Control of Gliotactin localization and levels by tyrosine phosphorylation and endocytosis is necessary for survival of polarized epithelia

Mojgan Padash-Barmchi, Kristen Browne, Kendra Sturgeon, Barbara Jusiak and Vanessa J. Auld*
Department of Zoology, Cell and Developmental Biology, University of British Columbia, Vancouver, Canada BC V6T 1Z3
*Author for correspondence (auld@zoology.ubc.ca)

Summary
The tricellular junction (TCJ) forms at the convergence of bicellar junctions from three adjacent cells in polarized epithelia and is necessary for maintaining the transepithelial barrier. In the fruitfly Drosophila, the TCJ is generated at the meeting point of bicellar septate junctions. Gliotactin was the first identified component of the TCJ and is necessary for TCJ and septate junction development. Gliotactin is a member of the neurexin family and associates with the PDZ protein discs large. Beyond this interaction, little is known about the mechanisms underlying Gliotactin localization and function at the TCJ. In this study, we show that Gliotactin is phosphorylated at conserved tyrosine residues, a process necessary for endocytosis and targeting to late endosomes and lysosomes for degradation. Regulation of Gliotactin levels through phosphorylation and endocytosis is necessary as overexpression results in displacement of Gliotactin away from the TCJ throughout the septate junction domain. Excessive Gliotactin in polarized epithelia leads to delamination, paired with subsequent migration, and apoptosis. The apoptosis and the resulting compensatory proliferation resulting from high levels of Gliotactin are mediated by the Drosophila JNK pathway. Therefore, Gliotactin levels within the cell membrane are regulated to ensure correct protein localization and cell survival.

Key words: Septate junction, Tricellular junction, Drosophila, Tyrosine phosphorylation, Endocytosis, JNK

Introduction
Establishment and maintenance of cellular permeability barrier is important for preservation of the integrity of epithelia. In epithelial cells of invertebrates, permeability barriers are formed by pleated septate junctions (SJs), which are functionally analogous to tight junctions in vertebrates (Noirot-Timothee et al., 1982; Tsukita et al., 2001). In Drosophila, SJs form ladder-like junctions between cells in a region just basal to the adherens junctions. The multi-stranded nature of SJs effectively prevents the paracellular flow of molecules. Several SJ proteins have been identified that are important for development of these junctional domains. Mutations in the genes encoding any of these components, including Neurexin-IV (Baumgartner et al., 1996), coracle (Fehon et al., 1994), Neuroglian and the Na+/K+ ATPase (Genova and Fehon, 2003; Paul et al., 2003), block the formation of SJ strands. Analysis of epithelial cells with electron microscopy has shown that the continuity of SJ strands is interrupted at the convergence point of three cells (Fristrom, 1982; Noirot-Timothee et al., 1982). This junction, known as the tricellular junction (TCJ), is also vital to create an intact permeability barrier to ensure that solutes fail to flow in the channel formed between the three cell corners. The permeability barrier at the TCJ is created by a series of diaphragms that might act as anchors for SJ strands (Fristrom, 1982; Noirot-Timothee et al., 1982). So far in Drosophila, only one protein, Gliotactin (Gli), is found specifically localized at the TCJ. In Gliotactin mutants, SJ strands form but fail to compact, and this, combined with a disrupted TCJ, results in a defective transepithelial barrier and embryonic lethality (Schulte et al., 2003). Gliotactin is a cholinesterase-like molecule with an extracellular serine esterase domain that lacks enzymatic activity (Gilbert et al., 2001; Gilbert and Auld, 2005). In the intracellular domain, Gliotactin contains a PDZ binding motif that has previously been shown to form a protein complex with discs large through a currently unknown linker protein (Schulte et al., 2006).

In addition to the PDZ binding motif, the cytoplasmic domain of Gliotactin also contains two predicted and highly conserved tyrosine phosphorylation sites at amino acids 766 and 790. Phosphorylation by tyrosine kinases plays an important role in internalization and endocytosis of many membrane proteins, including receptor tyrosine kinases (RTKs), insulin receptors and cell adhesion proteins such as E-cadherin (Ahmadian et al., 2004; Behrens et al., 1993; Ivanov et al., 2005; Takeda et al., 1995; Tanos and Pendergast, 2006). As such, endocytosis plays a crucial role in the downregulation of signaling and changes in membrane protein composition. For instance, tyrosine phosphorylation of cadherins is needed for endocytosis and destruction of adherens junctions during the epithelial–mesenchymal transition (Behrens et al., 1993). However, there was no prior evidence that tyrosine phosphorylation and endocytosis are important for SJ formation and maintenance.

Here, we show that phosphorylation of the conserved tyrosines in the intracellular domain of Gliotactin results in endocytosis and targeting to late endosomes and lysosomes for degradation. The expression level of Gliotactin and unique localization to the TCJ are regulated through phosphorylation and subsequent endocytosis. Tight control is necessary, as the displacement of Gliotactin away from the TCJ throughout the SJ domain leads to delamination, migration and apoptosis of columnar epithelial cells in imaginal discs. We show that the JNK pathway mediates the cell death and proliferation resulting from high levels of Gliotactin. Therefore,
Gliotactin levels within the cell membrane are regulated to ensure correct protein localization and cell survival.

