Drosophila melanogaster Guk-holder interacts with the Scribbled PDZ1 domain and regulates epithelial development with Scribbled and Discs Large

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Running title: Crystal structure of Scrib PDZ1-Gukh complex

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ABSTRACT

Epithelial cell polarity is controlled by components of the Scribble polarity module, and its regulation is critical for tissue architecture and cell proliferation and cell migration. In Drosophila melanogaster, the adaptor protein Guk-holder (Gukh) binds to the Scribbled (Scrib) and Discs Large (Dlg) components of the Scrib polarity module and plays an important role in the formation of neuromuscular junctions. However, Gukh’s role in epithelial tissue formation and the molecular basis for the Scrib-Gukh interaction remain to be defined. We now show using isothermal titration calorimetry that the Scrib PDZ1 domain is the major site for an interaction with Gukh. Furthermore, we defined the structural basis of this interaction by determining the crystal structure of the Scrib PDZ1-Gukh complex. The C-terminal PDZ-binding motif of Gukh is located in the canonical ligand binding groove of Scrib PDZ1, and utilizes an unusually extensive network of hydrogen bonds and ionic interactions to enable binding to PDZ1 with high affinity. We next examined the role of Gukh along with those of Scrib and Dlg in Drosophila epithelial tissues, and found that Gukh is expressed in larval-wing and eye-epithelial tissues and co-localizes with Scrib and Dlg at the apical cell cortex. Importantly, we show that Gukh functions with Scrib and Dlg in the development of Drosophila epithelial
tissues, with depletion of Gukh enhancing the eye- and wing-tissue defects caused by Scrib or Dlg depletion. Overall our findings reveal that Scrib’s PDZ1 domain functions in the interaction with Gukh and that the Scrib-Gukh interaction has a key role in epithelial tissue development in *Drosophila*.

**INTRODUCTION**

Cell polarity is a key property of tissue development, and manifests itself as the asymmetric organization of cellular components, such as proteins and lipids into distinct cellular domains. Correct establishment of cell polarity is important for tissue architecture, cell proliferation, cell migration and cellular fate, and its dysregulation has been recognized as a cancer hallmark, with ~70% of epithelial cancers displaying defects in polarity regulation (1-4). Cell polarity is regulated by the interplay between three key polarity modules, Scribble, PAR and Crumbs (5). Epithelial cell polarity (apico-basal polarity) is established and maintained by the antagonistic interactions between the Scribble module and the PAR and Crumbs complexes, resulting in the restriction of PAR and Crumbs complex components to the apical cortex and the Scribble module components to the basolateral cortex. In apico-basal cell polarity the three polarity modules function to specify the apical and basal membrane domains and to position the adherens and tight junctions, which are required for cell-cell contact, cell communication, epithelial tissue coherence and tissue growth regulation (6).

The Scribble module comprises three tumor suppressor proteins, Scribbled (Scrib), *Disc large* (Dlg) and *Lethal-2-giant larvae* (Lgl), which are highly conserved in structure and function from the vinegar fly, *Drosophila melanogaster*, to humans (7). Genetic analyses in *Drosophila* have provided valuable insight into their *in vivo* function (8,9), and revealed that Scrib, Dlg and Lgl, in addition to their role in cell polarity, are also involved in cell proliferation, differentiation and migration (8-12). In addition to the core Scribble module components, studies in *Drosophila* neuromuscular junctions revealed that the interaction between Scrib and Dlg is mediated by an adaptor protein, termed GUK-holder (Gukh) (13). However at a molecular level the precise interactions between these three tumor suppressors, and indeed the role of Gukh, are not well defined.

Scrib is a scaffold protein belonging to the LAP (LRR and PDZ) protein family, and contains 16 Leucine-rich repeats (LRRs), two LAP specific domains (LAPSADa and LAPSADb) and four PDZ domains. Whilst the N-terminal LRR domain is critical for Scrib’s cortical localization (14), the four PDZ domains are required for cell-cell junction localization (13) and are the major mediators of Scrib interactions with other proteins (15). hScrib (human Scrib) PDZ domains have been shown to bind a diverse set of cellular interactors including β-PIX, MCC, GIT1 and β-catenin (16-20), enabling Scrib to integrate a range of cellular cues for the establishment of apico-basal polarity, cell migration and cell signalling.

