an area showing the maximum level of PLA signals within the apterous side (the area is marked by a star in E).
C-C’: Gliotactin overexpression ($ap\text{-}Gli^{WT}$) led to increased PLA signals on the apterous side compared to the WT side.
D-D’: Co-expression of Csk-WT further increased the PLA signals on the apterous side.
E-E’: Reduction of PLA signals by co-expression of Csk-RNAi.
F-F”: Side projections showing the increase of PLA (Gli+pY) mostly at the apical side of the Gli-WT overexpression side (arrow).
G-G”: Side projections showing a further increase of amount of PLA (Gli+pY) in Gli-WT, Csk-WT coexpression. PLA signals were mostly at the apical side (arrow).
H: Statistical analysis of PLA signals (between Gli and pY) in $ap\text{-}Gli^{WT}$ and $ap\text{-}Gli^{WT}$, Csk-WT backgrounds. In each background, the mean ratios of PLA signal intensities in apterous side vs WT side are shown. n=11 discs in $ap\text{-}Gli$-WT and n=9 discs in $ap\text{-}Gli$-WT, Csk-WT. (**** P<0.0001). Bars represent the standard deviation (SD).
Figure 3.6: Co-expression of Csk suppresses deleterious phenotypes of a phosphorylation blocked form of Gliotactin (GliFF).
Third-instar wing imaginal discs with \( ap^{>\text{GliFF}} \) overexpression immunolabeled for Gliotactin (Gli, green), Dlg (red) and DAPI (blue). The apterous boundary is marked with dashed lines. Arrows indicate the leading edge of the Gliotactin expressing migratory cells. Each panel represents a single Z slice taken at the SJ$s$ level. Scale bars: 30 \( \mu \)m. \( n=6 \) in A and \( n=5 \) discs in B. Overexpression of a Gliotactin (\( ap^{>\text{GliFF}} \)) Gliotactin overexpressing cells delaminated and migrated from the apterous side with extra folds due to overproliferation.

B: Co-expression of Csk-WT with \( ap^{>\text{GliFF}} \) led to suppression of above phenotypes.

C-D: Schematic diagrams showing changes to endocytosis and signaling with changes to Gliotactin phosphorylation state as overexpressed Gliotactin forms oligomers or dimers with the endogenous Gliotactin. (C) Overexpressed Gliotactin phosphorylates and leads to both endocytosis and deleterious signaling. (D) A phosphorylation blocked form of Gliotactin (GliFF) has decreased endocytosis. When GliFF is dimerized with endogenous Gliotactin this slows endocytosis and increases deleterious signaling.

### 3.4.4 Csk mediated regulation of Gliotactin is Src independent

Csk is well established as a negative regulator of Src family kinases (Okada et al., 1991; Read et al., 2004; Okada, 2012). Further, Gliotactin can be phosphorylated on conserved tyrosine residues by activated Src using an \textit{in vitro} kinase assay (Padash-Barmchi et al., 2010). This led us to check whether the Csk phenotypes were generated through changes to levels of Src activity.

We tested whether the loss of Src in the Gliotactin overexpression could phenocopy the suppression of Gliotactin phenotypes observed with Csk co-expression. Src levels were reduced by using either loss of function mutants in either of the two \textit{Drosophila} Src genes (\textit{Src42A} and \textit{Src64B}) (Fig. 3.7) or RNAi lines to each (Fig. 3.8A-B). Reduction of Src levels did not rescue the Gliotactin overexpression phenotypes, rather a 50% reduction in the gene dose of each individual Src enhanced the Gliotactin overexpression phenotypes; \textit{Src64B}[\text{D404N}], \textit{Src64B}[\text{KO}] or \textit{Src42A}[\text{myri}] heterozygous mutants did not rescue (Fig. 3.7A, B, C) and thus did not phenocopy the Csk rescue of Gli-WT. We did not follow the enhancement we observed with Src mutants as this is likely due to an additive effect. Since the potential redundancy of the two isoforms of Src cannot be neglected, we also tested a double mutant line heterozygous for
each Src gene (*Src42A[myri], Src64B[D404N]*) in a Gli-WT overexpression background. Even the double mutant failed to suppress the Gli-WT phenotypes (Fig. 3.7D). The quantification of the Gliotactin cell migration ratios showed that reduction of Src activity levels either with mutants or RNAi lines in Gli-WT overexpression situation was not significantly different from Gli-WT overexpression alone (Fig. 3.7E and Fig. 3.8D). In contrast, if Src kinases phosphorylate Gliotactin *in vivo*, coexpression with Src-WT should increase the internalization of Gliotactin leading to a suppression of Gli-WT phenotypes. To test this, we coexpressed Src64B-WT with Gli-WT and found that the phenotypes were not suppressed, but rather enhanced (likely due to an additive effect) (Fig. 3.8C, D). Overall, these results suggest that Gliotactin phosphorylation and/or subsequent signaling events are not generated by Src kinase and the Csk effects on Gliotactin is Src independent.
Figure 3.7: Csk mediated regulation of Gliotactin is Src independent.

Third-instar wing imaginal discs with *ap>Gli-WT* overexpression in Src -/+ mutants immunolabeled for Gliotactin (Gli, green), Dlg (red) and DAPI (blue). Dashed lines mark the boundary between dorsal and ventral compartments with the apterous side on the left. Each panel represents a single Z slice. Scale bars: 30µm. n= 10 discs except n=18 in D.


E: Statistical analysis of cell migration ratios in Gliotactin overexpression alone (*ap>Gli-WT*) compared to expression in Src heterozygous mutant backgrounds. No significant (NS) differences were observed. Bars represent the standard deviation (SD). (n=15 discs).
Figure 3.8: Gliotactin phenotypes are not suppressed by Src loss or gain of function.

Third-instar wing imaginal discs with ap > Gli-WT overexpression in Src mutants immunolabeled for Gliotactin (green), Dlg (red) and Dapi (blue). The dashed lines represent the boundary between...
dorsal and ventral compartments of wing discs with the apterous side on the left. Arrows indicate the leading edge of the migratory cells. Each panel represents a single Z slice. Scale bars: 30µm. n= 10 discs in each genotype.

A-C: Coexpression of Gliotactin with Src64B-RNAi (ap>GLi-WT, Src64B-RNAi) (A), with Src42A-RNAi (ap>GLi-WT, Src42A-RNAi) (B), and with Src64B-WT (ap>GLi-WT, Src64B-WT) (C).

In all three genetic backgrounds, Gliotactin overexpressing cells were migrated to the wild type (ventral) side (A’, B’ and C’), extra folds were visible indicating cell overproliferation (stars) and Dlg was downregulated on the apterous sides (A”, B” and C”).

D: Statistical analysis of cell migration ratios in Gliotactin overexpression alone (ap>GLi-WT) compared to co-expression with Src-RNAi and Src-WT. No significant (NS) differences were observed with Src-RNAi coexpression. However, the Src-WT coexpression showed a significantly higher migration ratio. (****P<0.0001). Error bars represent standard deviation (SD). (n=10 discs).

3.4.5 Co-expression of Csk with Gliotactin increases the endocytosis of Ecad

Loss of Csk has varying effects depending if the reduction is over a broad area or over a discrete region neighboring wild type cells (Vidal et al., 2006). For instance, the knock-down of Csk within an entire tissue leads to mispatterning and overgrowth while blocking apoptosis. However, Csk knock-down in a discrete region of the wing imaginal disc leads to cell delamination, migration and cell death via apoptosis (Vidal et al., 2006). This has been attributed to increased Src activity leading to decreased levels of the adherens junction protein Ecad in Csk downregulated cells. Vidal et al., (2006) suggested that the relatively high levels of Ecad on the wild type side trigger the neighboring cells with low Ecad to undergo JNK-mediated apoptosis. As Gliotactin overexpression using the apterous driver leads to cell delamination, migration and JNK mediated-apoptosis (Padash-Barmchi et al., 2010), we tested for changes in Ecad levels at the interface between wild type and Gliotactin overexpressing cells. The overexpression of Gliotactin in the apterous side does not lead to a downregulation of Ecad in the apterous side (Padash-Barmchi et al., 2010). Instead, we observed that Ecad levels were often elevated in the apterous side (Fig. 3.9A”, B”). Interestingly, the along with elevated Ecad at the AJ, we observed
some Gliotactin vesicles were also positive for Ecad (arrows in Fig. 3.9A) suggesting that there is a change to Ecad endocytosis in Gliotactin overexpression. Ecad downregulation as well as upregulation can lead to cell migration depending on the context (Niewiadomska et al., 1999). Therefore, we tested whether there was a change in Ecad levels when the Gliotactin triggered phenotypes were suppressed by co-expression of Csk (Fig. 3.9C, D). In ap>Gli-WT, Csk-WT discs we observed that there was an increased level of endocytosis of Ecad (arrows in Fig. 3.9C) as well as the increased presence of Ecad within the membrane. The Ecad filled vesicles were also positive for Gliotactin. According to Vidal et al., (2006), increased Csk activity would lead to decreased Src activity reducing the Ecad endocytosis and this could explain the increased levels of Ecad we observed. The co-endocytosis with Gliotactin when Gliotactin is overexpressed, with or without Csk, may suggest that Ecad becomes internalized along with Gliotactin when Gliotactin spreads (Fig. 3.9C”, D”). Since there was an elevation in Ecad, we tested whether there was a change to another adherens junction protein using antibodies to Drosophila β-catenin (Armadillo (Arm)) and found that Arm immunolabeling intensities were the same in the apterous side compared to wild type side in ap>Gli-WT (Fig. 3.10A, B) as well as in ap>Gli-WT, Csk-WT (Fig. 3.10C, D).

To test if the changes to Ecad levels might be part of the suppression of the Gliotactin phenotypes, we reduced the shotgun (Drosophila Ecad) gene dosage by 50%. Specifically, Gliotactin was expressed (ap>Gli-WT) in conjunction with mutant alleles of Ecad (shotgun (shg); shg[E17B]/+ and shg[2]/+ separately (Fig. 3.10E). Conversely, we also increased the
Figure 3.9: Co-expression of Csk with Gliotactin increases the endocytosis of Ecad.

High-magnification images (60X) from the pouch area of third-instar wing imaginal discs with transgenes or RNAi lines expressed by apterous-GAL4 (ap>) driver. Discs were immunolabeled
for Gliotactin (green), Ecad (red) and DAPI (blue). The dashed lines represent the boundary between dorsal and ventral compartments of wing discs where the apterous side (dorsal compartment) is on the left. Arrows pointing to colocalization of Ecad with Gliotactin filled vesicles. Each en face panel represents a single Z slice taken at the septate junction level. Scale bars: 15µm. n=15 discs in each genotype.