**Results**

**Tyrosine phosphorylation sites in Gliotactin are necessary for endocytosis**

The intracellular domain of Gliotactin (Gli) contains a PDZ recognition sequence, shown to associate with Discs large (Dlg) in a complex at the TCJ, and two predicted tyrosine phosphorylation sites at amino acids 766 and 790 (Schulte et al., 2006). These tyrosine phosphorylation sites are strongly conserved, in particular Tyr766, which is found in all Gliotactin homologs (supplementary material Fig. S1B). To investigate the role of the tyrosine residues in Gliotactin, we used site-directed mutagenesis to alter these residues and generated transgenic lines that carry either Gliotactin with the two tyrosines mutated to phenylalanine or Gliotactin with the two tyrosines replaced by aspartic acid (supplementary material Fig. S1A). The former is a mutant version of Gliotactin that should produce a constitutive state of dephosphorylation (GliFF), and the latter is a form that should mimic phosphorylation and be in a constitutive state of phosphorylation (GliDD) (Cantarelli et al., 2007; Cuevas et al., 2001; Huh et al., 2004; Meyer et al., 2003).

As a control, we generated a wild-type Gliotactin construct (GliWT), and all three constructs were placed under the control of the GAL4 upstream activating sequence (UAS) sequence (Brand and Perrimon, 1993).

In order to see whether any of these transgenes are able to rescue the embryonic lethality of Gli mutants, we expressed each construct in a homozygous Gli null mutant background, GliAE2/H9004, using the daughterless GAL4 line (da–GAL4), which drives expression in all tissues. All transgenes including the wild-type form of Gli with intact tyrosine sites (GliWT) were able to fully rescue the embryonic lethality of GliAE2/H9004 mutants. Two independent lines were used for each to control for differences arising from the position of the transgene insertion. Rescued embryos developed into larval stages, but the majority of larvae became paralysed and died. Some progeny survived to adulthood, but adult flies exhibited malformed leg phenotypes and failed to inflate their wings (supplementary material Fig. S2).

To visualize the localization of Gliotactin in the rescued larvae, we used wing imaginal discs as they provide a powerful system to study the structure of both septate junctions and TCJs. In wild-type discs, Gliotactin is clearly localized to the TCJ of the columnar epithelial cells (Fig. 1A,B), as well as being found in intracellular vesicles (Fig. 1B insert, arrow). For each transgene, Gliotactin was localized to the TCJ in the rescued larva but also spread throughout the entire SJ owing to the elevated expression levels generated with the daughterless GAL4 driver (Fig. 1D–F). Interestingly, GliWT and GliDD were also found in large intracellular vesicles in comparison with those of GliFF, which was only present at the cell membrane (Fig. 1, arrows in D and E). These observations suggested that the tyrosine phosphorylation could be necessary for Gliotactin endocytosis but did not rule out the possibility that the presence of Gli-containing vesicles resulted from slow intracellular trafficking. We therefore immunolabeled rescued wing discs for Golgi markers and found no association of Gliotactin vesicles with the Golgi apparatus (supplementary material Fig. S3A–C). These results suggested that Gliotactin-containing vesicles are not blocked during trafficking to the plasma membrane. Rather, this suggested that the presence of Gliotactin-positive intracellular vesicles is due to endocytosis.

![Fig. 1. Gliotactin is endocytosed and targeted to late endosomes and lysosomes.](image)

Wild-type wing imaginal discs. (D–L) Rescued wing discs expressing Gliotactin transgenes under the control of daughterless-GAL4 (da–GAL4) in a Gliotactin-null (GliAE2/H9004) background. (A,B) Wild-type wing disc, immunolabeled for Gliotactin (red) and Dlg (green). Gliotactin is localized to the TJ. Inserts in (A) and (B) were digitally scaled by 100%. (C) Control wing discs with da–GAL4 driving the expression of the membrane protein mCD8GFP. mCD8–GFP localizes correctly to the plasma membrane, and no intracellular vesicles are detected. (D–F) Rescued wing discs expressing GliWT, GliDD and GliFF under the control of da–GAL4 in a Gliotactin-null (GliAE2/H9004) background. Owing to the strong expression of the da–GAL4 line, Gliotactin spreads through the SJ. GliWT and GliDD exhibit a greater amount of Gliotactin in intracellular vesicles (arrows in D and E) compared with GliFF. Inserts in C–F were digitally scaled by 100%. (G–I) A rescued wing disc expressing GliWT (red) under the control of da–GAL4 in a Gliotactin-null (GliAE2/H9004) background for the endosome marker Hrs (green). Many of the intracellular Gliotactin vesicles were Hrs positive (arrow in I). (J–L) A rescued wing disc expressing GliWT (red) under the control of da–GAL4 in a Gliotactin-null (GliAE2/H9004) background for the lysosomal marker LAMP1 (green). Association of LAMP1 with the large Gliotactin vesicles was observed (arrow in L). (M,N) Expression of a dominant-negative form of Rab5 (Rab5DN) with apterous–GAL4 in a wild-type wing disc. Endogenous Gliotactin is mislocalized away from the TJ and found in punctate structures throughout the SJ domain (arrows). Each panel represents a single Z slice. Scale bars: 15 μm.
and that the tyrosine residues of Gliotactin are necessary for this process.

**Gliotactin is phosphorylated at tyrosine residues in vivo and in vitro**

Our observations prompted us to investigate whether Gliotactin is endogenously phosphorylated at these tyrosine residues. We were unable to detect overlap between phosphotyrosine (phosphoTyr) and Gliotactin in immunolabeled wild-type discs. Therefore, we performed immunoprecipitations on embryos expressing UAS-GliWT under the control of the daughterless–GAL4 driver. Western analysis was carried out using immunofluorescent labeling for simultaneous detection of phosphoTyr residues and Gliotactin (Fig. 2A). A corresponding phosphoTyr band was detected in UAS-GliWT-expressing lines, indicating that Gliotactin is phosphorylated in vivo, possibly at both tyrosines.

