Gliotactin and Discs large are co-regulated to maintain epithelial integrity

Mojgan Padash-Barmchi, Kristi Charish, Jammie Que and Vanessa J. Auld*

Department of Zoology, Cell and Developmental Biology, University of British Columbia, Vancouver V6T 1Z3, Canada

*Author for correspondence (auld@zoology.ubc.ca)

Accepted 17 December 2012
Journal of Cell Science 126, 1134–1143

doi: 10.1242/jcs.113803

Summary

Establishment and maintenance of permeability barriers is one of the most important functions of epithelial cells. Tricellular junctions (TCJs) maintain the permeability barriers at the contact site of three epithelial cells. Gliotactin, a member of the Neuroligin family, is the only known *Drosophila* protein exclusively localized to the TCJ and is necessary for maintenance of the permeability barrier. Overexpression triggers the spread of Gliotactin away from the TCJ and causes epithelial cells to delaminate, migrate and die. Furthermore, excess Gliotactin at the cell membrane results in an extensive downregulation of Discs large (Dlg) at the septate junctions. The intracellular domain of Gliotactin contains two highly conserved tyrosine residues and a PDZ binding motif. We previously found that phosphorylation of the tyrosine residues is necessary to control the level of Gliotactin at the TCJ. In this study we demonstrate that the phenotypes associated with excess Gliotactin are due to a functional interaction between Gliotactin and Dlg that is dependent on both tyrosine phosphorylation as well as the PDZ binding motif. We further show that elevated levels of Dlg strongly enhance Gliotactin overexpression phenotypes to the point where tissue over-growth is observed. The exhibition of these phenotypes require phosphorylation of Dlg on serine 797, a known Par1 phosphorylation target. Blocking this phosphorylation completely suppresses the cell invasiveness and apoptotic phenotypes associated with Gliotactin overexpression. Additionally, we show that *Drosophila* JNK acts downstream of Gliotactin and Dlg to mediate the overgrowth and apoptosis caused by the functional interaction of Gliotactin and Dlg.

Key words: Septate junction, Tricellular junction, JNK, Discs large

Introduction

Establishment and maintenance of paracellular diffusion barriers is one of the most important tasks of epithelial cells. In *Drosophila*, permeability barriers are known as bicellular septate junctions, which are analogous to tight junctions in vertebrates (Noirot-Timothée et al., 1982; Tsukita et al., 2001). Bicellular septate junctions form between two neighboring epithelial cells and prevent paracellular diffusion. Mutations in any core components of the septate junctions, NrxIV (Baumgartner et al., 1996), Coracle (Genova and Fehon, 2003), Neuroglian and Na^+/K^+ ATPase (Yasuhara et al., 2000; Genova and Fehon, 2003; Paul et al., 2003) results in the disruption of septate junction formation. At the corners of epithelial cells the permeability barrier is established by the tricellular junction (TCJ), which is created by the convergence of three septate junctions (Fristrom, 1982; Noirot-Timothée et al., 1982). The only known protein exclusively found at the Drosophila tricellular junction to date is Gliotactin, a member of the Neuroligin family of choline esterase-like proteins (Schulte et al., 2003). Gliotactin is necessary for maintaining the permeability barriers at these contact sites as null mutants are paralyzed and die at late embryogenesis due to disruption of the TCJ and failure of the septate junction permeability barrier (Schulte et al., 2003).

Besides an extracellular choline-esterase-like domain, Gliotactin also contains an intracellular domain with two tyrosine phosphorylation residues and a PDZ binding motif, both found conserved in all Gliotactin homologues. Phosphorylation of the tyrosine residues are necessary for Gliotactin endocytosis, which is necessary to control the localization of Gliotactin to the TCJ. Blocking phosphorylation, disrupting endocytosis or overexpressing Gliotactin in imaginal disc epithelia causes Gliotactin to spread away from the TCJ and triggers a range of phenotypes including overproliferation, delamination and apoptosis (Padash-Barmchi et al., 2010). Tight regulation of Gliotactin and its restriction to the TCJ is necessary for cell survival, but the cellular pathways leading to these phenotypes and the mechanisms underlying the downregulation of Gliotactin has not been determined.

Gliotactin and Discs large (Dlg), a MAGUK scaffolding protein with three PDZ domains, colocalize to the TCJ and interact biochemically (Schulte et al., 2003; Schulte et al., 2006). Here we show that Dlg levels at the SJ domain are downregulated in conjunction with the presence of ectopic Gli and circumventing the reduction in Dlg results in extensive overgrowth of the imaginal disc epithelia and apoptosis. We find that both tyrosine phosphorylation and the PDZ binding motif of Gliotactin are necessary for this interaction as is phosphorylation of Dlg on Serine 797. Our results suggest that there is a specific mechanism to tightly control Gli localization to the TCJ and a reduction in Dlg is part of the cellular responses.

Results

Gliotactin interacts with Dlg in epithelial cells of the wing imaginal disc

In our previous study we showed that the level and localization of Gliotactin (Gli) is tightly controlled through tyrosine

phosphorylation, endocytosis and lysosome degradation. Overexpression of Gli results in the mislocalization of Gli away from the TCJ and a reduction in Dlg in entire septate junction domain (Schulte et al., 2006), and this spread leads to cell delamination and apoptosis (Padash-Barmchi et al., 2010). In this paper we set out to understand the molecular mechanism underlying the interaction between Gli and Dlg. We used the *Drosophila* wing imaginal disc as it provides a powerful system for studying cell junctions as well as for tissue specific expression using the GAL/UAS binary expression system (Brand and Perrimon, 1993). Expression of wild-type Gliotactin (GliWT) using the apterous-GAL4 driver in the dorsal compartment of the wing imaginal disc resulted in the spread of Gli away from the TCJ and into the bicellular septate junction (Fig. 1D–F). This was paired with a consistent reduction in Dlg immunolabeling, an effect that was not seen when the mCD8-GFP transgene was expressed (Fig. 1A–C′). Since overexpression increases the endocytosis of Gli (Padash-Barmchi et al., 2010), we wanted to see if the reduction in Dlg was due to co-endocytosis of Dlg with Gliotactin. By observing the GliWT overexpressing discs in greater detail, we found that Dlg was found colocalized with Gli in intracellular vesicles (Fig. 1G–I, arrowheads). Western analysis of wing imaginal discs expressing GliWT confirmed the reduction in Dlg protein levels observed with the immunolabeling (see later).

We have previously shown that phosphorylation on two conserved tyrosine residues in the intracellular domain of Gliotactin mediates endocytosis and degradation (Padash-Barmchi et al., 2010). To determine if tyrosine phosphorylation and endocytosis of Gliotactin played a role in the reduction of Dlg, we expressed a non-phosphorylated form of Gliotactin where the two tyrosines were mutated to phenylalanine, GliFF and assayed the effect on Dlg. GliFF is not endocytosed (Padash-Barmchi et al., 2010) and Dlg levels were not affected when GliFF was expressed (Fig. 1J–L). Conversely a Gliotactin transgene GliDD that mimics phosphorylation with both tyrosines mutated to aspartic acid and is extensively endocytosed (Padash-Barmchi et al., 2010) did result in the reduction in Dlg (Fig. 1M–O). Similar to GliWT, the intracellular vesicles formed by endocytosis of GliDD displayed high levels of Dlg colocalization (Fig. 1P,Q). These data suggested that tyrosine phosphorylation and the endocytosis of ectopically expressed Gliotactin is necessary to trigger the reduction in Dlg.

We attempted to block endocytosis to determine if co-endocytosis was the only means by which Dlg levels were reduced. However, coexpression of a dominant negative form of the small GTPase, Rab5 along with GliWT was cell lethal, most likely due to the persistence of excess levels of Gliotactin within the membrane (data not shown).

Other SJ proteins such as Coracle (Schulte et al., 2006) or E-cadherin (supplementary material Fig. S1) were not downregulated or endocytosed with Gliotactin in the presence of ectopic Gliotactin, indicating the interaction between Gliotactin and Dlg is specific. To determine the cellular consequence of blocking the reduction in Dlg, we coexpressed both GliWT and a Dlg-A isoform tagged with GFP (Budnik et al., 1996). When driven using apterous-GAL4, the wing imaginal discs exhibited dramatic tissue overgrowth (Fig. 2D–F, arrows), far more than observed with Gliotactin overexpression alone (Fig. 2B, arrow). Overexpression of Dlg-GFP alone had no morphological effect on the imaginal discs (Fig. 2C). Moreover,