A: ap>Gli-WT. On the apterous side, Gliotactin was found around the membrane and in internal vesicles (A and A’). Ecad was upregulated in the Gliotactin overexpressing apterous side (A”). Some of the Gliotactin vesicles were colocalized with Ecad in the cytoplasm (A’ and A”).

B: Corresponding side projections of the above panels. B’ showed the basolateral spreading of Gliotactin alone the lateral membrane and migration of Gliotactin positive cells to the wild type side. B” showed the up-regulation of Ecad in the apterous side in both apical and lateral cell membranes.

C: ap>Gli-WT, Csk-WT. Co-expression of Csk-WT led to an increased abundance of large vesicles in the apterous side (C and C’). There was an upregulation of Ecad level in the cell membranes of cell in apterous side (C”). In the cytoplasm, Ecad was colocalized with the majority of Gliotactin positive vesicles (C’ and C”).

D: Corresponding side projections of the above panels. D’ showed the suppression of cell migration of Gliotactin positive cells to the non-apterous side. D” showed the upregulation of Ecad in both apical and lateral cell membranes.

E: ap>Gli-WT, Csk-RNAi. Gliotactin was found around the membrane and formed only small vesicles (E’ and E”’). Changes to Ecad level or Ecad endocytosis in apterous side compared to wild type was not as clear as other two genetic backgrounds (A” or C”).

F: Corresponding side projections of the above panels.

Ecad level by co-expressing Ecad (UAS-Shg) with Gli-WT (Fig. 3.10F). However, neither loss of Ecad nor coexpression of Ecad lead to a suppression of the Gliotactin triggered phenotypes suggesting that the suppression of Gliotactin phenotypes by Csk is not mediated through changes to Ecad. Overall, these results suggest that the suppression of the Gliotactin overexpression phenotypes by Csk is independent of changes to both Src and Ecad.
Figure 3.10: Gliotactin phenotypes are independent of changes to the adherens junctions.
A-D: High-magnification images (60X) from the pouch area of third-instar wing imaginal discs with transgenes expressed using apterous-GAL4 (ap>) driver. Discs were immunolabeled for
Gliotactin (green), Arm (red) and DAPI (blue). The dashed lines represent the boundary between dorsal and ventral compartments of wing discs where the apterous side (dorsal compartment) is on the left. Each en face panel represents a single Z slice taken at the septate junction level. Scale bars: 15µm. n=10 discs in each genotype.

A: ap>Gli-WT. On the apterous side, Gliotactin was found around the membrane and in internal vesicles (A and A’). Immunolabeling of Arm was not changed with Gliotactin overexpression compared to wild type side (A”).

B-B”': Corresponding side projections of the above panels. Gliotactin was found along the lateral membrane and Gliotactin positive cells migrated along the basal surface into the wild type side of the wing disc (B’). Arm localization and levels were the same on the apterous side compared to wild type side (B”).

C: ap>Gli-WT, Csk-WT. Co-expression of Csk-WT led to an increase in the amount of large Gliotactin vesicles on the apterous side (C, C’). There was no upregulation of Arm in the apterous side (C”).

D-D”': Corresponding side projections of the above panels.

E-F: The low-magnification (20x) images of third-instar wing imaginal discs with ap>Gli-WT overexpression in different gene dosages of Shotgun (Shg, Ecad), immunolabeled for Gliotactin (green), Dlg or GFP (red) and DAPI (blue). Reduction of Shg (Ecad) in a Gliotactin overexpressing wing disc, ap>Gli-WT, shg[2]/+ (E) or co-expression of Shg (ap>Gli-WT, Shg::GFP) (F) did not change the Gliotactin overexpression phenotypes. Gliotactin positive cells still migrated to the wild type side and extra folds were formed indicating cell overproliferation (stars) (E’ and F’). Arrows indicate the leading edge of the migratory cells. Each panel represents a single Z slice. Scale bars: 30 µm. n=10 discs in each genotype.

3.4.6 Csk activity on Gliotactin might be indirect

Since our data suggested that the Csk activity on Gliotactin was Src and Ecad independent, we wanted to test if Csk was able to directly phosphorylate Gliotactin in vitro. Histagged fusion proteins of the Gliotactin intracellular domain (Gli-WT) (Padash-Barmchi et al., 2010) were tested in vitro using a Csk kinase assay (Fig 3.12A). While Csk was able to phosphorylate the positive control Src, Gli-WT was not phosphorylated (Fig. 3.12A). However, substrate recognition by Csk depends on both the correct three-dimensional structure along with the target amino acids (Ia et al., 2010). As the C-terminal domain used in our assays is unfolded in vitro (Zeev-Ben-Mordehai et al., 2003) it maybe that Gliotactin does not have the correct structural properties in vitro for recognition by Csk.
To determine if Gliotactin and Csk can associate in vivo, we tested for the distribution of Csk in wild type and Gliotactin overexpressing imaginal discs (Fig. 3.11; Fig. 3.12B-E). In wild type (w^{1118}), Csk immunolabeling was found throughout the cytoplasm with puncta-like labeling found colocalized with Ecad (Fig. 3.11B; Fig. 3.12B) and but not colocalized with Gliotactin (Fig. 3.11A,B). When Gliotactin was overexpressed, Csk levels at the apical side did not change (Fig. 3.11C”, D”) but increased Csk immunolabeling was now observed at the basal side of the epithelium in the areas of the delaminated Gliotactin expressing cells (Fig. 3.11D”). This immunolabeling pattern was different from that in wild type epithelium (Fig. 3.11B”) and suggested a redistribution of the endogenous Csk when Gliotactin was overexpressed. When overexpressed (ap>Csk-WT), Csk levels increased in the cytosol and at the membrane showing a clear colocalization with Ecad at the AJ (Fig. 3.11G, H; Fig. 3.12D). When coexpressed with Gliotactin (ap>Gli-WT, Csk-WT), Csk colocalized with Gliotactin both at the membrane and in the large Gliotactin vesicles (Fig. 311I”, J”, I”). Overall, the changes to the localization pattern of Csk with changes to Gliotactin and colocalization of overexpressed Csk with Gliotactin suggest that Csk could regulate Gliotactin in vivo.

Co-localization of both endogenous and overexpressed Csk with overexpressed Gliotactin (Fig. 3.11C, I) suggested that Csk may associate with Gliotactin. We carried out PLA assays to test if these two proteins were in close proximity (<40 nm) in vivo. We observed consistent PLA signals between Csk and Gliotactin when Gli-WT was expressed alone (Fig. 3.13A) and the PLA signals were concentrated at the AJ apical to the SJ (Fig. 3.13B”). When Gli-WT and Csk-WT were coexpressed, we observed increased PLA signals that were also concentrated at the apical side within the AJ (Fig. 3.13C”, D”). Of interest was that the concentration of the PLA signals were above the septate junction domain likely in the AJ
domain, even while both Gli-WT and Csk-WT were distributed over a much wider area. This suggests that the close proximity of Gli-WT and Csk-WT was limited to this region possibly due to the presence of a necessary intermediate protein or co-factor. Conversely when Gli-WT was coexpressed with Csk-RNAi, the PLA signals were reduced (Fig. 3.13E”, F”). Overall, these in vivo data show that when Gliotactin is overexpressed, Csk is in close proximity to Gliotactin but only in a discrete region of the epithelium and suggests that Csk may phosphorylate Gliotactin or a closely associated protein to Gliotactin within this domain.
Figure 3.11: Csk distribution.

High-magnification images (60X) from the wing pouch from wild type or with transgenes or RNAi lines expressed by apterous-GAL4 (ap>) driver. Discs were immunolabeled for Gliotactin (Gli, green), Csk (red) and Ecad or DAPI (blue). Dashed lines mark the apterous boundary where the apterous side (dorsal compartment) is on the left. Each en face panel represents a single Z slice taken at the septate junction level. Areas that are marked by boxes were digitally magnified by 28 times and placed at the end of the corresponding row. Arrowheads mark the adherens junction in side projections. Scale bars: 15µm except in 5µm digitally magnified panels. n=10 discs in each genotype.

A: Wild type disc with Gliotactin labeling at the corners of the cells and Csk puncta throughout the cytoplasm. Csk does not colocalize with Gliotactin (A’’).

B: Corresponding side projections with Gliotactin at the septate junction (B’). Csk labeling was throughout the cytoplasm, less abundant in the SJ domain and colocalized with Ecad in the adherens junctions (B”, B’’’).

C: ap>Gli-WT wing discs showed no clear difference in the Csk labeling in the Gliotactin overexpressing side.

D: Corresponding side projections with Csk labeling concentrated at the basal side (D’’) in the delaminated and migrating Gliotactin overexpressing cells (arrows) (D’ and D’’”). There was an increase in Ecad on the apterous side (D’’’).

E and G: ap> Csk-WT. The apical side of the wing disc with Csk distributed through the cytoplasm and at the membrane. Gliotactin distribution to the TCJ was unchanged (E’). Csk was at the membrane and concentrated at the adherens junctions level (G and G’). There was no change to the Ecad level or localization (G’).

F and H: Corresponding side projections showed an elevated level of Csk through the epithelia with a concentration at the AJ (F and H). In the Csk overexpressed apterous side, Gliotactin was spread further along the lateral membrane compared to the control side (F’). There was no difference in Ecad expression or distribution with Csk overexpression (H’).

I: ap> Gli-WT, Csk-WT. Gliotactin was concentrated in large vesicles (I’) along with Csk (arrow) (I”, I’’’).

J: Corresponding side projections showed elevated levels of Gliotactin and Csk and the spread from apical to basal in the apterous side (J’ and J’’”). In the apterous side Ecad was upregulated (J’’’).
Figure 3.12: Assays of Csk phosphorylation of Gliotactin *in vitro* and *in vivo*. 
A: Gliotactin is not phosphorylated by Csk in vitro. A Csk kinase assay using a Csk–GST fusion with His-tagged fusion proteins of the C-terminal domain of Gliotactin (Gli-WT) or whole human Src. Western blots were immunolabeled with a phospho-tyrosine antibody (pY, green) and Gliotactin (red). Src but not Gli-WT was phosphorylated in the presence of Csk.

B-E: High-magnification images (60X) from the wing pouch from wild type (w^1118) or from transgenes expressed by apterous-GAL4 (ap>) driver. All the panels were digitally magnified by 28x and are single Z slices taken at the level of adherens junction within the apterous side. Discs were immunolabeled for Gliotactin (Gli, green), Csk (red) and Ecad (blue). Scale bars: 5µm. n=10 discs in each genotype.