To test whether Gliotactin can be phosphorylated in vitro by a tyrosine kinase, we performed Src kinase assays using the human Src–GST fusion paired with purified intracellular domains of Gliotactin (Fig. 2C). Both the wild-type (GliCtermWT) and GliFF (GliCtermFF) intracellular domains were expressed as His-tagged proteins and purified. Tyrosine phosphorylation was detected only for GliCtermWT in the presence of Src. Our negative control in which no Src–GST was present did not show any phosphorylation, and nor did the GliCtermFF protein (Fig. 2C). These results suggest that Src can phosphorylate Gliotactin in vitro at one or both tyrosines.

**Gliotactin vesicles are targeted to late endosomes and lysosomes for degradation**

To determine whether Gliotactin is targeted for degradation after endocytosis, we immunolabeled wing discs from rescued third-instar larvae with a range of endosomal markers. Endocytosed proteins targeted for degradation traffic to late endosomes, where they are sorted into multivesicular bodies. Multivesicular bodies then fuse with lysosomes, delivering the contents for degradation (Raiborg and Stenmark, 2009). Hrs (Hepatocyte growth factor-regulated tyrosine kinase substrate) plays an important role in this process and is necessary for the maturation of endosomes into multivesicular bodies en route to lysosomes (Jekely and Rorth, 2003; Lloyd et al., 2002). Our immunofluorescence data show colabeling of Hrs with Gliotactin-containing vesicles in the rescued wing disc and some colabeling with the lysosomal marker LAMP1 (Fig. 1G–L, arrows).

In order to confirm the degradation of Gliotactin in our rescue experiments, we performed western blottings on extracts of rescued larvae from all three transgenic lines (Fig. 2B). GliWT and GliDD showed a considerable amount of degradation in comparison with GliFF, which had no degradation (Fig. 2B, arrows). We observed similar degradation products in our control lane, which contained endogenous wild-type Gliotactin. These observations suggest that the phosphoTyr residues in Gliotactin are necessary for endocytosis and might regulate the localization of Gliotactin to the TJ. In order to investigate this possibility further, we expressed a dominant negative form of Rab5, which has been shown to block endocytosis in a number of Drosophila tissues (Marois et al., 2006; Wucherpfennig et al., 2003). In Rab5DN-expressing cells, endogenous Gliotactin localization was not restricted to the TJ and was found throughout the cell membrane as punctate immunolabeling (Fig. 1M,N). These observations suggest that the tyrosine phosphorylation might regulate the amount of Gliotactin present in the membrane and ensure that localization is restricted to the tricellular corners.

**Overexpression of Gliotactin results in mislocalization and increased tyrosine phosphorylation**

The control of Gliotactin endocytosis by tyrosine phosphorylation suggests that epithelial cells have a mechanism to regulate Gliotactin protein levels. Therefore, we wanted to test the effects of excessive levels of Gliotactin and overexpressed each of the Gliotactin transgenes (GliWT, GliDD and GliFF) in wild-type wing discs using apterous–GAL4 (ap–GAL4). Throughout the overexpression studies, we observed similar phenotypes at both 25°C and 29°C; however, to increase the penetrance, we performed overexpression studies at both temperatures (29°C). The efficiency of GAL4 increases at 29°C, with a corresponding increase in the levels of Gliotactin-null background. Western blots of larval extracts were probed with antibodies to Gliotactin and actin, respectively. Daughterless–GAL4 (da) was used as a wild-type control. Endogenous Gliotactin had degradation products in the control lane comparable to those of GliWT and GliDD larval extracts. GliFF larval extracts had no degradation products. (C) Gliotactin can be phosphorylated at tyrosine residues in vitro. A Src kinase assay was used employing a Src–GST fusion paired with the C-terminal domain of Gliotactin, either GliWT or GliFF. Significant tyrosine phosphorylation was detected only for GliWT and Src itself (arrows) in the presence of Src. Neither the GliFF nor the control showed any tyrosine phosphorylation.
Overexpression of Gliotactin results in increased tyrosine phosphorylation and endocytosis. In all panels, apterous–GAL4 (ap>) was used to drive expression in wild-type wing imaginal discs. (A) ap>mCD8-GFP wing disc, a control disc in which no mCD8GFP was found in intracellular vesicles. (B–D) ap>GliWT, GliDD- and GliFF-expressing wing discs, respectively. Gliotactin was detected in intracellular vesicles in all three cases; however, GliDD had the greatest degree of intracellular vesicles and GliFF had very little. (E–G) ap>GliWT wing disc, immunolabeled for Gli (red) and phosphoTyr (green). PhosphoTyr levels were enhanced in the dorsal compartment expressing high levels of Gliotactin. High-magnification inserts (digitally scaled by 100%) show phosphoTyr associated with Gliotactin vesicles (arrows). (E’–G’) Side projections of the corresponding panels showing the basolateral spread of both GliWT and phosphoTyr (arrow). (H–J) ap>GliFF wing disc, immunolabeled for Gli (red) and phosphoTyr (green). PhosphoTyr levels were only slightly increased in the dorsal compartment. (H’–J’) Side projections of the corresponding panels. The normal localization of phosphoTyr at the adherens junctions is indicated (arrowhead). All enface panels represent a single Z slice. Scale bars: 15 μm.