Dlg is a member of the membrane-associated guanylate kinase homolog (MAGUK) scaffolding protein family, and comprises three PDZ domains, a Src homology 3 (SH3) domain, a Hook domain and a guanylate kinase-like (GUK) domain at its C-terminus. Four Dlg homologs have been found in mammalian systems, named: Dlg1 (hDlg/SAP97), Dlg2 (PSD-93/Chapsyn-110), Dlg3 (NE-Dlg/SAP102) and Dlg4 (PSD-95/SAP90) (3). In *Drosophila melanogaster*, the Dlg PDZ2 domain is required for its localization at basolateral (septate) junctions, whereas the SH3 and Hook domains are necessary for precise localization of Dlg to the cell membrane (21,22). The GUK domain (765-960 amino acids) is catalytically inactive and in *Drosophila* regulates Dlg-Scrib interactions via interaction with Gukh (1,13).
Gukh was identified in a yeast two-hybrid screen as a protein that bound to the
Drosophila Dlg GUK domain (13). Two orthologs of Drosophila Gukh have been
identified in humans, Nance-Horan syndrome (NHS) and NHSL1 (23). Nance-
Horan syndrome (NHS) is an uncommon X-linked disorder characterized by congenital
nuclear cataracts, dental irregularities, and craniofacial dysmorphisms, with mental
deficiencies also occurring in approximately 30% of the cases (24). The molecular details
of how NHS mutations cause Nance-Horan syndrome (NHS) are unclear although recent
studies have implicated it in regulation of epithelial junctions (25) as well as actin
remodelling (23,26).

Drosophila Gukh contains a GUK-holding domain at its C-terminus, which
directly binds the Dlg GUK domain (13,27). Additionally, another study showed that the
binding of Gukh to the GUK domain of Dlg occurs in a mutually exclusive manner via
the PDZ domain, only permitting Gukh interaction when the PDZ remains unbound
(28). However, others have reported that interactions between Gukh and Dlg require
the SH3-GUK domain of Dlg (29,30). This interaction is regulated via interdomain
interactions of PDZ3-SH3-GUK via a PDZ3 binding motif in a linker region enabling
dynamic regulation of ligand binding to Dlg PDZ3 (29,30).

Furthermore, in yeast two-hybrid assays the Gukh C-terminal region is able to
engage Scrib PDZ2, but not PDZ3-4 (13). Moreover, co-immunoprecipitation analysis
from Drosophila larval muscles showed that Scrib can form a complex with Dlg and
Gukh, and the interaction with Dlg is reduced in a Gukh mutant (13). These
genetic data suggest that Gukh is important for the formation of a ternary complex
between Scrib, Dlg and Gukh. Consistent with this notion, all 3 proteins co-localize at
Drosophila neuromuscular junctions and Dlg and Gukh are required for the correct
localization of Scrib (13). This interaction is likely to be evolutionarily conserved, as a
Zebrafish Gukh ortholog, Nhs1b also

physically interacts with Scrib and Dlg in
cultured cells, and nhs1b and scrib
genetically interact, in that double
heterozygotes show a strong neural cell
migration defect relative to single
heterozygotes (31).

Whilst Drosophila Gukh interacts with Dlg and Scrib in neuromuscular
junctions (13), its role in epithelial tissue formation and the molecular basis for the
Scrib-Gukh interaction remain to be defined. Here, we identify the Scrib PDZ1 domain as
the major interacting PDZ domain with the Gukh C-terminal peptide. Using X-ray
crystallography we then define the structural basis of Scrib PDZ1 interactions with the
Gukh C-terminal peptide. Furthermore, our studies reveal a novel role for Gukh in
epithelial development. We show that Gukh is expressed in larval wing and eye epithelial
tissue and co-localizes with Scrib and Dlg at the apical cell cortex. Importantly, we show
that Gukh functions with Scrib and Dlg in
Drosophila epithelial tissues, with depletion
of Gukh enhancing the eye and wing tissue
defects caused by Scrib or Dlg depletion. These findings provide the first evidence for a
role for Gukh in the Scribble module in the
control of epithelial cell polarity, and
provide structural and mechanistic insights
into the Scrib-Gukh interaction in
Drosophila.