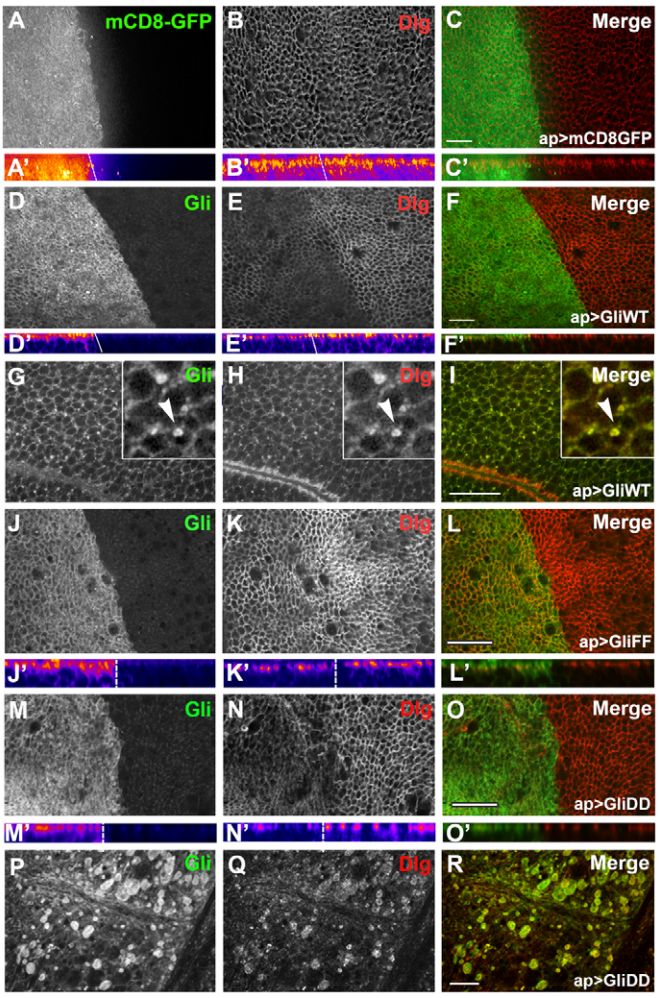

Fig. 1. Overexpression of Gli triggers a reduction in Dlg. Third-instar wing imaginal discs with apterous-GAL4 (ap-GAL4)-driven expression in the dorsal half, immunolabeled for Gli (green), Dlg (red) or marked with mCD8-GFP (green). (**A–C**) Control discs with apterous GAL4 (ap-GAL4) expression of mCD8-GFP has no effect on Dlg levels. (**A′–C′**) Side projections of A–C using the fire lookup table (A′,B′) to visualize the intensity of mCD8-GFP and Dlg immunolabeling. (**D–F**) Overexpression of Gli (ap>GliWT). Immunolabeling of Dlg is reduced in response to Gli overexpression compared with control. (**D′–F′**). Side projections using the fire lookup table to visualize the intensity of Gli and Dlg immunolabeling (D′,E′). (**G–I**) Higher magnification of the disc in D–F, indicating Dlg present with Gli in intracellular vesicles (arrowhead in inserts). (**J–L**) Overexpression of GliFF (ap>GliFF). Expression of a non-phosphorylated form of Gli (GliFF, green) results in no reduction in the levels of Dlg (red). (**J′–L′**). Side projections using the fire lookup table (J′,K′) to visualize the intensity of GliFF and Dlg immunolabeling. (**M–O**) Overexpression of GliDD (ap>GliDD). Expression of a form of Gli with aspartic acids to mimic phosphorylation (GliDD, green) does reduce the levels of Dlg (red). (**M′–O′**). Side projections of using the fire lookup table (M′,N′) to visualize the intensity of GliDD and Dlg immunolabeling. (**P–R**) Overexpression of GliDD (green) leads to increased endocyosis and colocalization with Dlg (red) in large intracellular vesicles. Scale bars: 15 μm (A–F′,J–R); 7.5 μm (G–I).

Dlg coexpressed with Gli was found colocalized with Gli in intracellular vesicles (Fig. 2J–L, arrowheads) identified as endosomes (Padash-Barmchi et al., 2010) (supplementary material Fig. S1). Dlg when overexpressed alone was not

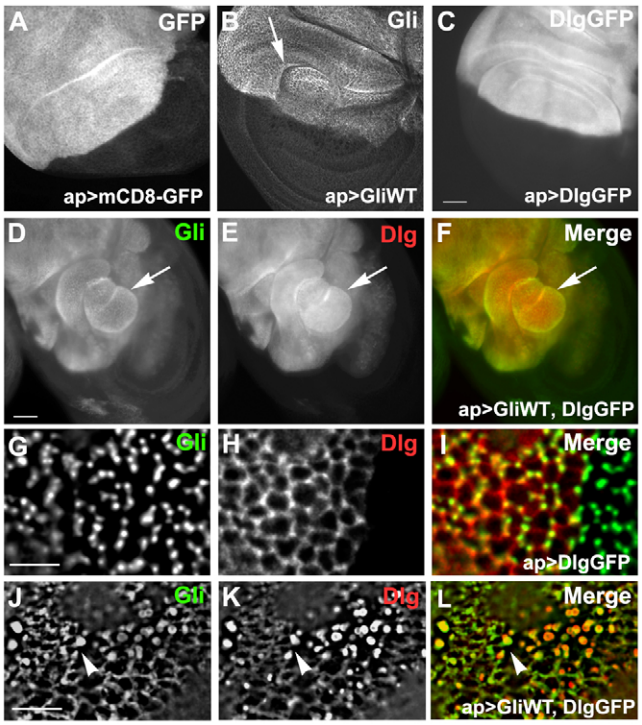

Fig. 2. Overexpression of Gli and Dlg triggers overgrowth. Third-instar wing imaginal discs expressing UAS constructs with apterous-GAL4 (ap-GAL4). (**A**) Control disc with ap-GAL4 driven mCD8-GFP (ap>mCD8GFP). Expression of mCD8GFP has no effect on the morphology of wing imaginal disc. (**B**) Overexpression of GliWT (ap>GliWT). Expression of GliWT in dorsal compartment results in the formation of ectopic folds (arrow). (**C**) Overexpression of Dlg tagged with GFP (ap>Dlg-GFP). Expression of Dlg has no effect on the morphology of the wing disc. (**D–F**) Overexpression of Gli and Dlg-GFP (ap>GliWT, Dlg-GFP). Coexpression of Gli and Dlg results in extensive overgrowth, with pockets of tissue protruding from the disc (arrows). (**G–I**) Overexpression of Dlg-GFP (ap>Dlg-GFP). Dlg is not found in intracellular vesicles when expressed alone. (**J–L**) Overexpression of Gli and Dlg-GFP (ap>GliWT, Dlg-GFP). Coexpression of Gli and Dlg results in the formation of intracellular Gli-Dlg vesicles (arrowheads). Scale bars: 40 μm (A–F); 15 μm (G–L).

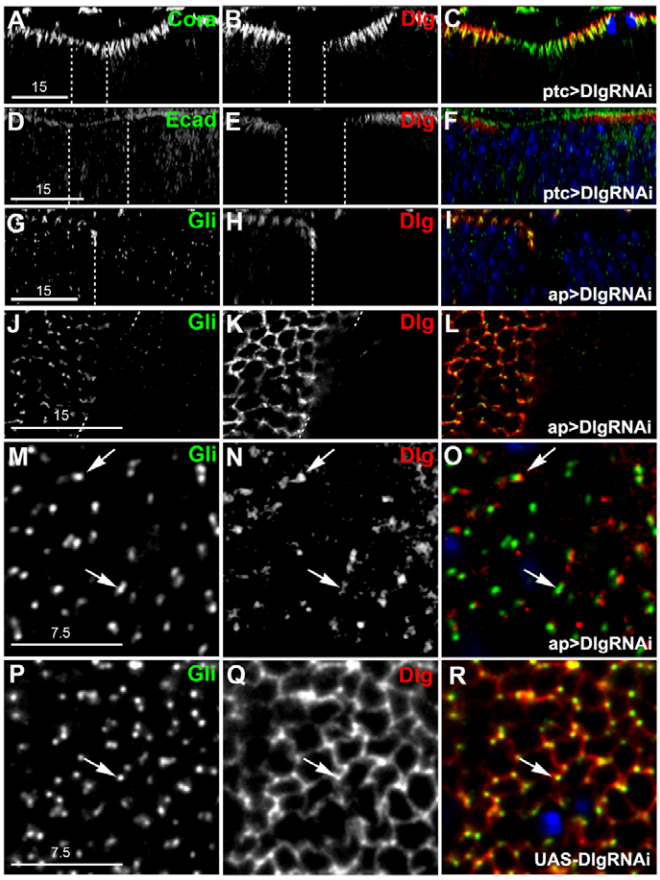

Fig. 3. RNAi mediated knockdown of Dlg affects Gli localization. Third-instar wing imaginal discs with apterous-GAL4 (ap) or patched-GAL4 (ptc) driving the expression of Dlg-RNAi. (**A–C**) DlgRNAi driven by ptc-GAL4 removes Dlg immunolabeling (red) but has little effect on Cora (green) immunolabeling (area highlighted by dashed lines). (**D–F**) DlgRNAi driven by ptc-GAL4 removes Dlg immunolabeling (red) but Ecad (green) is still present at the adherens junction (area highlighted by dashed lines). (**G–I**) DlgRNAi driven by ap-GAL4 removes Dlg immunolabeling (red) and Gli (green) is no longer localized to the SJ domain (dashed line marks the border between wild-type and Dlg RNAi). (**J–L**) Enface view of Gli (green) mislocalization when Dlg (red) is knocked down to the point where immunolabeling is no longer present (dashed lines indicate borders). (**M–O**) Enface view of the dorsal half of the wing disc with apterous-GAL4 driving Dlg RNAi. Gli (green) and residual Dlg immunolabeling (red) are retained at the tricellular corners (arrows). (**P–R**) Enface view of the ventral half of the wing disc in the non-apterous control region. Gli (green) and Dlg (red) are normally distributed with arrows indicating the wild-type tricellular corner. Scale bars: 15 μm (A–L); 7.5 μm (M–R).

localized to intracellular vesicles (Fig. 2G–I). These results further suggest that there is a strong association of ectopic Gli and Dlg beyond the TCJ such that Dlg is endocytosed along with Gli and that this ectopic interaction results in tissue overgrowth.