However, in wing discs expressing elevated levels of GliWT, GliDD or GliFF, Gliotactin localization was not restricted to the tricellular corners and was found throughout the entire SJ domain (Fig. 3B–D). Side projections of Gliotactin-overexpressing cells revealed the spread of Gliotactin in the basolateral membrane as well (Fig. 3E’–G’). In addition, a large quantity of Gliotactin was found in intracellular vesicles within the overexpressing cells. GliWT and GliDD had high numbers of internal vesicles, whereas very few Gliotactin-containing intracellular vesicles were detected in GliFF-overexpressing cells (Fig. 3D).

In our analysis of Gliotactin phosphorylation, we were unable to detect colocalization of phosphoTyr with endogenous Gliotactin in wild-type imaginal discs. However, when Gliotactin was overexpressed in the wing imaginal disc using apterous–GAL4, we observed colocalization of phosphoTyr and Gliotactin (Fig. 3E–G). In particular, the large intracellular vesicles were positive for both Gliotactin and phosphoTyr (Fig. 3G, arrows). In these discs, the phosphoTyr immunolabeling was increased and spread basolaterally, below its normal localization at the adherens junction (Fig. 3F, arrows). To determine whether the increase in phosphoTyr expression was due to the overexpression of Gliotactin, we analyzed discs overexpressing GliFF using apterous–GAL4 (Fig. 3H–J). In these cells, there was only a small increase in phosphoTyr immunolabeling in the GliFF-overexpressing cells. It is important to note that Gliotactin functions as a dimer or oligomer (Venema et al., 2004). As such, it is likely that the small increase in phosphoTyr observed with GliFF was due to the formation of dimers between GliFF and the endogenous Gliotactin.

Overexpression of Gliotactin results in excessive endocytosis and degradation
We then investigated the nature of the Gliotactin vesicles to find out whether they are endosomal or trafficking vesicles. Our immunolabeling data showed no association of Gliotactin vesicles with either the Golgi apparatus or endoplasmic reticulum (supplementary material Fig. S3D–F). Immunolabeling for the early endosomal marker EEA1, however, showed significant upregulation where high levels of Gliotactin were expressed (Fig. 4A–C).

Next, we investigated whether intracellular Gliotactin vesicles were ubiquitinylated as ubiquitination plays an important role in endocytosis, recycling and targeting of proteins for degradation (Dupre et al., 2001; Katzmann et al., 2001; Piper and Luzio, 2007; Purdy and Russell, 2007; Raiborg et al., 2002). Immunolabeling with an antibody against ubiquitin showed colabeling with internalized Gliotactin, suggesting that ubiquitination is a part of the process of Gliotactin internalization (Fig. 4D–F). Similarly the endosomal marker Hrs and lysosomal protein LAMP1 also showed colabeling with Gliotactin-containing vesicles (Fig. 4G–I; supplementary material Fig. S3G–I). The different Gliotactin transgenes suggest that these processes are dependent on the conserved tyrosine residues. GliDD overexpression resulted in a greater number of endocytic vesicles, many of which were colabeled by antibodies against LAMP1 and Gliotactin (Fig. 4J–L). GliFF had reduced levels of endocytic vesicles and little to no LAMP1 labeling (supplementary material Fig. S3J–L). The small degree of internalization observed with GliFF is again likely due to dimer or oligomer formation with the endogenous Gliotactin. Together, these data suggest that tyrosine phosphorylation of Gliotactin is necessary for endocytosis and targeting to lysosomes for subsequent degradation.

High levels of Gliotactin lead to cell delamination, migration and death
The controlled regulation of Gliotactin suggests that excessive levels of this protein might be deleterious to the cell. Therefore, we assayed overexpression of Gliotactin in the wing imaginal disc for changes in cell morphology and cell death. Using apterous–GAL4 to drive the expression of GliWT or GliFF in the presence of endogenous Gliotactin resulted in delamination of the columnar epithelial cells (Fig. 5B,D). We also observed that these cells had crossed from the apical expression boundary and invaded the ventral compartment of the disc (Fig. 5B,D, arrow). These phenotypes were dose dependent, showing the maximum...
penetrance at 29°C, with observable, but weaker, phenotypes at 25°C.

The delamination of Gliotactin-overexpressing cells was not due to disruption of the adherens junctions. The localization of DE-cadherin to the adherens junctions was not affected (Fig. 4M–O) and no other polarity defects were observed. For instance, immunolabeling for the SJ protein Coracle showed no effect (data not shown). Therefore the polarity of cells was not affected in the presence of high levels of Gliotactin. This effect was also specific to Gliotactin as overexpression of other SJ proteins had no effect on cell polarity (Schulte et al., 2006) and no effect on cell survival. For instance, overexpression of Dlg (supplementary material Fig. S5) or NrxIV (data not shown) using apterous–GAL4 did not result in cell delamination or cell death.