B: Wild type. The wing imaginal disc with weak Gliotactin labeling at the corners of the cells at this level and strong Ecad labeling of the adherens junction Csk puncta. Some of the Csk puncta overlapped with Ecad immunolabeling (B).

C: ap>Gli-WT. Wing discs showed no clear difference in the Csk labeling in the Gliotactin overexpressing side (C”). Overexpressed Gliotactin spread around the cell and apically and was colocalized with Ecad at the adherens junctions (C’ and C”’).

D: ap>Csk-WT. Csk is concentrated at the cell membrane and colocalized with Ecad at the level of adherens junctions.

E: ap>Gli-WT, Csk-WT. Gliotactin was found on the cell membrane and in large vesicles that were also positive for Csk and occasionally for Ecad (arrow).
Figure 3.13: Csk is in close proximity with overexpressed Gliotactin at the AJ.
High-magnification images (60x) from the wing pouch with transgenes or RNAi lines expressed by the apterous-GAL4 (ap>) driver. PLA signals between Gliotactin and Csk (red), immunolabeling for Mcr (green) and labeling for DAPI (blue) or Ecad (blue). Dashed lines mark the apterous boundary where the apterous side (dorsal compartment) is on the left. Each panel represents a single Z slice at the septate junction level. Scale bars: 15µm. n=10 except n=12 discs in A.

A: ap>Gli-WT. PLA signals for Gliotactin + Csk were increased compared to the wild type side (A and A”).

B: In the corresponding side projections, the PLA signals were concentrated at the apical side of the epithelium (arrow) (B”).

C: ap>Gli-WT, Csk-WT. Co-expression of Csk-WT increased the Gliotactin+Csk PLA in the apterous side (C and C”).

D: Corresponding side projections. PLA signals were concentrated in the apical side of the epithelium (arrow) (D”).

E: ap>Gli-WT, Csk-RNAi. PLA signals between Gliotactin + Csk were decreased when the Csk was knocked-down (E and E”). The apterous boundary is hard to determine and was not marked.

F: Corresponding side projections.

G: Statistical analysis of the PLA (between Csk and Gli) signal intensities in ap>Gli-WT and ap>Gli-WT, Csk-WT. The mean ratios of PLA intensities in the apterous side vs WT side are shown. n=12 discs in ap>Gli-WT and n=10 discs in ap>Gli-WT, Csk-WT. (**** P<0.0001). Error bars represent the standard deviation (SD).

3.4.7 Downregulation of Csk affects endogenous Gliotactin

Overall our results suggest that Csk associates with Gliotactin and triggers endocytosis either by directly or indirectly phosphorylating Gliotactin. Overexpression of Csk and Gliotactin increased the levels of endocytosis, whereas the downregulation of Csk decreased the levels of endocytosis. If Csk is able to regulate Gliotactin levels, we hypothesize that a loss of Csk in an otherwise wild type background would lead to a reduction of phosphorylation and endocytosis of endogenous Gliotactin. To test the loss of Csk on endogenous Gliotactin, we expressed Csk-RNAi using the apterous-GAL4 driver (Fig. 3.14). Prior work has found that the overall morphology of the wing disc is altered depending on the area where Csk is knocked-down. For instance, cell delamination and migration of delaminated cells are pronounced phenotypes in wing discs with patched-GAL4 driven Csk-RNAi (Vidal et al., 2006). While knock-down of Csk
in the entire wing pouch (with nub-GAL4) resulted in overproliferation as the key phenotype (Kwon et al., 2015). We observed that the knock-down of Csk with apterous-Gal4 resulted in overproliferation and extra folds of the wing discs without generating a clear cell delamination or migration phenotype (Fig. 3.14B). To detect whether the endogenous Gliotactin was affected due to loss of Csk, the least affected areas (areas still in the apterous side but that did not have many extra folds) were examined. Compared to the Gliotactin localization in the control (ap>NLS GFP) (Fig. 3.14A’, E”) it was clear that, even in the least affected areas, the tight localization of Gliotactin to the TCJ was lost when Csk was knocked-down (Fig. 3.14C”, D”’). Instead, Gliotactin spread away from the TCJ and formed extended ribbon-like structures through the septate junction (Fig. 3.14E, E’). In these areas, other septate junction markers, Dlg and Cora were not mislocalized and remained within the SJ domain (Fig. 3.14C”, D”). Similarly, Ecad was not mislocalized (Fig. 3.15A, B) suggesting that the effect on Gliotactin was not due to loss of either the SJs or AJs. These data suggest that Csk has a specific effect on controlling the localization and/or levels of endogenous Gliotactin. In total, our results suggest that Csk plays a specific role in limiting the expression of Gliotactin to the TCJ likely through phosphorylation and endocytosis of Gliotactin.
Figure 3.14: Downregulation of Csk affects endogenous Gliotactin.
Third-instar wing imaginal discs with transgenes or RNAi lines expressed by the apterous-GAL4 (ap>) driver. The apterous boundary is indicated with a dashed line with the apterous side on the left. Each panel represents a single Z slice at the septate junction level. Scale bars: (A-B 30µm), (C-D 15µm) and (E-E”) 5µm. n=10 discs except n=15 discs in C. 
A-B: Wing discs immunolabeled for Gliotactin (Gli, green), NLS-GFP (red) and DAPI (blue). Arrows indicate the leading edge of the migratory cells.
A: ap>NLS-GFP. There was no cell overproliferation or migration of control NLS-GFP positive cells compared to the non-apterous side.
B: ap>Csk-RNAi, NLS-GFP. Knock-down of Csk resulted in overproliferation resulting in extra folds. The entire disc was filled with NLS-GFP due to overproliferation making it hard to identify the apterous side.
C-D: Higher magnification images (60x) of ap>Csk-RNAi, NLS-GFP discs immunolabeled for Gliotactin (Green) and Dlg or Cora (red). The images were taken from least affected areas of least affected discs. C’ and D’ showed Gliotactin was spreading away from tricellular corners making circular or ribbon-like structures. C” and D” showed that the Dlg and Cora immunolabeling were not affected, respectively.
E and E’: 28 times digitally magnified images of areas marked by boxes in C’ and D’ respectively. Arrows showing that the tight regulation of Gliotactin at the TCJs was lost. In E”, the arrow is pointing to the punctate-like localization of Gliotactin at the TCJs in wild type wing disc (E”) at the same magnification as E and E’.

Figure 3.15: Downregulation of Csk does not lead to loss of polarity.
High-magnification images (60X) from the wing pouch expressing ap>Csk-RNAi, NLS-GFP. Discs were immunolabeled for Ecad (green), Gliotactin (red). DAPI or GFP is in blue. En face panels represent a single Z slice taken at the adherens junction level. Scale bars: 15µm. n=10 discs.
A: Ecad was localized to the apical side and was found around the cell membrane as in wild type epithelia (A’).
B: Corresponding side projections of the above panels showed that both Ecad (B’) and Gliotactin (B’’) localized apically and in extra folds (arrows indicate extra folds).

3.5 Discussion

The level and the correct localization of Gliotactin is tightly controlled by phosphorylation followed by endocytosis (Padash-Barmchi et al., 2010) and microRNA-mediated degradation of Gliotactin mRNA (SharifKhodaei et al., 2016). When overexpressed, the spread of Gliotactin away from the tricellular junction leads to aberrant phenotypes disrupting the entire epithelial architecture. Tyrosine phosphorylation of Gliotactin itself is a mechanism to control Gliotactin levels and localization (Padash-Barmchi et al., 2010). However, overexpression studies using different Gliotactin constructs (i.e., Gli-WT, GliDD and GliFF) in both wildtype and in Gli-/- null backgrounds Padash-Barmchi et al (2010) showed that Gliotactin phosphorylation also initiates the signaling pathway(s) that lead to the aberrant phenotypes. Here we demonstrate that a tyrosine kinase, C-terminal Src kinase (Csk) regulates Gliotactin through a Src-independent activity. Loss of Csk strongly enhanced the Gliotactin overexpression phenotypes, and co-expression of Csk suppressed the phenotypes. Loss of Csk also led to the spread of Gliotactin away from the TCJ when Gliotactin is expressed at normal physiological levels. Overall our data show that Csk controls both overexpressed and endogenous Gliotactin suggesting a novel role for Csk in controlling the protein components of the TCJ.

Our results point to a clear role for Csk in regulating Gliotactin phosphorylation, endocytosis and protein distribution. However, the effect of Csk on Gliotactin could be indirect through the actions of another kinase or direct where Csk itself phosphorylates Gliotactin. Csk is
a well-established regulator of Src kinase signaling and the intracellular domain of Gliotactin is phosphorylated by activated Src in vitro (Padash-Barmchi et al., 2010). This suggested Csk indirectly regulates Gliotactin through Src. However, we found that the effects of Csk on Gliotactin were independent of Src. Overexpression of Csk suppressed the Gliotactin phenotypes and a reduction of either or both Drosophila Src homologues did not. Overexpression of Src did not suppress the Gliotactin phenotypes, also supporting the conclusion that the in vivo regulation of Gliotactin by Csk is not mediated by Src. Evidence from our kinase assay suggested that the Csk activity on Gliotactin might not be direct either as Csk was not able to phosphorylate Gliotactin in vitro. However, the lack of phosphorylation by Csk in vitro maybe due to a lack of a specific co-factor for Csk activity. Alternatively, the recognition of a substrate by Csk is dependent on the native three-dimensional structure of the substrate and not on the sequence of the substrate protein (Ia et al., 2010). For instance, Src is not phosphorylated by Csk when a synthetic or truncated form of Src is provided, even when the targeted phosphorylation site is present (Ruzzene et al., 1997). Thus Gliotactin may not have the correct structural properties in vitro for recognition by Csk, especially as the C-terminal domain of Gliotactin is natively unfolded in vitro (Zeev-Ben-Mordehai et al., 2003).