Loss of Dlg results in the loss of Gliotactin from the TCJ

Gliotactin has a highly conserved PDZ binding motif and we have previously shown that Dlg and Gliotactin are found in the same protein complex but do not directly bind when tested *in vitro* (Schulte et al., 2006). While loss of Gliotactin has no effect on the localization of Dlg in the wing imaginal disc, Gliotactin is mislocalized in a Dlg null mutant (Schulte et al., 2006). Since epithelial polarity is disrupted in the Dlg null mutant, the loss of Gliotactin localization could be a consequence of this rather than the absence of Dlg. To test whether the interaction between Gliotactin and Dlg was specific, we used the patched- or apterous-GAL4 drivers to express an RNAi line known to effectively knockdown Dlg (Grzeschik et al., 2010; Brumby et al., 2011) (Fig. 3). By titrating the degree of Dlg knockdown, we determined that using Dlg RNAi without Dicer2 at 25℃ resulted in a downregulation of Dlg but no loss of cell polarity. Under these conditions Dlg levels were reduced but the septate junctions (Fig. 3A–C) and adherens junctions (Fig. 3D–F) remained intact. In all cases where Dlg was absent from the cell corners, Gliotactin was also lost (Fig. 3G–L). The last place residual Dlg immunolabeling was detected was at the TCJ and in those instances Gliotactin was retained at the TCJ (Fig. 3M–O). Therefore, it appears that Dlg functions to recruit or stabilize Gliotactin at the TCJ.

Gliotactin PDZ binding motif is necessary for the reduction in Dlg

As ectopic Gliotactin results in a downregulation of Dlg from the septate junction, we checked to see if the Gliotactin PDZ binding motif mediated the reduction of Dlg as this motif is recognized by PDZ domain proteins. A form of Gliotactin that lacked the PDZ binding motif (GliΔPDZ) (Schulte et al., 2003) was expressed under the control of apterous-GAL4 and had no effect on the levels or localization of Dlg (Fig. 4D–F). However, cells expressing the GliΔPDZ protein contained many Gliotactin-positive vesicles and Gliotactin levels at the cell membrane were greatly reduced compared to GliWT (Fig. 4I). While GliΔPDZ is normally trafficked to the plasma membrane (Schulte et al., 2003), our observations suggest that the lack of the PDZ binding motif triggers the increased endocytosis of ectopic Gliotactin. Therefore it appears that the reduction in Dlg at the membrane by ectopic Gliotactin requires the PDZ binding motif. In support of this conclusion, when GliΔPDZ was coexpressed with Dlg-GFP, Dlg was not colocalized with the Gliotactin-positive intracellular vesicles (Fig. 4J–L).

To confirm the higher levels of endocytosis of GliΔPDZ, we blocked endocytosis of GliΔPDZ by coexpressing a dominant negative form of Rab5 (Zhang et al., 2007a). GliΔPDZ was found and retained throughout the bicellular SJ domain, did not localize to intracellular vesicles (Fig. 4P) and Dlg levels remained unaffected (supplementary material Fig. S1). The absence of the PDZ binding motif does not block the tyrosine phosphorylation of Gliotactin as the GliΔPDZ protein at the membrane colocalized with phosphotyrosine immunolabeling (Fig. 4P–R). GliΔPDZ was also found to be tyrosine phosphorylated when immunoprecipitated from embryonic extracts expressing GliΔPDZ under the control of daughterless-GAL4 (Fig. 4S,T). A major degradation product was more prevalent in the GliΔPDZ immunoprecipitation (arrowhead) compared to full-length Gliotactin (arrow), and was strongly phosphotyrosine positive suggesting that GliΔPDZ is overall less stable than GliWT.

Overall these observations suggest that the Gli-induced Dlg reduction is mediated to a large extent by the co-endocytosis of Gliotactin and Dlg. Reducing either endocytosis using GliFF (Fig. 1J–L) or blocking a physical interaction between Gliotactin and Dlg using GliΔPDZ (Fig. 4) stops the reduction in Dlg.

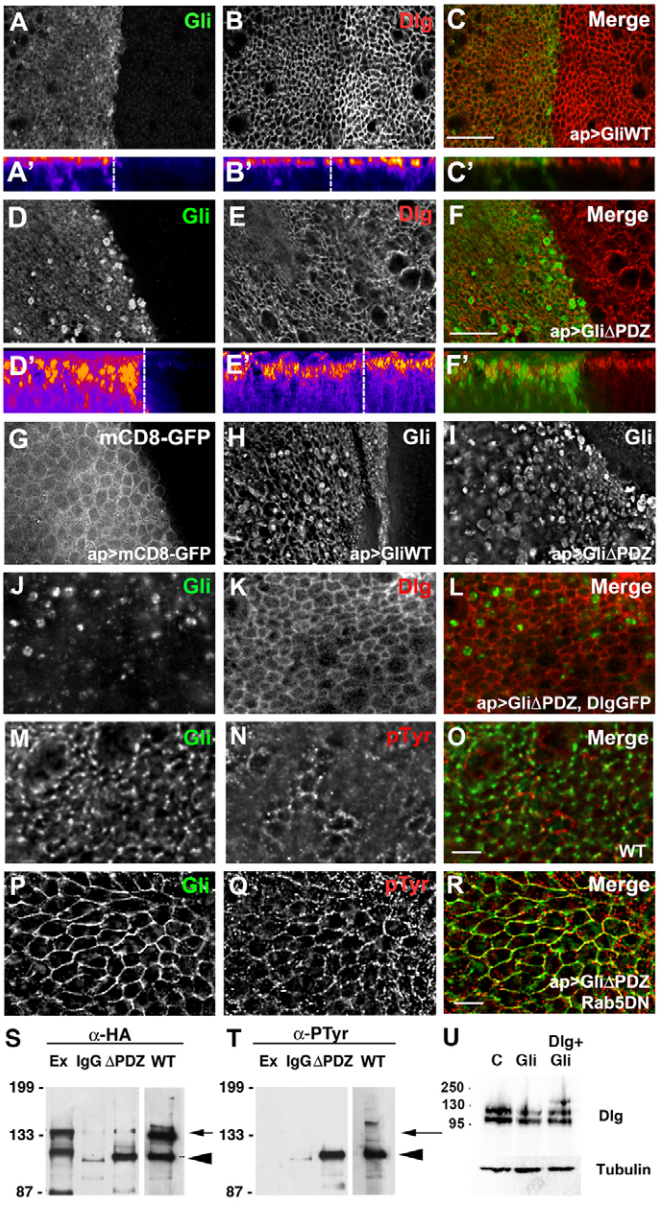

Fig. 4. The PDZ motif of Gli mediates the Dlg downregulation. Third-instar wing imaginal discs with apterous-GAL4 driven expression of different Gli constructs. All images were collected with a 60× objective except panels M–O, which were collected using a 20× objective. (**A–C**) Overexpression of GliWT (ap>GliWT). Expression of GliWT (green) results in a reduction in Dlg immunolabeling (red). (**A′–C′**) Side projections of A–C using the fire lookup table (A′,B′) to visualize the intensity of GliWT and Dlg immunolabeling. (**D–F**) Overexpression of GliΔPDZ (ap>GliΔPDZ). Expression of Gli lacking the PDZ binding motif (green) showed no reduction in Dlg immunolabeling (red). (**D′–F′**) Side projections using the fire lookup table (D′,F′) to visualize the intensity of GliΔPDZ and Dlg immunolabeling. (**G**) Expression of mCD8-GFP (ap>mCD8GFP) does not result in the formation of intracellular vesicles. (**H**) Overexpression of GliWT (ap>GliWT) shows the presence of Gli intracellular vesicles. (**I**) Overexpression of GliΔPDZ (ap>GliΔPDZ) generates extensive arrays of large Gli intracellular vesicles. (**J–L**) Coexpression of GliΔPDZ and Dlg-GFP does not result in the co-endocytosis of Gli and Dlg. (**M–O**) Gli (green) is found localized to the TCJ in wild-type (WT) columnar epithelia of the wing imaginal disc and does not normally overlap with phosphotyrosine immunolabeling (red), which is concentrated at the more apical adherens junctions. (**P–R**) Coexpression of GliΔPDZ and dominant negative Rab5 (ap>GliΔPDZ, Rab5DN). In the absence of the PDZ binding motif, Gli (green) is still phosphorylated and immunolabels with anti-phosphotyrosine (pTyr, red). (**S**,**T**) GliΔPZ is phosphorylated by phosphotyrosine. Immunoprecipitations of the HA epitope-tagged full-length Gli and GliΔPDZ expressed in embryos under the control daughterless-GAL4 and probed with anti-HA mAb (S) and anti-phosphotyrosine mAb (T). Full length Gli (arrow) and a major degradation isoform (arrowhead) are indicated. Ex, extracts; IgG, control mAb; ΔPDZ, GliΔPZ; WT, GliHA. (**U**) Western blot of protein extracts from wing imaginal discs expressing ap-GAL4 alone (control, C), ap>GliWT (Gli) and ap>GliWT, Dlg-GFP (Dlg+Gli). Dlg levels were detected using a Dlg mAb and were reduced in the presence of Gli. Multiple Dlg isoforms were observed similar to previous studies (Woods et al., 1996) including the higher molecular weight Dlg-GFP protein. Anti-tubulin was used as a loading control. Scale bars: 15 μm (A–L), 5 μm (M–R).