If delamination and migration result from the increased presence of Gliotactin in the plasma membrane, the GliFF mutation would be expected to have the greatest effect. To test this, we measured the distance that migrating cells had traveled from the dorsal–ventral boundary of apterous expression into the ventral compartment (Fig. 5). In GliFF-overexpressing discs, migrating cells had traveled a significantly longer distance in comparison with those overexpressing GliWT (Fig. 5B) (n=20 discs). Interestingly, unlike GliWT and GliFF, GliDD overexpression did not result in any cell delamination or migration phenotypes (Fig. 5C) (n=20 discs). These observations suggest that the presence of high levels of Gliotactin throughout the SJ domain might lead to signaling and the activation of apoptotic signals. By contrast, GliDD, which presumably mimics a steady state of tyrosine phosphorylation, is rapidly endocytosed and becomes degraded by lysosomes. The rapid endocytosis and degradation of GliDD might prevent the activation of apoptotic signals.

To test whether the migrating cells are in the process of elimination and death, we immunolabeled wing discs overexpressing each transgene for cleaved Caspase-3, the activated form of the effector caspase in apoptotic cells (Yu et al., 2002). For both GliWT and GliFF, activated Caspase-3 immunolabeling was concentrated in the wing pouch (Fig. 6F,N), corresponding to the...
area of delaminated and migrating cells on the basal side of the epithelium. Colabeling with the nuclear marker DAPI showed that the cells contain pyknotic nuclei, which is a hallmark of apoptosis (Fig. 6G, O). A side projection of the wing discs shows that the delaminated cells were below the plane of the epithelium and travelled along the basal membrane (Fig. 6M–P). GliDD overexpression, however, resulted in little or no cell death or delamination (Fig. 6I–L). These results suggest that the levels of Gliotactin are regulated in order to maintain the integrity of epithelial cells. The reduced amount of cell death in GliDD-overexpressing cells also indicates that mimicking tyrosine phosphorylation of Gliotactin likely accelerates the rate of endocytosis and speeds up the removal of excess Gliotactin from the cell membrane.

The preceding experiment tested the effect of overexpression of the different Gliotactin transgenes in the presence of endogenous Gliotactin. Given the ability of Gliotactin to form dimers or oligomers, we wanted to test the effect of each transgene alone in a Gli null background. Expression of GliWT and GliDD transgenes, using daughterless–GAL4, in GliΔ4545 mutant discs, resulted in delaminated cells that were positive for activated Caspase-3 plus contained pyknotic nuclei (supplementary material Fig. S6). These results suggest that, given its extensive endocytosis, the expression of GliDD was not sufficient to fully rescue the cell lethality observed in cells lacking Gliotactin (Venema et al., 2004) (K. Charish, personal communication). Conversely, the spread of GliWT or GliDD beyond the TCJ in these cells might be deleterious, similar to the situation for overexpression experiments. Surprisingly, the GliFF transgene, when expressed in the absence of endogenous Gliotactin, had no evidence of activated Caspase-3 or pyknotic nuclei (supplementary material Fig. S6). These results suggest that, in the absence of endogenous Gliotactin, the lack of endocytosis of GliFF is not deleterious. Collectively, these data suggest that, when overexpressed in a wild-type disc, GliFF blocks the endocytosis of endogenous Gliotactin, and the resulting retention and persistence of wild-type, phosphorylated Gliotactin in the plasma membrane results in cell death.

**High levels of Gliotactin increases cell proliferation**

In addition to apoptosis, overexpression of Gliotactin in wing discs also resulted in ectopic folds. In order to investigate further these extra folds, we checked whether the ectopic folds were a result of increased cell proliferation. Immunolabeling with phospho-histone3, which labels mitotic cells (Tapia et al., 2006), showed a significant increase in the number of dividing cells in the apertuous-GAL4 (dorsal) compartment of the wing disc (Fig. 7A–L). Statistical analysis for the different transgenes revealed that the overexpression of each in the presence of endogenous Gliotactin increased cell proliferation. Of note, GliFF had a higher ratio of cell proliferation compared with GliWT and GliDD, both of which showed similar levels of increased cell division (Fig. 7M–P). These observations suggest that the comparatively higher level of GliFF at the cell membrane leads to increased cell proliferation possibly through the retention of endogenous Gliotactin. An alternative possibility is that the increased number of apoptotic cells in GliFF-expressing discs send proliferation signals to the neighboring cell to compensate for cell loss and to maintain tissue homeostasis, a process known as compensatory proliferation (Fan and Bergmann, 2008).

**Overproliferation, cell migration and death resulting from Gli overexpression is mediated by Drosophila JNK**

One of the major signaling pathways involved in apoptosis, cell proliferation and motility is the Stress-activated Jun N-terminal
kinase JNK (Martin-Blanco et al., 2000; Mattila et al., 2005; McEwen and Peifer, 2005; Perez-Garrio et al., 2009; Ryoo et al., 2004). To test whether JNK is activated downstream of Gliotactin, we looked to see whether JNK signaling was increased when Gliotactin is overexpressed. Immunolabeling with an antibody against phosphoJNK, which detects the activated form of JNK, revealed increased phosphoJNK in the dorsal compartment when GliWT was driven with apterous–GAL4. A known target of JNK signaling is Mmp1 (encoding Matrix metalloproteinase 1) (Uhlirova and Bohmann, 2006), which enhances cell migration by degrading the extracellular matrix (McCawley and Matrisian, 2000; Page-McCaw et al., 2003). Immunolabeling showed increased Mmp1 in the dorsal compartment and in the migrating cells (Fig. 8A–F). Finally, we tested the effect of reducing puckered (puc), a phosphatase that negatively regulates JNK (Martin-Blanco et al., 1998). The puc
allele when heterozygous effectively increases JNK activity and enhanced the migratory behavior of the Gliotactin-overexpressing cells as well as the associated cell death (Fig. 8J–L), providing further evidence in support of JNK signaling in this process.