RESULTS
Molecular and structural basis of
Scrib:Gukh interaction
To understand the molecular basis of the
reported interaction between Scrib and
Gukh, we performed protein-ligand
interaction studies using individual
recombinant PDZ domains from Scrib and
C-terminal peptides derived from Gukh, and
determined binding affinities using
isothermal titration calorimetry (ITC) (Fig.
1). ITC experiments were conducted using
purified Drosophila Scrib PDZ1, PDZ2,
PDZ3 and PDZ4 domains with wild-type
Gukh C-terminal peptides. Raw heats of
titrations obtained for PDZ1 with Gukhwt
peptide revealed a tight interaction with a
calculated $K_D$ of 664 nM, whereas PDZ3 engaged Gukh$_{wt}$ peptide with only modest affinity with a $K_D$ of 27.8 $\mu$M. In contrast, PDZ2 and PDZ4 did not show any detectable binding to Gukh$_{wt}$ peptide, thus rendering the Scrib PDZ1 domain as the primary functional interaction site for the Gukh C-terminus.

We then examined the structural basis of the PDZ1:Gukh interaction by determining the crystal structure of a Scrib PDZ1:Gukh C-terminal peptide complex (Table 2). *Drosophila* Scrib PDZ1 adopts the typical PDZ fold consisting of six $\beta$-strands and two $\alpha$-helices that form a $\beta$-sandwich structure (Fig. 2A), and is highly similar to the previously determined human PDZ1 (hsPDZ1) structure (PDB ID 2W4F). The PDZ1 domain from the PDZ1:Gukh complex superimposes with the apo hsPDZ1 domain with an r.m.s.d. of 0.8995 Å over 92 Ca atoms, indicating that the binding of Gukh peptide to the PDZ1 domain does not substantially change the overall fold (Fig 2B).

In the PDZ1:Gukh structure, PDZ1 features an atypical $\beta_5$ displaying increased flexible in its geometry. PDZ1 interacts with Gukh peptide via its canonical ligand binding groove formed by the $\beta_2$ strand and helix $\alpha_2$ (Fig. 2A,C). Gukh binding to PDZ1 buries a combined total of 937 Å$^2$ of solvent accessible surface area, with the interface having a shape complementarity score of 0.78, indicating a very good fit. In the complex, the Gukh peptide faces the $\beta_2$ strand in an anti-parallel manner, with its N-terminus solvent exposed and its C-terminus stabilized by the PDZ1 $\beta_1$-$\beta_2$ loop. The complex is achieved via an extensive network of hydrogen bonds formed by Leu 741$^{PDZ1}_{}$:Leu 1788$^{Gukh}_{}$, Leu 743$^{PDZ1}_{}$:Leu 1788$^{Gukh}_{}$, Ile 745$^{PDZ1}_{}$:Thr 1786$^{Gukh}_{}$, Thr 753$^{PDZ1}_{}$:Pro 1782$^{Gukh}_{}$, Ser 764$^{PDZ1}_{}$:Glu 1785$^{Gukh}_{}$, His 796$^{PDZ1}_{}$:Thr 1786$^{Gukh}_{}$ as well as an ionic interaction between Arg 765$^{PDZ1}_{}$:Glu 1785$^{Gukh}_{}$. Furthermore, Gukh Leu 1788 is accommodated in a hydrophobic pocket comprising Leu 741, Leu 743, Val 800 and Leu 803. Hydrophobic interactions are also present between Gukh Phe 1784 and dmPDZ1 Gly 747 and Ser 751, with Phe 1784$^{Gukh}_{}$ being further stabilized by $\pi$-stacking with His 796$^{PDZ1}_{}$.

To validate the crystal structure we performed site-directed mutagenesis to target two key interactions, Arg 765$^{PDZ1}_{}$:Glu 1785$^{Gukh}_{}$ and His 796$^{PDZ1}_{}$:Phe 1784$^{Gukh}_{}$, as well as the floor of the canonical PBM binding site via G747W (Table 3). Mutation of R765 to an Ala resulted in a 5-fold reduction in affinity ($K_D = 5.1$ $\mu$M), whereas the H796A displayed no affinity for wt Gukh, suggesting that both residues make important contributions for Scrib PDZ1:Gukh interactions. Furthermore, introduction of the large hydrophobic Trp at G747$^{PDZ1}_{}$ also completely ablates the ability of PDZ1 to bind to the PBM of Gukh (Table 3).