Gliotactin phosphorylation is necessary for both apoptosis and endocytosis

Our observations with GliFF raised some interesting questions. Previously we observed that expression of GliFF in wild-type discs in the presence of endogenous Gliotactin resulted in a greater degree of apoptosis and overproliferation compared to GliWT (Padash-Barmchi et al., 2010). However, Gliotactin, similar to other Neuroligin family members, functions as a dimer or an oligomer (Venema et al., 2004). In the presence of endogenous Gliotactin, it is likely that any GliFF/Gliotactin dimers would be phosphorylated and the presence of the GliFF/Gliotactin hybrids could be the trigger of the deleterious cell phenotypes. Alternatively, as GliFF did not trigger a reduction in Dlg (Fig. 1J–L), the persistence of Dlg in the presence of the more abundant GliFF/GliFF dimers could be the trigger. To test the consequences of GliFF overexpression by itself, we carried out a rescue experiment in which a *Gli* null mutant was rescued by the expression of either GliWT or GliFF using daughterless-GAL4 (Fig. 5). Daughterless-GAL4 displayed a patchy expression pattern in the wing imaginal discs but drove expression of both proteins at high enough levels to trigger spread into the septate junction domain. GliFF when expressed in the absence of endogenous Gliotactin did not trigger apoptosis or delamination (Fig. 5K–N'). This is in contrast to GliFF overexpression in the presence of endogenous Gliotactin that does trigger apoptosis detected using activated Caspase-3 or the presence of delaminated pyknotic nuclei (Fig. 6I–L) (Padash-Barmchi et al., 2010). As expected, driving strong levels of GliWT overexpression in a *Gli* null mutant background resulted in apoptotic cell death and delamination (Fig. 5G–J′), similar to what we observed in a wild-type background. We also observed that GliFF in a $Gli^{-/-}$ background did not result in a reduction of Dlg (Fig. 5D–F) while GliWT did (Fig. 5A–C), confirming the reduction in Dlg is dependent on the tyrosine phosphorylation and endocytosis of Gliotactin.

These results suggest that the mixed dimers or oligomers of GliFF and endogenous Gliotactin lead to apoptosis. These results also point to a dual role of tyrosine phosphorylation in mediating Gliotactin endocytosis and triggering the apoptosis observed.

Gliotactin–Dlg interaction results in cell delamination and death

The endocytosis of ectopic Gliotactin is an important cellular response as an excess of Gliotactin at the bicellular septate junction triggers cell delamination, proliferation and apoptosis (Padash-Barmchi et al., 2010). Phosphorylation of Gliotactin on the two highly conserved intracellular tyrosine residues is necessary for endocytosis and perhaps also mediates the interaction observed between ectopic Gliotactin and Dlg. Our results also suggest that the interaction between phosphorylated Gliotactin and Dlg is deleterious to the cell, as GliFF alone does not result in the same phenotypes. To test this hypothesis, we overexpressed Dlg in the presence of ectopic Gliotactin by coexpressing Dlg tagged with GFP (Dlg-GFP) (Budnik et al., 1996). Coexpressing GliWT and Dlg-GFP resulted in a significant enhancement of the GliWT phenotypes (Fig. 6E–H)

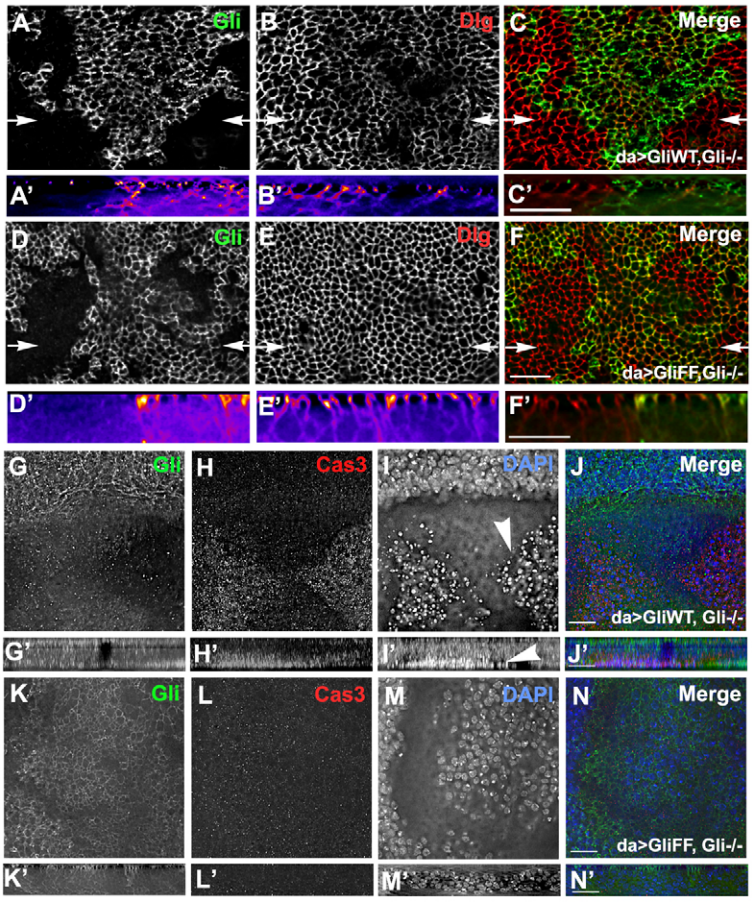

Fig. 5. Expression of Gli constructs in a *Gli* null. Third-instar wing imaginal discs from $Gli^{-/-}$ mutants rescued with daughterless-GAL4-driven expression of GliWT or GliFF constructs. All panels are a single *Z* slice of 0.2 µm. (**A–C**) Imaginal disc cells mutant for *Gli* expressing GliWT (da>GliWT, $Gli^{-/-}$) show spread of Gli (green) into the bicellular SJ domain and reduction in Dlg immunolabeling (red). (**A′–C′**) Side projections of A–C using the fire lookup table (A′,B′) to visualize the intensity of GliWT and Dlg immunolabeling across the region marked by arrows. (**D–F**) Imaginal discs cells mutant for *Gli* expressing GliFF (da>GliFF, $Gli^{-/-}$) show the spread of Gli (green) into the SJ domain but not the reduction of Dlg (red). (**D′–F′**) Side projections of D–F using the fire lookup table (D′,E′) to visualize the intensity of GliFF and Dlg immunolabeling across the region marked by arrows. (**G–J**) Wing imaginal discs mutant for *Gli* and expressing GliWT (da>GliWT, $Gli^{-/-}$) show apoptotic cell death, indicated by the presence of the cleaved Caspase-3 (Cas3, red) and pyknotic nuclei (arrowhead) labeled with DAPI (blue). The plane of this panel is at the basal side of the columnar epithelia. (**G′–J′**) Side view of G–J with an accumulation of delaminated cells with pyknotic nuclei on the basal of the columnar epithelia (arrowhead). (**K–N**) Wing imaginal discs mutant for *Gli* and expressing GliFF (da>GliFF, $Gli^{-/-}$) do not show apoptosis indicated by the absence of the activated Caspase-3 (Cas3, red) and pyknotic nuclei (DAPI, blue). The plane of this panel is at the basal side of the columnar epithelia. (**K′–N′**) Side view of K–N showing the lack of delaminated and pyknotic nuclei. Scale bars: 15 µm.