Our in vivo evidence pointed to an increase in phosphorylation of Gliotactin or a protein that are in close proximity to Gliotactin in the presence of increased Csk along with increased endocytosis. The positive PLA signals between Csk and Gliotactin when both proteins were co-expressed suggested that Csk can associate and perhaps directly phosphorylate Gliotactin. However, this only appears to occur when Gliotactin spreads away from the TCJ. In wild type wing discs, Csk is clearly absent from the TCJs but present at the adherens junctions (AJs) suggesting that Csk is normally excluded from contact with Gliotactin in wild type epithelia. The
PLA signals between overexpressed Gli and endogenous Csk were concentrated apical to the septate junction domain within the AJ. This suggests that the amount of Csk available to interact with Gliotactin is limited or spatially restricted. We observed that the PLA between Gliotactin and Csk was more concentrated apical to the SJ domain even when both overexpressed Gliotactin and Csk spread beyond their normal domains. This latter point suggests that Csk association with Gliotactin requires an intermediate whose distribution is limited to the adherens junction domain. If Csk directly phosphorylates Gliotactin, then the intermediate(s) could be associated with the adherens junction and be responsible for recruiting Csk and potentially activating Csk at the AJ. Alternatively, the recruitment of Csk to the AJ may activate another tyrosine kinase, which in turn phosphorylates Gliotactin.

Csk is a cytosolic tyrosine protein kinase (Cooper and Howell, 1993). Similar to Src, Csk contains amino-terminal Src homology, SH3 and SH2 domains, as well as a carboxyl-terminal catalytic domain. In contrast to Src, Csk is not myristoylated and thus other membrane proteins or lipids recruit Csk to the membrane and cell junctions (Hirao et al., 1997; Kwon et al., 2015). One mechanism is through the recruitment of Csk through binding of the SH2 domain to phospho-tyrosine residues of transmembrane protein and membrane-associated proteins. For instance, in vertebrate endothelia, Csk directly binds with phosphorylated VE-Ecad, further increasing VE-cadherin phosphorylation (Baumeister et al., 2005). Csk is recruited to focal adhesions through Pragmin in mammals and Paxillin in Drosophila (Rengifo-Cam et al., 2004; Senda et al., 2016). Pragmin has a tyrosine-phosphorylation motif which has a higher affinity towards Csk when phosphorylated (Senda et al., 2016). In platelets, the Junctional Adhesion Molecule-A (JAMs-A) act as a Csk binding protein when phosphorylated and recruits Csk to the integrin-Src complex to suppress Src activity (Naik et al., 2014). In addition, PAG1/CBP
(Phosphoprotein associated with glycosphingolipid-enriched microdomains 1/CSK binding protein) is localized to lipid rafts and functions to control activation of Src through recruitment of Csk (Okada, 2012). PAG has five Src binding sites as well as one Csk SH2 binding site (Okada, 2012). In Drosophila, dASPP (Ankyrin-repeat, SH3-domain, and proline-rich-region containing protein) is located at the AJ and functions as a Csk binding protein and as a positive regulator of Csk kinase activity (Langton et al., 2007). Given the localization of Csk to the AJ domain, it is possible that Ecad, dASPP or another AJ-associated protein could function as a potential intermediate between Csk and Gliotactin.

Another possibility is that Gliotactin can interact with Csk directly but only in the context of a tyrosine kinase that is located at the AJ. As outlined above, normal recruitment of Csk to the membrane involves binding of the Csk SH2 domain to a phospho-tyrosine residue. As Gliotactin has two highly conserved tyrosines (Y760, Y799), phosphorylation of one tyrosine could be necessary for binding to Csk and subsequent phosphorylation of the second residue. When overexpressed, Gliotactin is phosphorylated at one or both conserved tyrosine residues (Padash-Barmchi et al., 2010) and it is not known whether these sites are redundant or have different functions. It is possible that there is a second tyrosine kinase associated with the AJ that phosphorylates one residue to recruit Csk, which in turn phosphorylates the other. While the phosphotyrosine residues conserved in Gliotactin match predicted SH2 binding sequences for a range of proteins, they are not informative as whether they can recruit Csk as no consensus sequence for Csk binding has emerged (Ruzzene et al., 1997). However, the increase in Ecad level with the overexpression of Gliotactin supports a model where Gliotactin can recruit Csk and activate Csk, while our PLA data suggest that this can only happen within the AJ domain. The increase in Ecad levels at the membrane when Gliotactin and Csk are coexpressed along
with the increase in phosphor-Tyr and Gliotactin PLA at the AJ support this model. Whether Csk interacts with proteins in a phosphorylation independent mechanism is not known (Kwon et al., 2015). However, after the initial binding with phosphorylated Pragmin, Csk is known to phosphorylate un-phosphorylated Pragmin (Senda et al., 2016) suggesting Csk does not only interact with phosphoproteins. Similarly, Gliotactin may also bind with Csk at the adherens junctions and later being phosphorylated by Csk.

Our results determined that Csk directly or indirectly phosphorylates Gliotactin, and this mechanism does not involve either of the two Drosophila Src proteins (Src42A or Src64B). Csk can phosphorylate and regulate other kinases, in particular, LATS/Warts (Wts), which acts in the Hippo signaling pathway to suppress Yorkie (Yki) activation (Stewart et al., 2003; Kwon et al., 2015). dCsk has been proposed to phosphorylate LATS in a Src-independent fashion (Pedraza et al., 2004). However, Vidal et al., (2006) suggested that most or all of dCsk activity that is dependent on Lats also requires Src and argued against Src-independent links between dCsk and Lats. Wts is both cytosolic and co-localized with E-cadherin at the membrane (Sun et al., 2015). However, Wts is a serine/threonine kinase and not known to act as a dual specific kinase, hence it is unlikely that Csk controls Gliotactin tyrosine phosphorylation directly through Wts.

Among other kinases, Csk directly interacts with and phosphorylates c-Jun increasing the ubiquitination-mediated degradation of c-Jun and ultimately reducing cell proliferation, transformation and apoptosis (Zhu et al., 2006). Jun kinase (JNK) is a key regulator of the Gliotactin overexpression phenotypes, where blocking JNK activity with a dominant negative form suppresses the overexpression phenotypes (Padash-Barmchi et al., 2010; Padash-Barmchi et al., 2013). Therefore, it is possible that Csk is working through the JNK pathway as increased Csk would lead to a downregulation of Jun and thus suppress the Gliotactin phenotypes.
However, this model requires the involvement of another tyrosine kinase that is either Csk or JNK regulated as it is clear that the downregulation of Gliotactin requires tyrosine phosphorylation.

Regardless of whether Csk directly or indirectly phosphorylates Gliotactin, Csk is clearly regulating Gliotactin to control protein levels and localization to the TCJ. As Csk overexpression strongly suppressed the Gliotactin overexpression phenotypes, it is unlikely that Csk is responsible for the phosphorylation-dependent Gliotactin signaling. Rather Csk appears to control Gliotactin endocytosis to block the spread of Gliotactin from the TCJ such that when Csk is downregulated, endogenous Gliotactin is no longer tightly associated with the tricellular corners and spreads. It is possible that the effect on endogenous Gliotactin is due to disruption of other proteins known to regulate Gliotactin localization to the TCJ, Discs-large (Dlg) (Padash-Barmchi et al., 2013) and Bark/Anakonda (Byri et al., 2015). Loss of either leads to the spread of Gliotactin at the TCJ but also a dramatic down-regulation of Gliotactin on the membrane. With Csk knock-down, Gliotactin levels were not reduced and spread was not throughout the membrane suggesting that mechanism was not mediated by changes to either Dlg or Bark/Anakonda. Rather the spread of Gliotactin observed with the loss of Csk was similar to that seen when endocytosis was blocked using a dominant negative Rab5 (Padash-Barmchi et al., 2010). Overall our data suggests that the normal role of Csk is to control the phosphorylation of Gliotactin and endocytosis to ensure correct localization to the TCJ.

Csk is a cytosolic tyrosine kinase and a classic negative regulator of Src. Multiple other activities that are independent of controlling Src have been also reported. Csk null mouse embryos show various developmental defects indicating that Csk is an essential protein for development (Imamoto and Soriano, 1993). Further, the roles of Csk can be diverse as it has both
tumor suppressor as well as oncogenic properties (Okada, 2012; Yao et al., 2014). The role of Csk in controlling junctional proteins or signaling events at cell-cell or cell-matrix junctions either directly or through regulation of Src activity is known. However, a prior role for Csk in controlling proteins or signaling events in tricellular or bicellular septate junction is not known. We have uncovered a novel role for Csk as a potential regulator of tricellular junctions independent of the canonical function of Csk. It remains to be determined whether Csk activity on Gliotactin is direct or indirect, however, further investigation of the role of Csk in controlling TCJ targets could identify novel targets and functions for Csk. Control of the levels and distribution of tricellular junction proteins is important. For instance, overexpression of the vertebrate tricellular junctional protein Tricellulin leads to tumor formation in some tissues i.e., adenocarcinoma in human pancreatic ducts (Kojima and Sawada, 2012). Similarly, overexpression of Gliotactin in polarized epithelia leads to aberrant phenotypes including tissue overproliferation and migration of delaminated epithelial cells. Therefore, Gliotactin overexpression could be a useful model to understand the mechanisms and interactors associated with upregulation of tricellular junctional proteins beyond their normal distribution.
Chapter 4: Discussion and conclusions

Tricellular junctional proteins are tightly localized to tricellular corners and this correct localization seems to be critically important in both invertebrates and vertebrates. However, why the upregulation or mislocalisation of TCJ proteins are detrimental and which signaling pathways are activated are largely unknown, as are the cellular mechanisms necessary to restrict these proteins to the tricellular corners.

The focus of this thesis was on one of the key TCJ proteins in Drosophila, Gliotactin. Gliotactin was identified as a tricellular junction specific protein more than two decades ago and recent evidence suggest that in addition to barrier functions Gliotactin has other cellular roles as well. For instance, Gliotactin is suggested to regulate mitotic cell division in various ways including the orientation of cell division (Bosveld et al., 2016) and control of mitosis (Resnik- Docampo et al., 2016). However, the level and the localization of Gliotactin must be tightly regulated. If not, Gliotactin spreads to other junctional domains and has the potential to disrupt the entire epithelial architecture (Padash-Barmchi et al., 2010). There are at least two mechanisms to regulate Gliotactin protein levels and localization. One pathway involves regulation of Gliotactin mRNA levels through micro-RNA mediated degradation (SharifKhodaei et al., 2016). The other pathway involves tyrosine phosphorylation of Gliotactin followed by endocytosis mediated degradation (Padash-Barmchi et al., 2010). On the other hand, Padash-Barmchi et al (2010) also showed that the tyrosine phosphorylation of Gliotactin at the membrane is detrimental as this leads to deleterious phenotypes including cell delamination, cell migration, apoptosis and cell overproliferation and ultimately disruption of the epithelia. However, prior to this thesis, the signaling molecules involved in generation of Gliotactin overexpression-induced phenotypes were largely unknown. In addition to the tyrosine
phosphorylation sites Gliotactin has highly conserved predicted serine phosphorylation sites (Fig. 4.1) and the function of these phosphorylation sites or potential kinases associated with these have not been identified. The goal of this thesis was to investigate the kinase mediated control of the level, localization, and signaling events associated with tricellular septate junction protein Gliotactin. To identify phosho-regulators of Gliotactin, we carried out a RNAi screen on the Drosophila kinome and kinase associated proteins using the columnar epithelium of Drosophila wing disc.