Gukh is expressed in wing and eye epithelial tissue and co-localises with Dlg and Scrib in the apical cortex

Considering the previously established role for the Scrib-Gukh interaction in neuromuscular junction development, we next examined if Scrib and Gukh are involved in epithelial tissue development.

To determine whether Gukh was expressed in *Drosophila* larval epithelial tissues, we stained developing eye and wing epithelia from third instar larvae with an anti-Gukh antibody (13). Staining with the anti-Gukh antibody, and using expression of a *gukh* RNAi line in the wing epithelium or clones of a *gukh* loss-of-function P transposable-element mutant in the eye epithelium as controls, revealed that Gukh was expressed in the developing wing (Fig. 3A, quantified in 3C) and eye (Fig. 3B, quantified in 3D) tissues. We then examined the cellular distribution of Gukh relative to Scrib and Dlg (Fig. 4). In the larval wing epithelium, Gukh was localized cortically and enriched apically, co-localizing with Dlg and Scrib in the apical cortex, however cross-sections revealed that Gukh extended further apically and baso-lateral than Dlg and Scrib (Fig.
A similar co-localization between Gukh and Scrib or Dlg was observed in the larval eye epithelia, although Gukh exhibited a stronger staining of the apical cortex of photoreceptor cells, which are enriched in F-actin (Fig. 4B). Thus, Gukh shows overlapping localization with Scrib and Dlg in two larval epithelial tissues, although Gukh is also distributed more generally around the cell cortex, which is consistent with Gukh possessing a WH1 F-actin binding domain at its N-terminus (13,26).

Gukh genetically interacts with Scrib and Dlg in epithelial tissues

Previous studies revealed that Gukh GUK-holding domain binds to the Dlg GUK domain and that the C-terminal PDZ binding motif of Gukh interacted with Scrib PDZ domains (13). However, whether Gukh has a functional role with Scrib or Dlg in epithelial tissue is currently unknown. Consequently, we examined the genetic interaction between scrib and gukh in Drosophila eye and wing epithelial tissues. To manipulate the expression of several transgenes in a tissue specific manner, the UAS/GAL4 system was used to selectively knock down scrib, dlg or both genes using UAS-RNAi lines in the Drosophila eye or wing epithelial tissues, using the ey-GAL4 or dpp-GAL4 drivers, respectively. Genetic interactions with gukh expression/function knockdown were then examined relative to a control transgene (UAS-lacZ or UAS-GFP, to control for UAS element number).

Scrib knockdown using two RNAi transgenes (one on the 2nd an the other on the 3rd chromosome) via the ey-GAL4 driver in the Drosophila developing eye, resulted in slightly reduced and disorganized eye phenotype (termed a rough eye phenotype, Fig.5A) relative to control adult eyes (Fig.5B). To verify that this phenotype was modifiable, we knocked down Dlg using RNAi which showed as expected a robust enhancement of the eye roughness (Fig.5A) and a reduction in eye size (Fig.5F). Importantly, knockdown of Gukh using RNAi, enhanced the Scrib knockdown rough eye phenotype (Fig.5A), and led to a slight reduction in eye size (although this not statistically significant below P<0.05). We then examined the effect on the Scrib knockdown eye phenotype upon expression of the Gukh-C terminal region (Gukh-C), which lacks the important F-actin binding WH1/EVH1 domain at the N-terminus, and is expected to act in a dominant negative manner (13). When gukh-C was overexpressed with concurrent scrib knockdown, an enhancement of the eye roughness was also observed (Fig.5A). In contrast, individual overexpression of gukh-C or gukhRNAi expression showed normal eye size and arrangement of ommatidia relative to the wild type, however dglRNAi showed a slight decrease in eye size relative to the other genotypes (Fig.5B). Altogether, these results reveal a genetic interaction between scrib and gukh in the eye, although not as strong as that observed between scrib and dgl.

We next examined whether Gukh genetically interacts with Dlg. Since Dlg knockdown via the ey driver did not produce a strong phenotype (Fig.5B), we examined whether Gukh knockdown could modify the small rough eye phenotype observed upon Scrib and Dlg co-knockdown (Fig.5C,G). Strikingly, both gukhRNAi and gukh-C expression resulted in a strong enhancement of the small rough eye phenotype of ey>scribRNAi dglRNAi (Fig.5C,G) that were statistically significant (p=0.001 and 0.0003, respectively. Thus, Gukh genetically interacts with Dlg and Scrib in the eye, suggesting that Scrib, Dlg and Gukh function in the same genetic process in eye epithelial development.