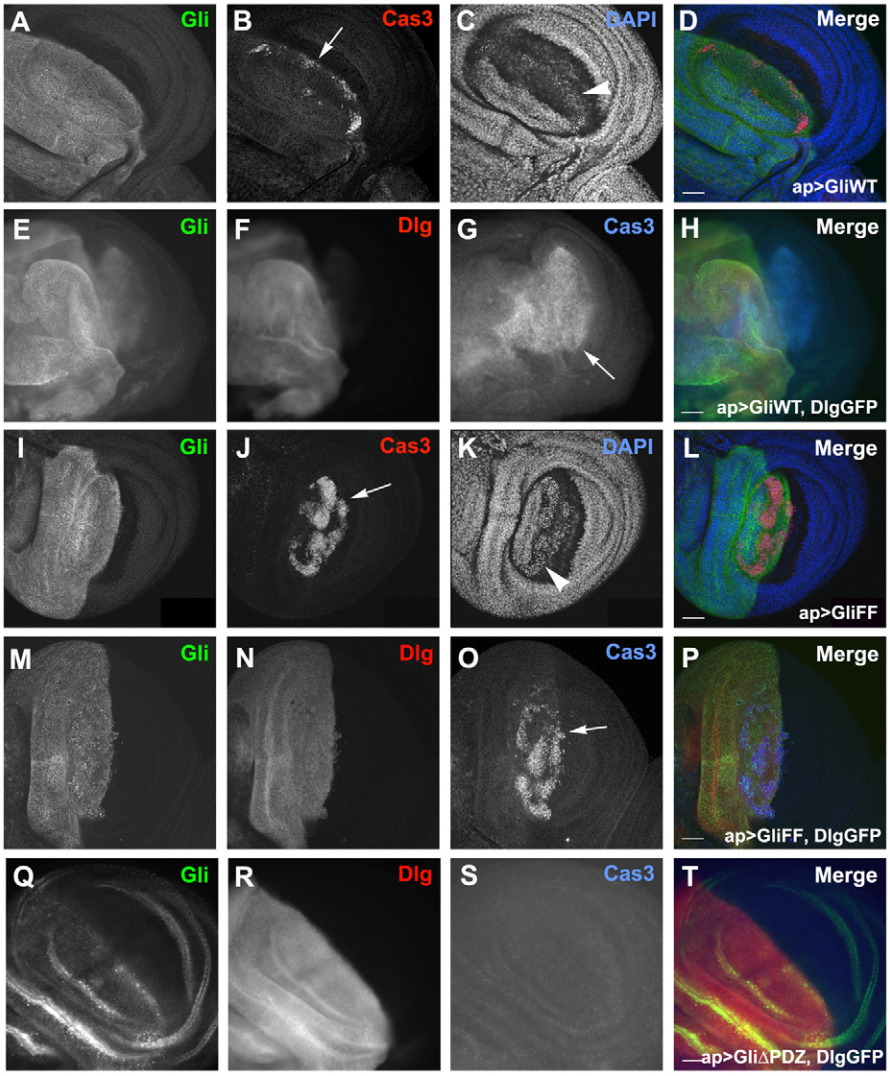

Fig. 6. Dlg enhancement of Gli-triggered apoptosis requires the phosphotyrosine and PDZ motifs. Third-instar wing imaginal disc with apterous-GAL4-driven expression of different Gli constructs. All images were collected with a 20× objective. (**A–D**) Overexpression of GliWT (ap>GliWT). Expression of GliWT (green) results in cell death, detected using immunolabeling with cleaved Caspase-3 (cas3, red, arrow) and pyknotic nuclei with DAPI labeling (blue, arrowhead). (**E–H**) Overexpression of GliWT and Dlg-GFP (ap>GliWT, DlgGFP). Coexpression of Gli (green) and Dlg (red) results in tissue overgrowth as well as increased apoptosis (cas3, blue, arrow) compared with GliWT alone. (**I–L**) Overexpression of GliFF (ap>GliFF). Expression of nonphosphorylated form of Gli (GliFF, green) results in cell death and increased cleaved Caspase-3 (cas3, red, arrow) and pkynotic nuceli (DAPI, blue, arrowhead). (**M–P**) Overexpression of GliFF and Dlg-GFP (ap>GliFF, DlgGFP). Coexpression Dlg (red) with nonphosphorylated form of Gli (GliFF, green) did not increase the amount of apoptosis (Cas3, blue, arrow). (**Q–T**) Overexpression of GliΔPDZ and Dlg-GFP. (ap>GliΔPDZ, DlgGFP). Coexpression of Dlg with GliΔPDZ did not result in apoptosis (cas3, blue) or ectopic folds. Scale bars: 40 μm.

compared to GliWT alone (Fig. 6A–D). Specifically, we observed increased ectopic folds and out-pockets of tissue growth, and increased apoptosis was detected using activated Caspase-3 or the presence of delaminated pyknotic nuclei. This interaction is specific to Dlg as the overexpression of other polarity proteins, Scribble (Scrib) or Lethal giant larvae (Lgl) did not enhance the GliWT phenotypes (supplementary material Fig. S2). To test the requirement of phosphotyrosine signaling and the Gli PDZ binding motif, Dlg-GFP was coexpressed with either GliFF or GliΔPDZ in imaginal discs. Coexpression of Dlg-GFP had no significant effect on the GliFF expressing cells (Fig. 6M–P) compared to GliFF alone (Fig. 6I–L). Similarly, coexpression of Dlg had no effect on GliΔPDZ expressing cells (Fig. 6Q–T) such that no apoptosis or tissue overgrowth was detected. These observations support our hypothesis that phosphotyrosine signaling paired with a Gliotactin/Dlg interaction plays a role in regulating more than just the endocytosis of Gliotactin and Dlg. These results suggest that it is the association of ectopic GliWT and Dlg that triggers the deleterious consequences observed in when Gliotactin is overexpressed.

Phosphorylation of Dlg at serine797 is necessary for mediating overgrowth and apoptosis

Dlg can be regulated by phosphorylation and the serine/threonine kinases PAR-1 and CaMKII regulate Dlg functions important for synaptic plasticity and growth (Koh et al., 1999; Zhang et al., 2007b). We next asked if the overgrowth and cell death phenotype triggered by coexpression of ectopic GliWT and Dlg required the phosphorylation of Dlg. Two serine phosphorylation sites in Dlg (Ser48 and Ser797) that are phosphorylated by *Drosophila* CaMKII and PAR-1 respectively were tested (Zhang et al., 2007b; Koh et al., 1999). Coexpression of GliWT with DlgS797A, a Dlg mutant with Ser797 replaced by an Alanine to block phosphorylation, completely abolished the overgrowth, delamination and apoptosis induced by coexpression of Dlg and GliWT (compare Fig. 7I–L and Fig. 7E–H). However, Dlg was still colocalized with Gliotactin in intracellular vesicles suggesting that phosphorylation of Dlg at S797 is necessary for the induction of overgrowth and apoptosis but not for the association of Gliotactin and Dlg (Fig. 7Q–S). Conversely, coexpression of GliWT with DlgS797D, an isoform that mimics phosphorylation, induced a similar tissue overgrowth

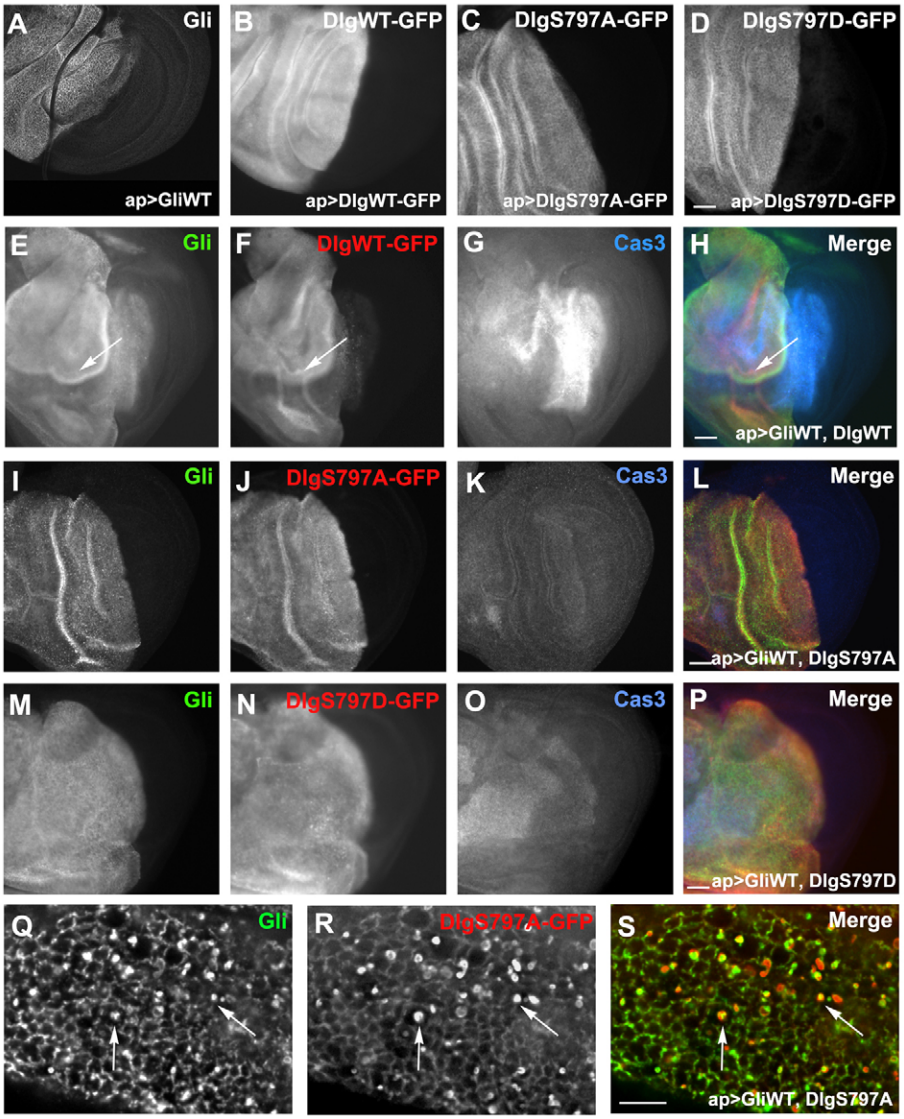

Fig. 7. Phosphorylation of Dlg is crucial to the Gli–Dlg interaction. Third-instar wing imaginal discs with apterous-GAL4 (ap-GAL4) driving the expression of Gli and/or Dlg constructs. (**A–D**) Overexpression of GliWT, Dlg-GFP, DlgS797A-GFP or Dlg-S797D-GFP has little or no effect on the morphology of the wing imaginal disc. (**E–H**) Coexpression of Gli (green) and Dlg-GFP (red) (ap>GliWT, DlgWT) results in overgrowth phenotype (arrows) plus apoptosis detected with immunolabeling for activated Caspase-3 (Cas3, blue). (**I–L**) Coexpression of Gli (green) and a nonphosphorylated form of Dlg, DlgS797A-GFP (red) (ap>GliWT, DlgS797A) suppresses the overgrowth (loss of ectopic folds) and apoptosis (loss of activated Caspase-3) (Cas3, blue) seen when Gli is coexpressed with wild-type Dlg. (**M–P**) Coexpression of Gli (green) and a phosphomimic form of Dlg, DlgS797DGFP (red), (ap>GliWT, DlgS797D) results in an overgrowth phenotype with activation of apoptosis (Cas3, blue) similar to that observed with GliWT and Dlg-GFP. (**Q–S**) When coexpressed, GliWT (green) and DlgS797A-GFP (red) colocalize to intracellular vesicles (arrows), similarly to GliWT and Dlg-GFP. Scale bars: 40 μm (A–P); 15 μm (Q–S).

phenotype and apoptosis seen with coexpression of wild-type Dlg and Gliotactin (Fig. 7M–P). We also tested another known Dlg serine phosphorylation site (DlgS48), normally phosphorylated by *Drosophila* CaMKII, and found that changes to this site still gave the same overgrowth and apoptosis phenotypes as the wild type Dlg-GFP when coexpressed with GliWT (data not known). These results point to a role for serine phosphorylation of Dlg at S797 but not S48 in mediating the overgrowth and apoptosis phenotypes observed when Gliotactin and Dlg are coexpressed.