In the study presented in chapter two, we identified kinases and some kinase associated proteins that act as modifiers, including both suppressors and enhancers of Gliotactin overexpression phenotypes. We concluded that the Gliotactin phenotypes are generated through the involvement of at least two signaling pathways. One pathway is through Jun kinase (JNK) through the newly identified TNF receptor Grindewald (Grnd) and in a Tak1, TAB2 and TRAF6 dependent manner. The other pathway is activated through PI3K-Akt. We also concluded that BTK29A is a signaling component that may be activated in a Gliotactin overexpression background. In addition to these signaling pathways, we identified Csk, PI4KIII-α, Sktl, and Pkaap as candidates for further investigation for their potential to regulate endogenous Gliotactin protein level and/or localization. These are likely involved in regulation of endocytosis or trafficking of Gliotactin to the correct junctional domain as the knock-down of these proteins led to mislocalisation of Gliotactin.

In the study presented in chapter three, we investigated C-terminal Src kinase (Csk), one of the modifiers of Gliotactin phenotypes identified in the primary screen. Knock-down of Csk enhanced the Gliotactin-mediated phenotypes whereas co-expression of Csk suppressed
Figure 4.1: Conserved phosphorylation sites of Gliotactin.
Sequence alignment of part of Gliotactin C-terminus of Arthropods. Two conserved tyrosine residues are shown in blue and conserved serine residues are in red.
phenotypes. We demonstrated that Csk suppressed Gliotactin phenotypes, most likely by increasing Gliotactin phosphorylation and endocytosis. We found that Csk controls the level and/or localization of endogenous Gliotactin as well. Interestingly, our data show that the control of Gliotactin phenotypes by Csk was independent of Src and changes to Drosophila E-cadherin, different from the normal role of Csk on Src and Ecad regulation. (what is marked in yellow is not certain).

4.1 The kinase control of TCJ protein localization

Similar to Gliotactin, tight regulation of TCJ proteins seems to be a global requirement as the downregulation, upregulation and/or mislocalisation of vertebrate tricellular junctional proteins also have been reported in a range of cancers (Kondoh et al., 2011; Patonai et al., 2011; Korompay et al., 2012; Shimada et al., 2016; Takasawa et al., 2016). However, details about how TCJ proteins are localized to the tricellular corners are largely unknown. The phosphorylation followed by endocytosis is one mechanism to regulate this. Therefore, we hypothesized that kinases play a key role in achieving this restricted localization and identified Csk as likely a tyrosine kinase that controls the Drosophila TCJ protein Gliotactin. The caveats of the experiments, potential future experiments, the possible mechanisms and intermediates that involve with Csk in regulating Gliotactin as well as whether it is possible that Csk also controls vertebrate TCJ are discussed below.

The C-terminal Src kinase is well known for its negative regulatory function in Src family kinases (Okada et al., 1991). As shown in this thesis, changes to Csk protein level change the Gliotactin overexpression phenotypes and downregulation of Csk in an otherwise wild type background leads to spread of Gliotactin away from TCJs, showing a strong relationship between
Gliotactin and Csk. Further, the PLA signals between phosphotyrosine and Gliotactin, as well as the number of Gliotactin positive large vesicles were increased when Gliotactin was co-expressed with Csk (ap > Gli-WT, Csk-WT), suggesting Csk changes the phosphorylation and subsequent endocytosis of Gliotactin. However, the kinase assay showed that Csk was not able to directly phosphorylate the C-terminal end of Gliotactin in vitro suggesting Csk might not directly phosphorylate Gliotactin.

If Csk is not the kinase responsible for direct phosphorylation of Gliotactin, the kinase directly phosphorylating Gliotactin leading to endocytosis has yet to be identified. While our RNAi screen covered all tyrosine kinases, the RNAi lines used might have not been strong enough and generated false negatives. The coexpression of Dicer2 (Zhang et al., 2004) to increase the effectiveness of RNAi knock-down, simultaneous use of more than one RNAi to the same target or use of more independent RNAi to each would address this to some extent. On the other hand, since the restricted localization of Gliotactin is critical for survival there may be more than one kinase to regulate Gliotactin localization. Therefore, it is possible that we failed to identify a direct kinase due to potential redundancy. Doing a secondary screen with simultaneous knock-down of more than one identified kinases in various combinations would be an approach to solve this problem. Alternatively, it is also possible that these phosphorylation sites are targeted by a dual specific kinase (Douville et al., 1994) not by a tyrosine kinase. Therefore, all the other serine/threonine kinases that are known to act as duel specific kinases might be interesting to further screen in a secondary screen.

Further, in addition to tyrosine phosphorylation, control there is a microRNA (miR-184) mediated regulation that is sensitive to elevated Gliotactin protein levels (Sharifkhodaei et al., 2016). Therefore, miRNA mediated degradation of the Gliotactin may be upregulated to
compensate for loss of the kinase that regulates Gliotactin protein levels and mask the effect of kinase-mediated knock-down. To avoid this in future experiments, the kinase knock-down can be done with in conjunction with a miRNA sponge (Esau, 2008) to block miR-184 or in the miR-184 null mutant background. Overall, further investigations have to be carried out to fully identify other phospho-regulators of Gliotactin.

Csk regulates Gliotactin level and/or localization, however, the mechanism of this control yet to be investigated. Tyrosine phosphorylation of one or both highly conserved tyrosine residues is important to regulate level and/or localization of Gliotactin (Padash-Barmchi et al., 2010). While it was unclear whether Csk acts directly or indirectly on Gliotactin, our results suggest that the physical or functional interaction between Csk and Gliotactin occurs at the AJ level. The increased phosphorylation of Gliotactin and the PLA between co-expressed Gliotactin and Csk was localized close to the AJ level. If Csk does not directly phosphorylate Gliotactin, it is still possible that the SH2 domain of Csk interacts with one of phosphotyrosine residues of Gliotactin phosphorylated by another kinase. This interaction may lead to the activation of Csk which is usually present at the level of the AJ domain, signaling for the subsequent endocytosis and degradation of Gliotactin. Alternatively, knowing the negative regulatory activity of Csk, it is possible to hypothesize that Csk functions to block deleterious signaling from Gliotactin. However, it is more likely that Csk controls Gliotactin localization and protein levels through activation of endocytosis as Csk knockdown caused endogenous Gliotactin to spread away from the TCJ similar to that observed when endocytosis was blocked.

If Csk does not directly phosphorylate Gliotactin, another kinase at the AJ level might be involved. Src was a likely candidate yet we demonstrated that Src was not involved in regulation of Gliotactin phenotypes. In our study, we observed that phosphorylated Ret kinase localized to
AJs, and an enhancement of the Gliotactin phenotypes when Ret kinase was knocked down. However, the endogenous Gliotactin was not affected by loss of Ret kinase so Csk might not act through Ret kinase. Further, we identified Btk29A kinase as another regulator of Gliotactin phenotypes. The Btk29A kinase belongs to Tec family kinases, which is structurally very similar to Src family kinases. Therefore, it is appealing to explore the activity of Btk29A kinases on Gliotactin and a potential link to the Csk in this context. If Csk inhibits Btk29A then increased Csk would inhibit Btk29A and suppress Gliotactin overexpression phenotypes. Since the Btk29A RNAi also suppresses these phenotypes, this could suggest that Csk inhibits Btk29A. However, a direct action of Csk on Btk29A has not been identified so far. If Csk acts through Btk29A kinase, it would be through an inhibitory context as Btk29A knock down suppressed the Gliotactin overexpression phenotypes. Therefore, the other tyrosine kinases and dual specific kinases that are known to act at AJs are candidates for further investigations to find out the mechanism of Csk activity on Gliotactin.

To understand the mechanism that regulates the Csk-Gliotactin interaction, not only AJ associated kinases but also known interactors of Csk at the AJ are candidates for further investigation. In particular, Ankyrin-repeat, SH3-domain, and proline-rich-region containing protein (ASPP) regulates the localization of Csk to the AJ domain and perhaps the activation of Csk (Langton et al., 2007). To test whether the Csk regulation of Gliotactin is also dependent on ASPP, ASPP can be knocked down or overexpressed in a Gliotactin overexpression background. If ASPP is involved, the ASPP overexpression should mimic the Csk co-expression with Gliotactin and suppress the Gliotactin-induced phenotypes. Loss of ASPP should also remove the interaction between Csk and Gliotactin and thus remove the ability of Csk to suppress the Gliotactin overexpression phenotypes. Whereas knockdown of ASPP should mimic the Csk
knockdown and enhance the Gliotactin overexpression phenotypes. It is also important to look at whether the knock-down of ASPP in otherwise wild type wing epithelia has an effect on endogenous Gliotactin protein level and localization similar to the Csk knock-down.

While the mechanism of Csk activity yet to be identified, our data showed that Csk controls level and/or localization of Gliotactin. In addition to Csk, the localization of Gliotactin to the TCJ is dependent on Bark/Anakonda the other identified TCJ protein in Drosophila (Byri et al., 2015), however, that mechanism is also not yet known. The kinase-mediated control of Gliotactin at the TCJ may occur through Bark/Anakonda. Similar to Gliotactin, the mechanism of Bark/Anaconda localization is largely unknown. Bark is also thought to be regulated in an endocytosis dependent mechanism as Bark colocalizes with Rab5, Rab7 and Rab11 positive endosomes (Hildebrandt et al., 2015). Therefore, the effect of Csk knock-down on Bark must be examined to test whether both Gliotactin and Bark are under the control of the same regulators. However, we do not think that the effect of Csk knock-down on endogenous Gliotactin is solely through a loss of Bark as the loss of Bark leads to loss of Gliotactin at the membrane with global downregulation, while loss of Csk leads to spread of Gliotactin away from TCJs. Overall, we think that Csk controls Gliotactin independent of Bark.