Next we asked whether inhibition of JNK signaling is sufficient to block the cell migration and death in the Gliotactin-overexpressing cells. In order to address this question, we coexpressed a dominant-negative form of the Drosophila JNK, basket (Bsk\textsuperscript{DN}) with GliWT under the control of apterous–GAL4. Strikingly, inhibition of JNK signaling by expression of Bsk\textsuperscript{DN} completely blocked the invasiveness as well as the cell death caused by overexpression of Gliotactin (Fig. 8M–O). These results suggest that high levels of Gliotactin lead to activation of the JNK pathway. The end result is an increase in mitotic cell division, cell delamination, migration and apoptosis.

Blocking apoptosis with p35 in Gliotactin-overexpressing wing discs results in overgrowth

Given the apoptosis of those cells overexpressing GliWT and GliFF, we wanted to determine the consequences of blocking apoptosis using the baculovirus caspase inhibitor p35 (Hay et al., 1994). Expression of p35 alone using apterous–GAL4 did not affect the normal development of wing imaginal discs (Fig. 9A). However, blocking apoptosis with p35 in wing discs expressing high levels of Gliotactin resulted in overgrowth of the apterous–GAL4 compartment (Fig. 9B). Specifically, we observed increased folding and an absence of cell migration into the ventral compartment. Blocking apoptosis in GliFF-overexpressing discs (Fig. 9C) resulted in dramatically enhanced overgrowth compared with GliWT coexpressed with p35 (Fig. 9B). The more severe overgrowth phenotype in those cells expressing both GliFF and p35 could be due to a lack of endocytosis and the persistence of Gliotactin-mediated signaling at the plasma membrane. Conversely, the greater number of apoptotic cells observed in the GliFF-overexpressing discs could contribute to a higher level of compensatory proliferation. Blocking apoptosis with p35 has been shown to keep the dying cells in an ‘undead’ state such that these cells continuously send compensatory proliferation signals to the neighboring cells, resulting in tissue overgrowth (Martin et al., 2008).

Overgrowth after Gli overexpression is mediated by JNK signaling

Previous studies have reported a role for Drosophila JNK in compensatory proliferation (Fan and Bergmann, 2008; Perez-Garrijo et al., 2004; Ryoo et al., 2004). JNK is the main regulator of compensatory proliferation, and it induces the activity of Protein wingless (Wg) and Protein decapentaplegic (Dpp) (Perez-Garrijo et al., 2009). The ectopic expression of these mitogens when apoptosis is blocked results in overgrowth. Given the role of JNK signaling downstream of Gliotactin, we checked for ectopic Dpp, Wg or Protein decapentaplegic (Dpp) (Perez-Garrijo et al., 2009). The ectopic expression of these mitogens when apoptosis is blocked results in overgrowth. Given the role of JNK signaling downstream of Gliotactin, we checked for ectopic Dpp, Wg or JNK activation in wing discs expressing both GliWT and p35 under the control of apterous–GAL4 (Fig. 9). Immunolabeling with an antibody against phosphoMad, which detects the activated form of Mad, a target of Dpp (Telemann and Cohen, 2000), showed ectopic expression in the dorsal compartment overexpressing...
Gliotactin and p35 (Fig. 9G–I). Similar observations were made with wingless–lacZ and puckered–lacZ reporters, which reflect the activation of endogenous Wg and JNK, respectively (Fig. 9J–O) (McEwen and Peifer, 2005). In addition, the JNK target Mmp1 also showed ectopic activation in the dorsal compartment overexpressing Gliotactin and p35 (Fig. 9P–R). These observations further support the view that JNK signaling and its targets are activated by overexpression of Gliotactin. The dramatic overgrowth seen with GliWT and GliFF in the presence of p35 suggests that JNK might also be initiating the compensatory proliferation pathway in these cells.

Discussion

Tricellular junctions, in spite of their importance in the formation and maintenance of SJs and the establishment of permeability barriers, have received very little attention. Currently, in Drosophila, Gliotactin is the only protein uniquely located to the TCJ complex (Schulte et al., 2003). However, little is known about the localization, interaction with other SJ components and the function of individual domains of Gliotactin. Here, we demonstrate that the two conserved tyrosine residues of Gliotactin, Y766 and Y790, are necessary for the endocytosis and degradation of Gliotactin. Mutation of these residues to phenylalanine blocks the endocytosis of Gliotactin and its subsequent degradation. Conversely, mutation of these residues to aspartate increases endocytosis and promotes degradation.

Phosphorylation by tyrosine kinases and subsequent endocytosis is a key mechanism for downregulation of signaling molecules (Chen et al., 2007) and adherens junctions (Behrens et al., 1993). However, little is known about the regulation of junctional complexes that form at the septate or tricellular permeability barrier. Our data suggest that tyrosine phosphorylation, endocytosis and degradation might be a mechanism to localize and restrict Gliotactin to the TCJ. Blocking endocytosis with dominant-negative Rab5 expression resulted in the spread of Gliotactin beyond the TCJ into the bicellular SJ. This points to the existence of an endocytic mechanism to remove excess Gliotactin from the SJ domain.