PAR-1 and JNK are not necessary for phosphorylation of Dlg

To see whether Dlg mediated induction of tissue overgrowth and apoptosis requires phosphorylation by PAR-1, we expressed a kinase dead form of PAR-1 in cells coexpressing wild-type Gli and Dlg. PAR-1 KD can generate dominant-negative effects and block endogenous signaling when overexpressed (Sun et al., 2001; Biernat et al., 2002). We found that expression of PAR-1KD had no effect on the tissue overgrowth, delamination and apoptosis associated with Dlg, Gli coexpression (Fig. 8A–D). These results suggested that either endogenous PAR-1 signaling was not affected or that Dlg phosphorylation by a protein kinase other than PAR-1 is important for defects caused by interaction of Gli and Dlg. The serine/threonine kinase JNK has been shown to phosphorylate Dlg in mammalian cells in response to osmotic shock (Massimi et al., 2006) and Dlg was shown to interact with JNK in a *Drosophila* proteomic study (Bakal et al., 2008).

To test if JNK was responsible for the phosphorylation of Dlg, we expressed a dominant negative form of *Drosophila* JNK, BskDN, in cells coexpressing wild-type Gli and Dlg. Remarkably, blocking JNK completely suppressed the enhanced apoptosis and overgrowth caused by Gli/Dlg coexpression (Fig. 8E–H). However, this result did not determine if JNK was acting upstream or downstream of Dlg. In order to see if JNK was necessary to phosphorylate Dlg, we expressed BskDN in cells coexpressing GliWT and a Dlg construct that mimics phosphorylation, DlgS797D. We found that expression of BskDN completely suppressed the tissue overgrowth as well as the apoptotic cell death phenotype caused by GliWT plus DlgS797D coexpression (Fig. 8I–K). Altogether our results suggest that it is a kinase other than PAR-1 that is necessary for Dlg phosphorylation and although JNK is involved, JNK is likely to function downstream of Dlg.

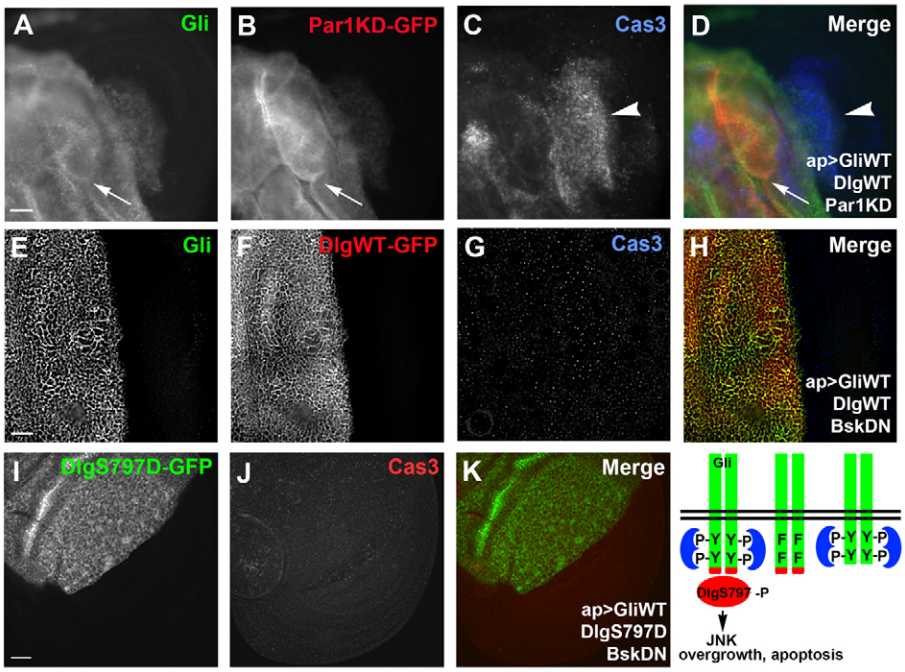

Fig. 8. JNK but not Par1 plays a role in the Gli–Dlg interaction. Third-instar wing imaginal discs with apterous-GAL4 (ap-GAL4) driving the expression of Gli, Dlg plus Par1-KD or DN JNK. (**A–D**) Wing imaginal discs coexpressing a dominant negative form of Par-1 (ParKD, red) with GliWT (green) and Dlg-GFP. Blocking Par-1 has no effect on the Gli–Dlg coexpression defects and fails to block the generation of ectopic folds (arrows) and apoptosis (arrowhead) (Cas3, blue). (**E–H**) Wing imaginal discs coexpressing a dominant negative form of *Drosophila* JNK, (BskDN) with GliWT (green) and Dlg-GFP (red). Blocking JNK completely suppresses the Gli-Dlg coexpression defects. (**I–K**) Wing imaginal discs coexpressing GliWT, a phosphomimetic form of Dlg (DlgS797D, green) and a dominant negative form of Bsk. *Drosophila* JNK blocked the formation of ectopic folds as well as apoptosis (Cas3, red). Diagram shows the Gli dimers (green) with the two tyrosine residues (Y) or the phenylalanine mutants (F) and the PDZ binding motif (red). In our model only the wild-type Gli dimer is able to recruit both phosphotyrosine binding proteins (blue) and Dlg (red). It is the convergence of this protein complex that leads to the activation of JNK and the resulting overgrowth and apoptosis. Scale bars: 20 μm (A–H); 40 μm (I–K).

Discussion

Correct localization of proteins to distinct subcellular locations is crucial for proper function of cells as protein mislocalization can have severe consequences. We have previously shown that tight control of Gliotactin levels and localization is important for survival of the polarized epithelial cells in the wing imaginal disc (Padash-Barmchi et al., 2010). Loss of tyrosine phosphorylation or blocking endocytosis causes Gliotactin to spread away from the TCJ into the SJ domain and beyond. In this paper we demonstrated that the presence of high levels of ectopic Gliotactin results in an interaction with the septate junction associated protein Dlg, which in turn leads to tissue overgrowth and apoptosis. Here we discuss the nature of this interaction and how it may lead to tissue overgrowth.

Ectopic Gliotactin leads to a reduction in Dlg

Dlg is a MAGUK scaffolding protein with three PDZ domains that is found at both the bicellular septate junction and the tricellular junction. Dlg is present in the same protein complex as Gliotactin at the tricellular junction (Schulte et al., 2006) and we found that Gliotactin appears to be recruited to the TCJ by Dlg. However when tested *in vitro*, Gliotactin and Dlg do not directly bind suggesting the presence of an intermediary protein or proteins. At the TCJ, the Gliotactin/X/Dlg interaction may function to stabilize Gliotactin at the TCJ perhaps by inhibition of endocytosis or retention of the protein at the TCJ. For instance, it is possible that tyrosine phosphorylation of Gliotactin only occurs outside the TCJ and that the Gli/X/Dlg interaction at the TCJ blocks access to tyrosine kinases. The relative instability of GliΔPDZ supports a role for a PDZ domain protein in this process as loss of the PDZ motif dramatically increased endocytosis and protein degradation.

The reduction in Dlg, but no other SJ proteins in the presence of ectopic Gliotactin, suggests that this interaction is specific. Ectopically expressed Gliotactin colocalized to endocytic vesicles with Dlg demonstrating their interaction is not restricted to the TCJ. Moreover, ectopic Gliotactin resulted in the reduction of Dlg from SJ domain in a manner that was dependent on Gliotactin tyrosine phosphorylation and the PDZ binding motif. These results suggest that Dlg directly or indirectly binds Gliotactin through a PDZ mediated interaction and the two proteins are then subsequently endocytosed.

Given the colocalization of Gli and Dlg in vesicles, the most likely mechanism for ectopic Gli induced Dlg reduction is co-endocytosis of Gli and Dlg away from the plasma membrane. It is possible that ectopic Gli could also trigger proteasomal degradation of Dlg as has been shown for human hDlg (Mantovali et al., 2001). In vertebrates, the proteosome SCF β-TrCP recognizes a destruction motif (DSG/DDG/EEG/SSGXXS/E/D motifs) where β-TrCP binds the non-phosphorylated Asp/Glu or phosphorylated Ser residues (Frescas and Pagano, 2008). This destruction motif is highly conserved in all Dlg proteins and corresponds to DDGXXS in the insect homologues (V.J.A. unpublished data). Another possibility is that ectopic Gli could induce the transcriptional downregulation of Dlg. A final possibility comes from recent researcher that found mouse β-catenin increases the proteosome-mediated turnover of Dlg through a direct interaction between Dlg and the PDZ binding motif of β-catenin (Subbaiah et al., 2012). The overexpression of Gliotactin can result in the ectopic spread into the adherens junction domain (Padash-Barmchi et al., 2010), suggesting that if Gliotactin recruits Dlg into this domain this would allow for the interaction of Dlg and β-catenin and lead to the reduction of Dlg.

Regardless of the mechanism, the presence of ectopic Gliotactin at the septate junction results in the reduction of Dlg. Mutations in Dlg have been implicated in tissue overgrowth (Woods et al., 1996; Bilder et al., 2000) and in apoptotic cell death (Papagiannouli and Mechler, 2009) so it is possible that the overgrowth and cell death caused by excess Gliotactin is due to the reduction of Dlg at the septate junction. However this is unlikely since ectopic Gliotactin mediated Dlg reduction is only moderate compared to reduction caused by levels of Dlg RNAi

knockdown, which still did not disrupt epithelial polarity. Since blocking Dlg reduction in the Gliotactin overexpressing cells resulted in a stronger phenotype than when Gli was expressed alone, it is more likely that reduction of Dlg is a reflection of the endocytic mechanism to reduce the effects of a Gli–Dlg interaction. In support of this conclusion, circumventing the loss of Dlg by coexpression of Dlg and Gli resulted in overgrowth paired with enhanced apoptosis.