Similar to Gliotactin, it will be interesting to test whether the vertebrate TCJ proteins are also under Csk control. Some tight junction proteins and tricellular tight junction proteins are also under the control of kinases for their localization, level and/or functions (Wong, 1997; Takasawa et al., 2013). However, details such as the kinases involved, and the mechanisms are largely unknown. For, instance, the vertebrate TCJ protein, Tricellulin (TRIC) has a number of conserved threonine phosphorylation sites (Takasawa et al., 2013) and TRIC may have multiple phosphorylated states (Ikenouchi et al., 2005). The phosphorylation of Tricellulin is Ca²⁺
dependent and proposed to be involved in TRIC trafficking, internalization and turnover (Mariano et al., 2011; Takasawa et al., 2013). However, details on the functional significance of these phosphorylation sites, relative roles to each other, or interacting kinases are not known. The related tight junction protein, Occludin is phosphorylated by various kinases to regulate the function and the localization of the protein (Takasawa et al., 2013). As the C-terminal end of Tricellulin is homologous to Occludin, it is predicted that TRIC can also be phosphorylated by multiple kinases (Takasawa et al., 2013). JNK could be a kinase that regulates TRIC as activation of JNK increases phosphorylation of TRIC (Takasawa et al., 2013) and TRIC expression is increased by JNK (Kojima and Sawada, 2012). In general, the kinases that control vertebrate and invertebrate TCJ proteins are largely unknown. Csk functions are conserved between vertebrates and invertebrates and Csk regulates AJ in both vertebrates and invertebrates, therefore, it is worth checking whether vertebrate TCJ proteins are also controlled by Csk and Csk-associated proteins.

4.2 Signaling and endocytosis of Gliotactin may have an antagonistic relationship

In the study presented in chapter three, we showed that the co-expression of Csk with Gliotactin leads to suppression of phenotypes leading to increased tyrosine phosphorylation associated with Gliotactin and making large endocytic vesicles full of Gliotactin. This led us to conclude that Csk functions to increase Gliotactin endocytosis. In the study presented in chapter two, the knock-down of the Grnd-JNK pathway or the PI3-Akt components suppressed the Gliotactin phenotypes leading us to conclude that those were signaling components required for the formation of Gliotactin overexpression phenotypes. Interestingly, the knock-down of these later signaling components also leads to formation of large vesicles. Gliotactin is phosphorylated
at one or both conserved tyrosine residues, Tyr760 or Tyr799 (Padash-Barmchi et al., 2010). However, it was not determined whether these sites are redundant or have distinct functions. This point to two potential models for further investigation,

1. The tyrosine phosphorylation sites are redundant. However, the kinases involved in Gliotactin signaling for deleterious phenotypes have an antagonistic effect on kinases involve in Gliotactin endocytosis (Fig. 4.2).

2. The two highly conserved tyrosine residues have distinct functions i.e., one phosphorylation site is required for endocytosis of Gliotactin and the other for activation of the signaling pathways responsible for deleterious phenotypes seen with Gliotactin overexpression (Fig. 4.2). If that is the case, the above observation can be explained by assuming phosphorylation of one residue has an antagonistic effect on the phosphorylation of the other tyrosine.

Single phosphomimetic and phosphorylation-blocked mutants could be investigated including transgenes for: GliY760D, GliY799D, GliY760F and GliY799F. A more rigorous approach would be to use CRISPR-mediated genome editing to introduce mutations into Y760 and Y799 in the endogenous gene to test the effect without overexpression of Gliotactin. In this way, double mutants that mimic phosphotyrosine at one residue and block the phosphorylation at the place of other tyrosine can be used.

If the phosphorylation sites are not redundant, the antagonistic effect could be at the level of kinases. So, the antagonistic effect of Csk and the signaling components identified in Chapter 2 have to be investigated. i.e., whether Btk29A, PI3K or TNF pathway components have a negative regulatory effect on Csk. Further, a direct action of Csk leading to degradation of c-jun has been reported (Zhu et al., 2006). Epistasis analysis can be carried out between Csk and some
identified signaling components in Gliotactin overexpressing background to determine the hierarchy between the different signaling components.

![Figure 4.2: Models for Gliotactin activity in the context of Gliotactin overexpression.](image)

Schematics show that if Gliotactin spreads away from the tricellular junctions Gliotactin phosphorylates at highly conserved tyrosine phosphorylation sites leading to endocytosis and activation of deleterious signaling pathways though an unidentified mechanism.

Model 1: This model assumes that the two tyrosine phosphorylation sites are redundant. However, the kinases that are involved in Gliotactin signaling for deleterious phenotypes are different from the kinases that are involved in Gliotactin endocytosis.

Model 2: This model assumes that two conserved tyrosine residues have distinct functions. One phosphorylation site is required for endocytosis of Gliotactin and the other for activation of the signaling pathways responsible for deleterious phenotypes seen with Gliotactin overexpression.

### 4.3 The TCJ as a signaling center

Tricellular junctional proteins are well known for their permeability barrier function. However, research in both vertebrate and invertebrates suggest that they are involved in other important cellular processes. This leads to the hypothesis that the TCJ proteins have the potential to interact with various signaling pathways. Overexpression of the TCJ protein Gliotactin signals for detrimental phenotypes including cell delamination, cell migration, apoptosis and overproliferation. These phenotypes mimic cancer-like conditions and disrupt the epithelial architecture. Consistently, in about 1 out of 30 wing discs, we observed secondary tumor-like formation in areas beyond the Gliotactin overexpression domain (Fig. 4.2). A link between
cancers and vertebrates TCJ protein, Tricellulin has been reported in some cases including pancreatic ductal carcinoma and some hepatic cancers. However, the exact mechanism of TCJ protein involvement is not clear as both upregulation and downregulation of TRIC has been reported in these cancers. Similar to TRIC, changes in the level of the other vertebrate TCJ

![Figure 4.3: Formation of secondary growths in Gliotactin overexpressing context.](image)

A-A”: Third-instar wing imaginal discs with Gliotactin-WT driven by apterous-GAL4 (ap>), immunolabeled for Gliotactin (Gli). The dashed lines mark the apterous boundary between the dorsal and ventral compartments with the apterous side (dorsal compartment) on the top. Arrows indicate the Gliotactin overexpressing secondary tumor-like growths outside of the apterous expression domain. Scale bars: 30 µm.

protein, LSR, is also detrimental. LSR downregulation and TRIC upregulation and mislocalisation to bicellular tight junctions have been reported in cancers in the endometrium (Shimada et al., 2016). These data suggest that the regulation of level and localization of TCJ is a global requirement for all the tricellular junction proteins. To further test this, Bark/Anakonda, the other tricellular junctional protein identified in *Drosophila*, can be easily tested by overexpressing in the wing imaginal disc epithelium. If the Bark overexpression phenotypes are different, that would lead us to conclude that the overexpression phenotypes are unique to Gliotactin and dissect the functions of Gliotactin from Bark to some extent. In contrast, if Bark
also interacts with signaling pathways similar to Gliotactin, we would expect to see similar detrimental phenotypes. If so, the identified modifiers of the Gliotactin can be further tested to identify evidence for any differential interactors of Bark and Gliotactin. For instance, this will provide us with another way to check whether Csk controls Bark overexpression phenotypes as well.

While the potential signaling role of Bark remains to be determined, it is certain that Gliotactin, and the vertebrate TCJ protein TRIC, have signaling roles. Gliotactin can modulate events in mitosis and act as a special landmark for future junctions in rounded dividing cells (Bosveld et al., 2016). In the midgut epithelia of old flies, Gliotactin is downregulated in the differentiated cell population leading to an increase in the number of the undifferentiated cells (Resnik-Docampo et al., 2016) (Fig. 4.3). Similarly, in pancreatic cancers, Tricellulin is upregulated in differentiated cancer cells and downregulated in dedifferentiated cancer cells showing a link between Tricellulin and differentiation. Moreover, a study done on mosquitos, Aedes aegypti, showed that Gliotactin changes the permeability of junctions related to changes in the salinity in the environment (Jonusaite et al., 2017). Taken together, this evidence points to a signaling role associated with these TCJ proteins. Therefore, understanding of signaling pathways activated by Gliotactin overexpression will have implications for understanding the signaling as well as the cellular and tissue level processes associated with tricellular junctions in general. These are important to understand how tricellular junctions are associated with human cancers.
Figure 4.4: Barrier dependent and independent potential signaling of Gliotactin.

Schematic showing a model for potential signaling of Gliotactin. At TCJs the tyrosine residues are masked by an unidentified Protein ‘A’, preventing interaction with kinases. At TCJs, Gliotactin signals for differentiation, orientation of cell division and changes for permeability through unidentified pathways.

If Gliotactin spreads away from TCJs, protein ‘A’ dissociates from Gliotactin, exposing the tyrosine residues. At bicellular contacts, Csk directly or indirectly phosphorylates Gliotactin leading to endocytosis-mediated degradation. Phosphorylation of Gliotactin by another unidentified kinase (Kinase ‘X’) activates signaling pathways that lead to deleterious phenotypes including cell death and overproliferation. The mechanism(s) of these pathways activation are not known. The Kinase X may be a kinase in one of the identified pathways responsible for deleterious phenotypes (TNF-JNK, PI3K-Akt or Btk29A).

4.4 Gliotactin overexpression activates multiple signaling pathways through unidentified mechanism(s)

The overexpression of Gliotactin leads to deleterious phenotypes including cell delamination, migration, apoptosis and overproliferation. Padash-Barmchi et al (2010) identified
Gliotactin phenotypes that were mediated through JNK, including apoptosis and increased cell proliferation. This thesis identified that cell delamination observed with overexpression of Gliotactin still occurred when JNK was blocked. However, JNK has to be blocked in otherwise wildtype tissues to see whether this is caused by loss of JNK. In parallel, JNK has been identified as a regulator of TCJ proteins associated with some cancers in vertebrates. Upregulation of JNK and changes to TCJ proteins, TRIC level and localization have been observed in these cancers (Takasawa et al., 2016). There, JNK seems to increase the TRIC protein expression level, however, the mechanisms underlying the link between JNK and TCJ protein-associated cell transformation in vertebrates are not known. The study presented in this thesis provides a substantial amount of information on potential pathways that can be involved in Gliotactin overexpression and perhaps overexpression of TCJ proteins in general. However, the mechanisms of these pathway activations still remain to be investigated as Gliotactin is not known as a classic signaling molecule. Gliotactin might leads to ectopic interactions and interference with signaling pathways when localized to ectopic locations.