Why is there a mechanism to remove excess Gliotactin? Our previous work indicated that high levels of Gliotactin in the bicellular SJ resulted in Gliotactin binding to other SJ components such as Neurexin IV (NrxIV) and reduced the level of Dlg (Schulte et al., 2006). One possible explanation could be that the interaction of Gliotactin with SJ components might block the normal interactions of these components and disrupt cell polarity or junction assembly, resulting in cell death. However, overexpression of Gliotactin did not lead to changes in cell polarity or loss of cell adhesion. Another possibility is that overexpression of SJ proteins in general might result in cell death. However, the overexpression of Dlg or NrxIV had no effect on cell viability. Alternatively, the spread of Gliotactin into the SJ domain might trigger a signaling cascade that is regulated by the rapid endocytosis of Gliotactin. In support of this, GliDD, which mimics phosphorylation of Gli, when overexpressed resulted in more endocytic vesicles and induced little or no cell delamination or apoptosis. The increased delamination and cell death observed with the overexpression of GliFF supports the idea that phosphorylation of Gliotactin residues is part of a mechanism to remove Gliotactin rapidly from bicellular SJs. This also suggests that the increased presence of Gliotactin within the SJ or the plasma membrane triggers a cell signal that needs to be controlled. The contrasting results we observed between the expression of GliFF alone in a null background compared with the expression of endogenous Gliotactin point to a signaling mechanism that is controlled by one or both of the conserved tyrosine residues in Gliotactin. Gliotactin, like other members of the neuroligin family, forms dimers or oligomers (Venema et al., 2004). Thus, we suggest that overexpression of GliFF blocks the endocytosis of endogenous Gliotactin proteins, resulting in persistent Gliotactin signaling in the bicellular SJ domain through the phosphoTyr domains.

A strong candidate for a signaling pathway downstream of ectopic Gliotactin is the JNK pathway. Overexpression of Gliotactin resulted in the upregulation of phospho-JNK and JNK targets such as Mmp1. Blocking JNK signaling completely suppressed the cell migration and death induced by overexpression of Gliotactin. Conversely, increasing JNK activity enhanced the overexpression phenotypes of Gliotactin, including an increase in cell death and migration.

One of our interesting findings was the increase in number of mitotic cells and the generation of ectopic folds in the presence of high levels of Gliotactin. The enhanced proliferation in the presence of excess Gliotactin could reflect a signaling pathway occurring downstream of Gliotactin in mediating cell proliferation. Alternatively, the cell death associated with ectopic Gliotactin could stimulate compensatory proliferation, which is also under the control of the JNK pathway (Perez-Garijo et al., 2009).

Coexpression of Gliotactin and p35 to block apoptosis resulted in enhanced overgrowth, especially in the case of GliFF. It has been shown previously that, if apoptosis is blocked by p35, the remaining ‘undead’ cells continue to secrete compensatory proliferating signals to their neighboring cells, resulting in tissue overgrowth (Fan and Bergmann, 2008; Martin et al., 2008). When p35 was coexpressed with Gliotactin, we observed an upregulation of Dpp and Wg signaling, and overall our results mirror the effects downstream of JNK in the compensatory proliferation pathway (McEwen and Peifer, 2005). Therefore, it is likely that the high level and the subsequent spread of Gliotactin away from the TCJ leads to the activation of JNK, resulting in delamination and apoptosis. Downstream of JNK, compensatory proliferation is induced to make up for the loss of imaginal disc cells. It still remains to be investigated how these processes communicate to each other and why the spread of Gliotactin away from the TCJ in the membrane leads to the activation of the JNK pathway.

Materials and Methods

Fly stocks

The following fly strains were used: Gliotactin null alleles, GliF<sup>2A47</sup> and Gli<sup>C2</sup> (Venema et al., 2004), UAS lines including UAS-GliWT, UAS-GliDD, UAS-GliFF (generated below), UAS-mCD8GFP, UAS-Rab5DN, UAS-p35 and NrxIV<sup>EP604</sup> from the Bloomington stock center, UAS-Bsk<sup>DN</sup> from the DGRC Kyoto stock center, UAS-DlgGFP from D. Bilder. The daughterless–GAL4 and apertorous–GAL4 lines from the Bloomington stock center were used as GAL4 drivers. Puckered–lacZ A251.1F3 from Bloomington stock center and wingless–lacZ were used as reporter lines for Drosophila JNK, and Wg, respectively. The <i>puc</i> allele, an imprecise excision in <i>puckered</i> (Kirchner et al., 2007), and w<sup>1118</sup>, used as wild type, were from the Bloomington stock center.

Generation of transgenic lines

For generation of different Gliotactin transgenic lines, the N-terminus of Gliotactin (GliNter), the extra intracellular coding sequence and the transmembrane domain was amplified from a Gliotactin cDNA (AE27.41) (Auld et al., 1995) using the following primers: GliNter5 (5’-AGATCTACAGGTGAGAGTTCGCTGCGC-3’) and GliNter3 (5’-GAATTCGACTAGTCATGATGCAATGCAATC-3’). Similarly, the C-terminus of Gli (GliCter) was amplified using the following primers: 5’-GGATCCGCAGATGCGGAGATGAGATGACG-3’ and 5’-GTCGAGGGAAGTGCAGCTTCCAACATCGATCTTGCGGCTGCGGCTGCGGCTGCGGCTGCGGCTGCGGCTGCGGCTGCGGCTGCGGCTGGCAAGCCACATC-3’.
using the Quik Change Kit (Stratagene). The following primers were employed for generation of different tyrosine residue mutations: 

- Y790D mutation – GliY1D1 (5'-CCAGGGGGATATCATCAGTGCTGCACGACTCC-3') and GliY2D1 (5'-GGGAGAGTCGCCATGCAATATTATCCCTG-3'); 
- Y790F mutation – GliY1F1 (5'-CCGGGGAATATATCCTTGACGACCGACTCC-3') and GliY2F1 (5'-GGGAGACCTTGCACTGGAAGATGTCTT-3').