Activation of the JNK pathway and phosphorylated JNK was found associated with endocytic vesicles in scrib−/− mutant clones in concert with Eiger endocytosis (Igaki et al., 2009). Furthermore, JNK activation in surrounding wild-type cells promotes elimination of scrib or dlg mutant cells through PVR activation of a phagocytic pathway (Ohsawa et al., 2011). We were unable to detect elevated phospho-JNK in the Gliotactin-positive vesicles nor were increased concentrations of phospho-JNK observed along the dorsal/ventral border between wild-type and apterous-GAL4 expressing cells (data not shown). For these reasons, and from our epistasis analysis, it suggests that JNK functions downstream of Gliotactin and Dlg.

Tyrosine phosphorylation of Gli may have a signaling function beyond endocytosis

The ectopic spread of Gli into the SJ domain results in increased proliferation and apoptosis mediated by JNK (Padash-Barmchi et al., 2010). Previously we observed that expression of GliFF resulted in increased apoptosis paired with a significant increase in proliferation in the presence of endogenous Gli. However, the homologues of Gliotactin in vertebrates, Neuroligins, have been shown to dimerize or oligomerize via their extracellular serine-esterase-like domain (Ichtchenko et al., 1995; Ichtchenko et al., 1996) and genetic data also suggests *Drosophila* Gliotactin dimerizes (Venema et al., 2004). Thus apoptosis in wild-type wing imaginal discs expressing GliFF is likely due to its coupling with endogenous Gliotactin that can still be phosphorylated. Consistent with this model, no apoptosis or overproliferation was observed when GliFF was expressed in *Gli* null wing discs.

We favour a model in which the phosphorylation of Gliotactin only occurs outside of the TCJ for a number of reasons. Firstly we have been unable to observe a colocalization of phosphotyrosine immunolabeling with Gliotactin at the TCJ. Secondly the expression of GliFF alone in a Gli null mutant was able to rescue to adult stages, albeit with significantly malformed wings.

If phosphotyrosine is signaling more than endocytosis of Gliotactin then one possibility is that tyrosine phosphorylation generates a signal that recruits an unknown intermediary protein (Fig. 8L). Gliotactin has a highly conserved SH2 domain binding sequence at the second tyrosine residue pointing to the involvement of an SH2 domain adapter protein in recruiting Dlg to the complex. An alternate possibility is that tyrosine phosphorylation contributes/induces conformational changes in Gliotactin necessary for the formation of an ectopic Gli/Dlg complex.

The interaction between Gli and Dlg is dependent on the phosphorylation of Dlg at Serine 797, as blocking this phosphorylation completely suppressed the overgrowth and excessive cell death associated with coexpression of Gliotactin and Dlg. However, phosphorylation of Dlg is not necessary for Dlg/Gli co-endocytosis suggesting the phenotype is due to another, separate effect and phosphorylation of Dlg is only necessary for mediating the ectopic Gli–Dlg interaction leading to cell death. PAR-1 phosphorylates Dlg at Serine 797 and negatively regulates its targeting to the postsynaptic region of the neuromuscular junction in *Drosophila* (Zhang et al., 2007b). We found that a kinase dead form of PAR-1 did not have an effect on the Gli–Dlg coexpression phenotypes. JNK phosphorylates hDlg in mammalian cells (Massimi et al., 2006). However, our epistatic analysis placed JNK downstream of Dlg and the JNK sites identified in hDlg do not correspond to those known in Drosophila Dlg. Expression of dominant negative Bsk completely suppressed the Gli–DlgS797D coexpression phenotype suggesting that phosphorylation of Dlg at Serine 797 is likely independent of JNK activity. Recently *Drosophila* adducin, hts, was shown to regulate levels of PAR-1 and CAMKII at the NMJ and, as a result, the degree of Dlg phosphorylation (Wang et al., 2011). Whether adducin/hts plays a role in regulating other kinases and the phosphorylation of Dlg at serine 797 in the wing imaginal disc remains to be determined.

In conclusion the tight regulation of Gliotactin and the deleterious effects that spread of Gliotactin away from the TCJ indicates that a cellular mechanism exists to ensure its restriction. This leads to the question of why ectopic Gliotactin is deleterious. Our results suggest the reason is to limit the association of Gliotactin and Dlg within the bicellular septate junction domain. Circumventing the regulation of the Gli/Dlg association results in overgrowth and apoptosis through a mechanism that involves phosphorylation of Ser797 on Dlg and the activity of JNK. What the exact nature of this interaction is remains to be elucidated but it is clear regulation of Gliotactin and Dlg are essential for the survival of the cell.

Materials and Methods

Fly stocks

The following fly strains were used: Gliotactin null alleles, *GliΔE2Δ45* and *GliCR2* (Venema et al., 2004), UAS lines including UAS-GliWT, UAS-GliFF, UAS-GliΔPDZ(HA), UAS-GliHA (Schulte et al., 2006), UAS-mCD8GFP, UAS-Rab5DN:YFP (Zhang et al., 2007a) from the Bloomington stock center, UAS-DlgRNAi (VDRC 41134) (Grzeschik et al., 2010; Brumby et al., 2011) from the VDRC, UAS-DlgS797D-GFP and UAS-DlgS797A-GFP (Zhang et al., 2007b) from Dr Bingwei Lu, UAS-GFP-DlgS48D and UAS-GFP-DlgS48A (Koh et al., 1999) from Dr V. Budnik, UAS-Par-1KD from Dr Anne Ephrussi, UAS-Lgl-GFP (Tian and Deng, 2008) from Dr Wu-Min Deng, UAS-DlgGFP (Koh et al., 1999) from Dr Dave Bilder and UAS-BskDN (Weber et al., 2000) from the DGRC Kyoto stock center. The daughterless-GAL4, patched-Gal4 and apterous-GAL4 lines from the Bloomington stock center were used as GAL4 drivers.

Biochemistry

Membrane preparations and immunoprecipitations were carried out essentially as described previously (Schulte et al., 2006). Membrane preparations were isolated and immunoprecipitated from: wild-type (OregonR) embryos (150 µg), *daughterless-GAL4, UAS-GliHA* embryos (40 µg) or *daughterless-GAL4, UAS-GliΔPDZ* embryos (40 µg). Immunoprecipitations were carried out using Protein G agarose beads (Invitrogen) prebound with mouse anti-HA (BabCO), or mouse anti-actin as control (Jackson ImmunoResearch). Samples were incubated in 200–250 µl lysis buffer (10 mM Tris-HCl, 4 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.5% Triton X-100 or 0.2% NP-40, 1 mM PMSF, 2 ug/ml leupeptin, 2 ug/ml pepstatin). Precipitated complexes were washed four to five times in 200 µl of lysis buffer, run on a 6% SDS-PAGE gel and subjected to western blot analysis using the following primary antibodies: mouse anti-HA (1:1000) (BabCO), or mouse anti-pTyr (1:3000) (4G10). Secondary antibodies used included HRP-conjugated goat anti-mouse (1:10,000).

For western analysis of Dlg downregulation, wing imaginal discs from *apterous-GAL4*; *apterous-GAL4, UAS-GliWT*; or *apterous-GAL4, UAS-GliWT, UAS-Dlg-GFP* third-instar larvae were isolated and homogenized (10 mM HEPES, pH 7.4, 137 mM NaCl, 1 mM EDTA, 0.1% NP-40 plus protease inhibitor cocktail from Roche, Indianapolis, IN). Homogenates were run on a 10% SDS-PAGE gel, transferred to nitrocellulose membranes, blocked in 5% milk buffer and probed with mouse anti-Dlg4F3 antibody at 1:500. Bands were detected using an HRP-conjugated anti-mouse secondary antibody at 1:4000 (Jackson Laboratories) and ECL detection method. The blot was stripped with stripping buffer (Thermo) and probed with an anti-tubulin antibody (Sigma) for internal control.

Immunolabeling

Wing imaginal discs of third-instar larvae were stained as described previously (Schulte et al., 2006). The following primary antibodies and their dilutions were used:

mouse anti-Gli IF6.3 at 1:200 (Auld et al., 1995), rabbit anti-Gli at 1:300 (Venema et al., 2004), mouse anti-Dlg 4F3 at 1:200 (DSHB) (Parnas et al., 2001), mouse anti-phosphoTyr (4G10) at 1:200 (a gift from M. Gold), rat anti-DE-Cadherin at 1:50 (DSHB), rabbit anti-activated Caspase-3 (Cell Signaling), mouse anti Coracle (9C and C615-16B cocktail) at 1:100 (DSHB) (Fehon et al., 1994) and DAPI. The monoclonal antibodies listed from the Developmental Studies Hybridoma Bank (DSHB) 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 Fluor 488 at 1:300, goat anti-mouse Alexa Fluor 568 at 1:300, goat anti-rat Alexa Fluor 647 at 1:300, goat anti-rabbit Alexa Fluor 568 at 1:300, goat anti-mouse Alexa Fluor 488 at 1:300 (Molecular Probes).

Imaging

High resolution images were collected with an Olympus IX70 confocal with a 60× oil-immersion lens (1.4 NA) or using a DeltaVision Spectris microscope (Applied Precision, Issaquah, WA) with a 60× (1.4 NA) oil immersion lens using a CoolSnap HQ digital camera. Data from all wavelengths was collected for each 0.2 micron optical section before the next section was collected. For Deltavision images, SoftWorx (Applied Precision) software was used for deconvolution of 10–15 iterations using a point-spread function calculated with 0.2 micron beads conjugated with Alexa Fluor 568 (Molecular Probes) mounted in Vectashield. Images were then exported to Photoshop 7 for generation of figures. False colouring using the fire LUT (look up table) was done on individual channels saved in grayscale and imported into ImageJ. Lower magnification images were collected with a 20× objective on a Zeiss Axioskop using Northern Eclipse software.