All the Gliotactin phenotypes might result from activation of a cell death pathway or have independent routes of origins. When the cell death was blocked by coexpressing P35 (ap> Gli-WT, P35) the cell migration was also suppressed (Padash-Barmchi et al., 2010) indicating that the cell migration phenotype occurs as a part of cell death. However, formation of secondary growths in the wildtype side of the wing disc (Fig. 4.2) suggests that at least some cells migrate via a cell death independent mechanism or escape the cell death pathway afterwards. The cell migration phenotype must be further characterized in future studies. In a classic epithelial to mesenchymal transition (EMT), the proteins like, Twist, NCad and MMPs are upregulated and the polarity proteins like Crumbs and Ecad are downregulated (Lamouille et al., 2014; Klymenko
et al., 2017). Gliotactin overexpression leads to increased MMP1 expression (Padash-Barmchi et al., 2010). To further check whether EMT occurs in Gliotactin overexpression background, the identity of migrating cells can be tested by using mesenchymal markers like Twist and for expression of NCad. If all the phenotypes resulted from the activation of cell death, the big question is why the Gliotactin overexpressing cells are dying. As discussed before in section 1.8.1, the cell death is less likely due to an unfolded protein stress response caused by overexpressed Gliotactin (Padash-Barmchi et al., 2010). However, since the endocytic pathway and autophagy do share common components, it is important to check various markers for autophagy in Gliotactin overexpression background. However, the cell death in Gliotactin overexpression is less likely due to an induction of a stress response through autophagy, as whenever there was a prevalence of large vesicles filled with Gliotactin, the Gliotactin overexpression phenotypes were suppressed. Cell death may result simply due to ectopic interactions by Gliotactin outside its native domain or due to cell cycle defects, as Gliotactin is reported as a key component in the regulation of orientation of cell division (Bosveld et al., 2016). However, cell division proceeds with the correct orientation when Gliotactin is overexpressed (unpublished data). Alternatively, Gliotactin phenotypes might result from ectopic interactions when Gliotactin spreads outside its native domain. If so, overexpressed Gliotactin may signal to JNK through the pathways we identified and cell overproliferation is triggered by apoptosis-induced proliferation (AiP) by JNK. However, the cell delamination process seems to be independent of JNK-activated cell death as this was still present in JNK blocked background. To test whether these delaminated cells have defects in cell division, various cell cycle markers can be tested in this cell death blocked (ap>Bsk-DN, Gli-WT) background.
The potential link between Gliotactin and Gliotactin-induced phenotypes with some of the identified signaling molecules/pathways in our study are further discussed in subsequent paragraphs.

Figure 4.5: Gliotactin overexpression activates multiple signaling pathways through unidentified mechanism(s)

A: Schematics showing pathways that can be activated if the TCJ protein Gliotactin is overexpressed. The knockdown of pathway components shown in red boxes leads to suppression of all the deleterious phenotypes seen in the context of Gliotactin overexpression. Gliotactin overexpression leads to somehow activation of TNF-JNK, PI3K-Akt, and Btk29A. The relationship between pathways, the position of Btk29A and the mechanisms of activation of these pathways in the context of Gliotactin overexpression are not known.
4.4.1 TNF-JNK pathway

Our data suggests that when Gliotactin is overexpressed, the TNF-JNK pathway is somehow activated through Grnd, TRAF6, TAB2 and Tak1 (Fig. 4.5). Many signaling pathways can activate JNK and one well-known pathway is the TNF pathway. In Drosophila only one form of JNK (Basket, Bsk) is present, however, the understanding of the activation and functions of JNK is complicated as many signaling pathways including growth factors, cytokines and stress factors are known to be involved (Dhanasekaran and Reddy, 2008). Therefore, it is possible that some other pathways/molecules that are known to activate JNK are also simultaneously involved with the TNF pathway. Based on our data, we propose that the PI3K-AKT pathway and a pathway that involves Btk29A also signal to JNK in Gliotactin overexpression background.

In addition, within the JNK pathway itself also there are at least six JNKK kinases (JNKKK) in flies that are involved in linking upstream signaling with downstream core components in the JNK pathway (Stronach, 2005). To have a better understanding of JNK activation, it is important to test the involvement of these components as well as signal specificity and the outcome depends on the JNKKK involved (Stronach, 2005). The six known JNKKKs in flies are; Tak1, Tak1-like 2 (Takl2), Wallenda, misshapen (msn), PK92B, Slipper (slpr) and Mekk1. In our study, in addition to Tak1 we tested Wallenda and Pk92B in Gliotactin overexpression background. The knock-down of Pk92B did not have an effect on Gliotactin phenotypes. However, the knock-down of Wallenda showed a partially suppressed phenotype where the cell migration was suppressed but not the cell overproliferation. This suggests that in addition to Tak1 other JNKKKs may also have a role in generating Gliotactin phenotypes. We did not test for the involvement of slpr. However, the suppression by Tak1-CT (only the C-
terminal side of Tak-1) coexpression in Gliotactin overexpression background provides an indirect evidence that the slpr pathway components are not involved (Stronach et al., 2014). Testing for the other three JNKKKs that we did not test (msn, Tak1-like-2, Mekk1) would give a clearer understanding of the other JNK pathway components involved in Gliotactin overexpression. We found that blocking JNK completely blocked the cell death induced by overexpressed Gliotactin, suggesting that JNK is the only mediator of apoptosis in this context. However, as outlined above, the involvement of some other potential JNK pathway components as well as the mechanism of JNK activation need to be determined. Using phosphomimetic forms of Gliotactin and Dlg, Padash-Barmchi et al (2013) reported that the JNK activation is downstream of Gliotactin and Dlg phosphorylation. However, the other pathway components identified in this study also must be tested to see if any of these acts upstream of Gliotactin and/or Dlg phosphorylation.

4.4.1.1 The involvement of Eiger in Gliotactin overexpression is not certain

One means by which the overexpression of Gliotactin leads to JNK activation was through the activation of TNF pathway components. However, the mechanism of TNF pathway activation in this context remains to be determined. TNF is well known as a pathway involved in activation of the extrinsic pathway of apoptosis (Locksley et al., 2001) in which usually neighboring cells send the signal to activate cell death in aberrant cells. This is largely TNF ligand dependent as TNF ligands can be secreted as a soluble form. However, in the context of Gliotactin overexpression, whether the ligand (Eiger) is involved or not was uncertain. In Drosophila, Eiger (Egr) is the only known TNF ligand homolog (Igaki et al, 2002; Moreno et al, 2002; Kauppila et al, 2003) and there are two TNF receptors, Wengen (Wgn) (Kanda et al, 2002;
Kuranaga et al., 2002; Kauppila et al., 2003) and the recently identified Grindelwald (Grnd) (Andersen et al., 2015). Knock-down of the TNF receptor Grnd suppressed the Gliotactin phenotypes, while the knock-down of the sole TNF ligand, Eiger, and the other TNF receptor Wengen did not suppress.

Eiger is one way to activate the JNK pathway in Drosophila and overexpression of Eiger is sufficient to induce cell death through the activation of the JNK pathway (Igaki et al., 2002; Moreno et al., 2002; Kauppila et al., 2003). Overall, Eiger-JNK signaling controls a range of cellular processes such as tissue growth, cell proliferation in addition to host defense, pain sensitization and canalization (Igaki and Miura, 2014). In our study, the Gliotactin phenotypes were not suppressed using two independent Eiger-RNAi lines, suggesting that the phenotypes were Eiger independent. However, simultaneous use of both RNAis or use of RNAi in Dicer2 expressing background would increase the effectiveness of Eiger knock-down and would eliminate the possible false negative results associated with RNAi approach (Dietzl et al., 2007). Similar to mammalian TNF, Eiger can be cleaved and released as a soluble ligand (Wang et al., 2006a) and is known to act in a cell autonomous as well as cell non-autonomous manner (Andersen et al., 2015). Therefore, in our RNAi experiments, it is also possible that still there was a supply of Eiger from the non-apterous wild type side, given the ability of Eiger to diffuse once secreted. In future experiments, we should first address whether the neighboring cells trigger the cell death in Gliotactin overexpressing cells. The creation of loss of function mutations only in discrete patches (somatic null clones) is an attractive way to study the effect of neighbors on cell elimination and hence the effect of cell non-autonomous activity of Eiger. The ability of neighboring cells to eliminate mutant cells has been demonstrated for mutants in Discs large (DlG), and Scribbled (scrib), which are septate junction associated proteins involved in
maintenance of cell polarity of epithelia (Perrimon, 1988; Gardiol et al., 2002; de Vreede et al., 2014). Somatic clones that are deficient for either scrib or dlg (scrib/ or dlg/) are eliminated by Eiger-JNK dependent apoptosis. If the neighboring cells induce cell death in the clones of Gliotactin overexpressing cells, we predict small clones. Another approach to study whether the neighbors have a role in inducing cell death of Gliotactin overexpressing cells would be the use of the gene called Flower (Few) (Soldini et al., 2010). Flower is a cell membrane protein with three isoforms and the level and the localization of these isoforms are different in aberrant cells compared to wild type neighboring cells and lead to the loss of the aberrant cells through cell competition. Testing the expression of Flower in Gliotactin overexpression cells would help to identify whether cell competition by wild type neighbors plays a role in the cell death of Gliotactin overexpressing cells.

Eiger can also be activated in fat bodies and transported by hemolymph to act on the insulin producing cells as a nutrition response of the brain (Agrawal et al., 2016). Production and secretion of Eiger from various sources, i.e., from fat bodies, hemocytes as well as from the tumor itself have been reported in tumors in wing discs caused by loss of function mutation in dlg/ or scrib/- In those contexts, the Eiger secreted from hemocytes required for the cell death in tumor cells in wing discs (Parisi et al., 2014). These studies show that Eiger can crosstalk between tissues. Therefore, other experiments should be carried out to confirm whether the activation of TNF pathway in Gliotactin overexpression is Eiger dependent or not. One could involve simultaneous knock-down of Eiger in wing discs and fat bodies in Gliotactin overexpression background. If the Eiger is provided externally this should shut down the external sources of Eiger confirming whether Gliotactin phenotypes are Eiger dependent or not.
4.4.1.2 The mechanism of Grnd activation when Gliotactin is overexpressed remains to be investigated.