The resulting plasmids were sequenced for verification, and each individual construct was then treated with a second restriction enzyme to generate the necessary double mutations GliDD (Y766D, Y790D) and GliFF (Y766F, Y790F).

The two parts of Gliotactin were then subcloned into a modified pUAST vector (pUAS-linker). GliNter was introduced using the BglII, EcoRI digest to generate pUAS-GliNter, and each GliCter product (WT, DD or FF) was introduced using an EcoRI, XhoI digest to generate pUAS-GliWT, pUAS-GliDD or pUAS-GliFF, respectively. Each construct was sequenced to verify accuracy and then sent to Genetics Services (Cambridge, MA) for generation of transgenic lines.

**Biochemistry**

**Generation of His-tagged Gliotactin C-terminal constructs**

The Gliotactin C-terminal domain with and without the Y766F and Y790F mutations were subcloned from pGEX4T Gliotactin C-terminal constructs into pET28a (Novagen) using EcoRI and XhoI sites. The final construct was transformed into BL21DE3 pLysS competent cells (Invitrogen). Protein expression was induced using 1 mM IPTG for 45 min, and the cells were lysed in 40 mM imidazole, 300 mM NaCl, 50 mM Tris-HCl pH 8) and dialyzed using Slide-A-Lyzer 7000MWCO cassettes (Pierce) into Kinase Buffer (25 mM Tris-HCl pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM sodium orthovanadate, 10 mM MgCl₂).

**Kinase assay**

A 50 µl Kinase Reaction [1 µg GliCter in pET28, 1 µg GST–Src (Cell Signaling, Technologies), 1% Kinase Buffer, 1 mM ATP] was incubated at 30°C for 1 h. One quarter of this reaction was loaded for SDS-PAGE and western blot analysis.

**Immunoprecipitations**

Approximately 20 adult flies overexpressing GliWT, GliFF or GliDD with daughterless-GAL4 were homogenized in 20 mM Tris-HCl pH 8, 137 mM NaCl, 10% Glycerol, 1% NP-40, 2 mM EDTA, 1/100 Complete (Roche) and 1/100 PhosStop (Roche). Lysates were spun at 12,000 × g, for 10 min at 4°C. Supernatants were added to 20 µl of Protein G agarose (Calbiochem), previously incubated with ~1 µg of mouse anti-Gliotactin IF6.3 antibody (Auld et al., 1995). The beads were washed four times with 20 mM Tris-HCl pH 8, 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA and once with PBS. The bound proteins were eluted in loading buffer.

**Loric western blot analysis**

Samples were run on 10% SDS-PAGE gels then transferred to nitrocellulose membranes. Blots were blocked using 5% Odyssey blocking buffer (LI-COR) diluted in PBS for LI-COR Odyssey fluorescent detection systems. The primary antibodies rabbit anti-Gli IF6.3 and mouse anti-phosphoTyr 4G10 (a gift from M. Gold, mouse anti-PhosphoH3 at 1:500 (Abcam), rabbit anti-PhosphoBioT at 1:200 (Aigle, rabbit anti-PhosphoNK at 1:200 (a gift from M. Gold), mouse anti-Mmp1 at 1:100 (Page-McCaw et al., 2003), rabbit anti-phosphoMAD at 1:300 (a gift from D. Allan), rabbit anti-RGα (Abcam), mouse anti-Golgi at 1:200 (Calbioche), rabbit-anti-Cnhexin at 1:300 (a gift from I. R. Nabi), the monoclonal antibodies listed from the Developmental Studies Hybridoma Bank were developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology. The following secondary antibodies and dilutions were used: goat anti-rabbit Alexa 488 at 1:300, goat anti-mouse Alexa 568 at 1:300, goat anti-rat AlexaA-647 at 1:300, goat anti-mouse Alexa 488 at 1:300 (Molecular Probes).

**Imaging**

Image stacks were collected with a DeltaVision Spectris microscope (Applied Precision), on an Olympus IX70 with >×60 oil-immersion lens (NA 1.4), using 0.2 µm z sections. The image stacks were deconvolved with SoftWorx (Applied Precision), based on measured point-spread functions from 0.2 µm fluorescent beads (Molecular Probes) mounted in Vectashield. Side projections were created using the SoftWorx program. Images were exported to Photoshop for compilation. For lower magnification, images were collected with a ×20 objective on a Zeiss Axioskop using Northern Eclipse software.

**Statistical analysis**

The distance travelled by migrating cells from the D–V boundary was measured using the linear tool in Northern Eclipse. Overproliferation was measured for each of the apterous-Gli transgenes, GliWT, GliDD and GliFF as well as for the apterous-mCDGPFP discs. For each, comparable areas from the dorsal and ventral compartments of the wing pouch were selected, and the number of objects (PH3 cells) was counted digitally using Northern Eclipse software. For statistical analysis, GraphPad Prism software was used to perform a paired t-test on the two sets of data to be compared.

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**Supplementary material available online at** [http://ecbiologists.org/cgi/content/full/129/23/4052/DC1](http://ecbiologists.org/cgi/content/full/129/23/4052/DC1)

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