Acknowledgements

We would like to thank: Dave Bilder, Vivian Budnik, Wu-Min Deng, Anne Ephrussi and Bingwei Lu for fly stocks; Barb Jusiak and Kendra Sturgeon for technical help; Bing Zhang for support; the Bloomington, VDRC, and DGRC Stock Centers for fly stocks.

Author contributions

M.P.-B. contributed to all aspects of the manuscript, including writing and editing of the manuscript, study design, data collection, analysis and interpretation. K.C. contributed data collection, analysis and contribution plus editing of the manuscript. J.Q. contributed data collection, interpretation and analysis. V.J.A. contributed study conception and design, data interpretation, writing, and editing of the manuscript.

Funding

This study was supported by the Canadian Institutes of Health Research [grant number MOP-82862 to V.J.A.].

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.113803/-/DC1

References

Auld, V. J., Fetter, R. D., Broadie, K. and Goodman, C. S. (1995). Gliotactin, a novel transmembrane protein on peripheral glia, is required to form the blood-nerve barrier in Drosophila. *Cell* **81**, 757-767.

Bakal, C., Linding, R., Llense, F., Heffern, E., Martin-Blanco, E., Pawson, T. and Perrimon, N. (2008). Phosphorylation networks regulating JNK activity in diverse genetic backgrounds. *Science* **322**, 453-456.

Baumgartner, S., Littleton, J. T., Broadie, K., Bhat, M. A., Harbecke, R., Lengyel, J. A., Chiquet-Ehrismann, R., Prokop, A. and Bellen, H. J. (1996). A Drosophila neurexin is required for septate junction and blood-nerve barrier formation and function. *Cell* **87**, 1059-1068.

Biernat, J., Wu, Y. Z., Timm, T., Zheng-Fischhöfer, Q., Mandelkow, E., Meijer, L. and Mandelkow, E. M. (2002). Protein kinase MARK/PAR-1 is required for neurite outgrowth and establishment of neuronal polarity. *Mol. Biol. Cell* **13**, 4013-4028.

Bilder, D., Li, M. and Perrimon, N. (2000). Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. *Science* **289**, 113-116.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brumby, A. M., Goulding, K. R., Schlosser, T., Loi, S., Galea, R., Khoo, P., Bolden, J. E., Aigaki, T., Humbert, P. O. and Richardson, H. E. (2011). Identification of novel Ras-cooperating oncogenes in Drosophila melanogaster: a RhoGEF/Rho-family/JNK pathway is a central driver of tumorigenesis. *Genetics* **188**, 105-125.

Budnik, V., Koh, Y. H., Guan, B., Hartmann, B., Hough, C., Woods, D. and Gorczyca, M. (1996). Regulation of synapse structure and function by the Drosophila tumor suppressor gene dlg. *Neuron* **17**, 627-640.

Fehon, R. G., Dawson, I. A. and Artavanis-Tsakonas, S. (1994). A Drosophila homologue of membrane-skeleton protein 4.1 is associated with septate junctions and is encoded by the coracle gene. *Development* **120**, 545-557.

Frescas, D. and Pagano, M. (2008). Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. *Nat. Rev. Cancer* **8**, 438-449.

Fristrom, D. K. (1982). Septate junctions in imaginal disks of Drosophila: a model for the redistribution of septa during cell rearrangement. *J. Cell Biol.* **94**, 77-87.

Genova, J. L. and Fehon, R. G. (2003). Neuroglian, Gliotactin, and the Na+/K+ ATPase are essential for septate junction function in Drosophila. *J. Cell Biol.* **161**, 979-989.

Grzeschik, N. A., Parsons, L. M., Allott, M. L., Harvey, K. F. and Richardson, H. E. (2010). Lgl, aPKC, and Crumbs regulate the Salvador/Warts/Hippo pathway through two distinct mechanisms. *Curr. Biol.* **20**, 573-581.

Ichtchenko, K., Hata, Y., Nguyen, T., Ullrich, B., Missler, M., Moomaw, C. and Sudhof, T. C. (1995). Neuroligin 1: a splice site-specific ligand for beta-neurexins. *Cell* **81**, 435-443.

Ichtchenko, K., Nguyen, T. and Sudhof, T. C. (1996). Structures, alternative splicing, and neurexin binding of multiple neuroligins. *J. Biol. Chem.* **271**, 2676-2682.

Igaki, T., Pastor-Pareja, J. C., Aonuma, H., Miura, M. and Xu, T. (2009). Intrinsic tumor suppression and epithelial maintenance by endocytic activation of Eiger/TNF signaling in Drosophila. *Dev. Cell.* **16**, 458-465.

Koh, Y. H., Popova, E., Thomas, U., Griffith, L. C. and Budnik, V. (1999). Regulation of DLG localization at synapses by CaMKII-dependent phosphorylation. *Cell* **98**, 353-363.

Mantovani, F., Massimi, P. and Banks, L. (2001). Proteasome-mediated regulation of the hDlg tumour suppressor protein. *J Cell Sci.* **114**, 4285-4292.

Massimi, P., Narayan, N., Cuenda, A. and Banks, L. (2006). Phosphorylation of the discs large tumour suppressor protein controls its membrane localisation and enhances its susceptibility to HPV E6-induced degradation. *Oncogene* **25**, 4276-4285.

Noirot-Timothée, C., Graf, F. and Noirot, C. (1982). The specialization of septate junctions in regions of tricellular junctions. II. Pleated septate junctions. *J. Ultrastruct. Res.* **78**, 152-165.

Ohsawa, S., Sugimura, K., Takino, K., Xu, T., Miyawaki, A. and Igaki, T. (2011). Elimination of oncogenic neighbors by JNK-mediated engulfment in Drosophila. *Dev. Cell* **20**, 315-328.

Padash-Barmchi, M., Browne, K., Sturgeon, K., Jusiak, B. and Auld, V. J. (2010). Control of Gliotactin localization and levels by tyrosine phosphorylation and endocytosis is necessary for survival of polarized epithelia. *J. Cell Sci.* **123**, 4052-4062.

Papagiannouli, F. and Mechler, B. M. (2009). discs large regulates somatic cyst cell survival and expansion in Drosophila testis. *Cell Res.* **19**, 1139-1149.

Parnas, D., Haghighi, A. P., Fetter, R. D., Kim, S. W. and Goodman, C. S. (2001). Regulation of postsynaptic structure and protein localization by the Rho-type guanine nucleotide exchange factor dPix. *Neuron* **32**, 415-424.

Paul, S. M., Ternet, M., Salvaterra, P. M. and Beitel, G. J. (2003). The Na+/K+ ATPase is required for septate junction function and epithelial tube-size control in the Drosophila tracheal system. *Development* **130**, 4963-4974.

Schulte, J., Tepass, U. and Auld, V. J. (2003). Gliotactin, a novel marker of tricellular junctions, is necessary for septate junction development in Drosophila. *J. Cell Biol.* **161**, 991-1000.

Schulte, J., Charish, K., Que, J., Ravn, S., MacKinnon, C. and Auld, V. J. (2006). Gliotactin and Discs large form a protein complex at the tricellular junction of polarized epithelial cells in Drosophila. *J. Cell Sci.* **119**, 4391-4401.

Subbaiah, V. K., Narayan, N., Massimi, P. and Banks, L. (2012). Regulation of the DLG tumor suppressor by β-catenin. *Int. J. Cancer* **131**, 2223-2233.

Sun, T. Q., Lu, B., Feng, J. J., Reinhard, C., Jan, Y. N., Fantl, W. J. and Williams, L. T. (2001). PAR-1 is a Dishevelled-associated kinase and a positive regulator of Wnt signalling. *Nat. Cell Biol.* **3**, 628-636.

Tian, A. G. and Deng, W. M. (2008). Lgl and its phosphorylation by aPKC regulate oocyte polarity formation in Drosophila. *Development* **135**, 463-471.

Tsukita, S., Furuse, M. and Itoh, M. (2001). Multifunctional strands in tight junctions. *Nat. Rev. Mol. Cell Biol.* **2**, 285-293.

Venema, D. R., Zeev-Ben-Mordehai, T. and Auld, V. J. (2004). Transient apical polarization of Gliotactin and Coracle is required for parallel alignment of wing hairs in Drosophila. *Dev. Biol.* **275**, 301-314.

Wang, S., Yang, J., Tsai, A., Kuca, T., Sanny, J., Lee, J., Dong, K., Harden, N. and Krieger, C. (2011). Drosophila adducin regulates Dlg phosphorylation and targeting of Dlg to the synapse and epithelial membrane. *Dev. Biol.* **357**, 392-403.

Weber, U., Paricio, N. and Mlodzik, M. (2000). Jun mediates Frizzled-induced R3/R4 cell fate distinction and planar polarity determination in the Drosophila eye. *Development* **127**, 3619-3629.

Woods, D. F., Hough, C., Peel, D., Callaini, G. and Bryant, P. J. (1996). Dlg protein is required for junction structure, cell polarity, and proliferation control in Drosophila epithelia. *J. Cell Biol.* **134**, 1469-1482.

Yasuhara, J. C., Baumann, O. and Takeyasu, K. (2000). Localization of Na/K-ATPase in developing and adult Drosophila melanogaster photoreceptors. *Cell Tissue Res.* **300**, 239-249.

Zhang, J., Schulze, K. L., Hiesinger, P. R., Suyama, K., Wang, S., Fish, M., Acar, M., Hoskins, R. A., Bellen, H. J. and Scott, M. P. (2007a). Thirty-one flavors of Drosophila rab proteins. *Genetics* **176**, 1307-1322.

Zhang, Y., Guo, H., Kwan, H., Wang, J. W., Kosek, J. and Lu, B. (2007b). PAR-1 kinase phosphorylates Dlg and regulates its postsynaptic targeting at the Drosophila neuromuscular junction. *Neuron* **53**, 201-215.