To understand the mechanism of activation of TNF pathway by Gliotactin, the observation that the activation of the JNK pathway occurs through Grindelwald in an Eiger independent manner was unexpected. Grnd is a homologue of the TNF receptor (TNFR). Unlike the other Drosophila TNFR, Wengen, Grnd has a conserved dTRAF2 (TRAF6) binding motif, a feature common with many TNFR receptors and Grnd physically binds with dTRAF2 (TRAF6) (Andersen et al., 2015). The Eiger-JNK activation is part of the mechanism to remove cells with polarity defects such as those generated in scrib or dlg null mutant clones. Loss of Grnd in this context reduces the death of scrib mutant clones suggesting that Eiger functions through Grnd to activate JNK induced cell death. Including an oncogenic Ras oncogenic activity in this background changes the Eiger-JNK role to tumor promoting and tumor invasion (Igaki et al., 2009; Andersen et al., 2015). Interestingly, Grnd promotes the invasiveness of these tumors through Eiger-dependent Matrix metalloprotease 1 (MMP1) expression (Andersen et al., 2015) showing that Grnd also function to promote cell migration which is another prominent Gliotactin overexpression phenotype. Therefore, identification of Grnd as a component responsible for the Gliotactin phenotypes fits well. Grnd functions through dTRAF2 (vertebrate homolog, TRAF6), TAB2, Tak1 to activate JNK to induce cell death (Andersen et al., 2015) and to induce AiP (Fogarty et al., 2016). Consistently, our results show that same regulatory elements are involved with Grnd in generating cell delamination, cell migration, cell death, and perhaps overproliferation phenotypes in Gliotactin overexpressing context. However, mechanism of Grnd activation in overexpressed Gliotactin background is not clear.
If the activation of Grnd is Eiger independent, the possibility of Gliotactin and Grnd interactions must be investigated. Immunostaining for Grnd shows that Grnd localizes apical to both septate and AJs and colocalizes with apical protein Crumbs (Crb) (Andersen et al., 2015). When Gliotactin is overexpressed Gliotactin spreads around the cell membrane as well as along the lateral cell membrane both basally and laterally. Therefore, it is possible that apical spread of Gliotactin leads to an ectopic interaction with Grnd. To get evidence for this, PLA assays can be carried out using antibodies to Gliotactin and Grnd and interactions between these proteins can be confirmed using immunoprecipitation assays. If the activation of Grnd is not through an association with Gliotactin, this points to the activation of Grnd through an intermediate protein. A possible link to Grnd and Dlg should also be investigated as Gliotactin phenotypes are dependent on phosphorylation of Dlg as well. Atypical PKC (aPKC) is also known to activate JNK dependent on Grnd (Andersen et al., 2015) and thus aPKC is also a potential candidate for the Dlg phosphorylation. Therefore, aPKC knock-down in a Gliotactin overexpression would be an initial step to test for a potential aPKC- Dlg -Grnd association in Gliotactin overexpression background.

4.4.2 The simultaneous activation of JNK and PI3K-Akt pathways and their relationship with Gliotactin phenotypes.

In addition to the TNF-JNK pathway, the other pathway we identified in Gliotactin overexpressing background involved PI3K-Akt, which is well known as a cell survival pathway (fig. 4.5). The simultaneous activation of both JNK and PI3K-Akt pathways has been observed in various cancers (Vivanco et al., 2007; Zhao et al., 2015). However, why the both pathways are activated, the order of activation (i.e., which pathway activates first) and their relative roles in
cancers are not yet known. Overexpressed Gliotactin might first signal for cell proliferation due to ectopic activation of the PI3K-Akt pathway. Usually, JNK is activated to suppress the malignant formation in many epithelial tissues in TNF pathway dependent manner (Andersen et al., 2015). Therefore, in Gliotactin overexpression background the JNK activation might be downstream of activation of the PI3K-Akt pathway and overproliferation. However, JNK itself might also responsible for generating Gliotactin phenotypes as JNK has both apoptotic and tumor formation properties and a sustained activation of JNK usually leads to apoptosis and acute and transient activation of JNK leads to cell proliferation or survival pathways. Epistasis analysis of the role of JNK using BskDN or Bsk mutants would be able to place where exactly these components in the signaling pathways.

4.4.3 Role of Btk29A in the context of Gliotactin overexpression remains to be investigated

We identified Btk29A kinase as another modifier of Gliotactin phenotypes. It is important to find out the relationship between the PI3K-Akt pathway and TNF-JNK pathways, and whether there is a role of Btk29A in crosstalk between these pathways in a Gliotactin overexpression background (Fig. 4.5). Btk (Bruton’s tyrosine kinase) is a non-receptor tyrosine kinase and a member of Tec family kinases, which are involved in a plethora of cellular processes including growth, proliferation, differentiation, cell migration and apoptosis (Hussain et al., 2011). However, how and why Btk29A is activated in this context is not clear. Btk kinases are activated by a wide range of signals including antigen receptors, cytokine receptors, growth factor receptors, G-protein coupled receptors and integrins. Further, Btk family kinases interact with all known families of non-receptor tyrosine kinases except Abl (Qiu and Kung, 2000).
Knockdown of Btk29A suppresses Gliotactin overexpression phenotypes including cell migration, cell death, and cell overproliferation. One key functional feature of both mammalian and fly Btk is binding to F actin and reorganization of cytoskeleton by targeting downstream components such as Rho family GTPases (Nore et al., 2000; Qiu and Kung, 2000). This, coupled with the ability to interact with focal adhesion kinase (FAK) and integrins makes Btk a regulator of cell migration and cell shape changes. Therefore, Btk29A might be a key regulator in cell migration phenotype in Gliotactin overexpressing background.

The members of the Btk family of kinases are also known to act as major regulators of apoptosis (Qiu and Kung, 2000). Paradoxically, both anti-apoptotic and pro-apoptotic functions of Btk family kinases have been reported (Qiu and Kung, 2000; Uckun et al., 2007). For instance, overexpression of Btk inhibits TNF-alpha receptors, and Fas-mediated apoptosis by phosphorylating and removing a protein called Fas-associated protein with death domain (FADD) from the death domain of the Fas receptor (Uckun 1998). The anti-apoptotic function also could be via the activation of Akt kinase or NF-kB (Qiu and Kung, 2000). In contrast, Btk is also known to activate apoptosis and one mechanism is through STAT in the JAK/STAT pathway directly or indirectly downstream of Src kinases (Qiu and Kung, 2000). There are no publications showing Btk29A as a part of Grnd-Tak1-JNK pathway. However, Btk29A can be upstream or downstream of PI3K and Btk29A can activate JNK (Kawakami et al., 1997; Kawakami et al., 1998; Mao et al., 1998). Overall, how Btk29A is activated as well as the role of Btk29A in generating the Gliotactin overexpressing phenotypes are not clear. To understand the role of Btk29A in Gliotactin overexpression background, epistasis analyses can be performed to see whether the activation of Btk29A is upstream or downstream of PI3K kinase. Further, to check the contribution of Btk29A in JNK activation immunolabeling for pJNK can be performed.
in Btk29A co-expression background with Gliotactin. The same analysis can be performed with
the loss of PI3K to see the contribution of PI3K on JNK activation. To test, whether the Btk29A
is activated independent of PI3K and TAK1 pathways, both Tak1 and PI3K can be knocked-
down simultaneously in Gliotactin overexpression background and look for the level and
localization of Btk.

4.5 Conclusions

The work done in this thesis study identified a number of kinase and kinase-associated
modifiers of Gliotactin overexpression phenotypes. This study led us to conclude that at least
two signaling pathways are activated when Gliotactin is overexpressed. One pathway involves
the TNF-JNK signaling through Grnd, TAB2, TRAF6 and TAK1. The other pathway involves
PI3K-Akt. Further, we concluded that Btk29A is a signaling component involved in a Gliotactin
overexpression context. The position of Btk29 in relation to the other identified pathways
remains to be determined. We also identified a novel role of Csk in controlling the tricellular
junctional protein Gliotactin independent of Src. It remains to be identified whether Csk activity
on Gliotactin is direct or indirect. Further, this thesis highlights the usefulness of Gliotactin
overexpression as a model to investigate consequences and signaling pathways elicited by TCJ
protein upregulation.
Bibliography


Banerjee, S., A.M. Pillai, R. Paik, J. Li, and M. a Bhat. 2006a. Axonal ensheathment and septate


doi:10.1074/jbc.M500496200.


doi:10.1158/0008-5472.CAN-06-0410.


doi:10.1038/nature11127.


Kondoh, A., K. Takano, T. Kojima, T. Ohkuni, R. Kamekura, N. Ogasawara, M. Go, N. Sawada,


Nishio, M., K. Otsubo, T. Maehama, K. Mimori, and A. Suzuki. 2013. Capturing the mammalian


for septate junction function and epithelial tube-size control in the Drosophila tracheal

doi:10.1038/sj.onc.1207635.

Pereira, S.F.F., L. Goss, and J. Dworkin. 2011. Eukaryote-like serine / threonine kinases and

Pérez-Garijo, A., Y. Fuchs, and H. Steller. 2013. Apoptotic cells can induce non-autonomous

Perrimon, N. 1988. The maternal effect of lethal(1)discs-large-1: a recessive oncogene of


Piontek, J., L. Winkler, H. Wolburg, S.L. Müller, N. Zuleger, C. Piehl, B. Wiesner, G. Krause,

61. doi:10.1038/sj.onc.1203958.

Quan, C., and S.J. Lu. 2003. Identification of genes preferentially expressed in mammary
epithelial cells of Copenhagen rat using subtractive hybridization and microarrays.

Turner. 2010. Tight junction–associated MARVEL Proteins MarvelD3, Tricellulin, and


doi:10.1371/journal.pgen.1003253.


doi:10.1038/ncb3454.


Sarkar, S. 2013. Regulation of autophagy by mTOR-dependent and mTOR-independent pathways: autophagy dysfunction in neurodegenerative diseases and therapeutic application of autophagy enhancers. 41:1103–1130. doi:10.1042/BST20130134.


Seth, A., P. Sheth, B.C. Elias, and R. Rao. 2007. Protein phosphatases 2A and 1 interact with occludin and negatively regulate the assembly of tight junctions in the CACO-2 cell


Tan, J., and J.A. Brill. 2014. Cinderella story: PI4P goes from precursor to key signaling


doii:10.1242/jcs.129031.


Tateno, M., Y. Nishida, and T. Adachi-yamada. 2016. Regulation of JNK by Src during Drosophila Development Linked references are available on JSTOR for this article:

Regulation of JNK by Src During Drosophila Development. 287:324–327.


doii:10.1146/annurev.genet.35.102401.091415.


Zeev-Ben-Mordehai, T., E.H. Rydberg, A. Solomon, L. Toker, V.J. Auld, I. Silman, S. Botti,


**Appendix**

**Appendix 1: list of RNAi lines screened in the primary screen presented in Chapter 2**

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Stock numbers are given for the RNAi lines from the Vienna Drosophila RNAi Center (VDRC) or Bloomington (BL) stock centers.