To extend these result, we used the dpp-GAL4 driver to knockdown scrib or dgl in another epithelial tissue, the wing epithelium, and examined the interactions with gukhRNAi and gukh-C. The Dpp driver is expressed along the anterior-posterior boundary in the developing wing, which constitutes the region between the 3rd and 4th wing vein of the adult wing. Expression of
gukh-RNAi or gukh-C via the dpp driver did not affect the wing phenotype (Fig 5D), but dpp>scribRNAi resulted in a severe wing phenotype and less than 6% survival (data not shown) and therefore the interaction with gukh could not be examined. However, dpp>dlgRNAi resulted in a normal wing phenotype, except for the truncation of the 3rd wing vein in 25% of cases (Fig.5E). Importantly, knockdown of gukh or overexpression of gukh-C together with dlgRNAi resulted in a truncation of the 3rd wing vein in 57% and 67% (respectively) of flies examined. This was greater than a two-fold increase in comparison with the 25% seen in the GFP control (Fig.5E,H). Thus, gukh genetically interacts with dlg in the wing epithelium, suggesting that Gukh and Dlg function in a common genetic pathway in wing development.

DISCUSSION

Epithelial tissues are highly polarized, and the Scribble polarity module is a critical regulator of epithelial tissue organization and polarization in Drosophila and in mammals. In addition to the core components of the Scribble module, Scrib, Dlg and Lgl, an important regulatory role has been emerging for the adaptor protein Gukh. Notably, in Drosophila neuromuscular junctions (13) Gukh is a crucial adaptor protein that enables assembly of a functional ternary complex of Scrib, Dlg and Gukh, thereby allowing correct synaptic localization of Scrib. However, neither the molecular basis for Scrib:Gukh interactions nor a role for Gukh in epithelial tissue structure or function have previously been described. We now show that the Scrib PDZ1 domain is the major high affinity interaction site for Gukh, and define the molecular basis for this interaction. Furthermore, we now provide the first description of Gukh expression and function in Drosophila larval epithelial tissues. We show that Gukh is expressed in the larval wing and eye epithelial tissues and that Gukh is generally cortically localized and overlaps with Scrib and Dlg at the apical cell cortex. Importantly, our studies have revealed that Gukh together with Scrib and Dlg is a key mediator of epithelial tissue development in Drosophila, with loss of Gukh in combination with loss of Scrib and Dlg leading to morphological and differentiation defects in eye and wing tissues.

The expression and localization of Gukh in epithelial tissue

Our expression analysis of Gukh protein in the eye and wing epithelium, revealed co-localization with Scrib and Dlg at the apical cortex, however Gukh was also distributed more apically as well as baso-laterally around the cell cortex, and in the differentiated region of the eye epithelium strong staining was observed in the apical region of the photoreceptor cells, where F-actin accumulates. Since Gukh/NHS1, via its WH1 domain regulates the WAVE/SCAR-ARP2/3-branched F-actin pathway (26,31), Gukh's general cortical localization might be commensurate with this role in F-actin biogenesis, which conceivably might also function independently of its function with Dlg and Scrib. However, our genetic data in Drosophila showing that expression of Gukh-C (which can bind to Dlg and Scrib, but lacks the F-actin regulatory WH1 domain), phenocopies knockdown of gukh in its interaction with scrib and dlg in the eye and wing, suggests that the Gukh WH1 domain and regulation of F-actin is essential for Scribble module function in epithelial development.

The function of Gukh in larval epithelial tissues

In the eye, knockdown of Scrib or Scrib with Dlg via the ey driver gave rise to tissue growth and patterning defects, most likely due to the disruption of epithelial cell polarity and the deregulation of the Hippo and JNK signaling pathways (14,32). The enhancement of the Scrib and Scrib Dlg phenotypes by impairment of Gukh function (via RNAi or the dominant negative transgene) suggests that Gukh functions in
the same genetic pathway as Scrib and Dlg in epithelial cell polarity and cell signaling. The more robust enhancement observed by Gukh impairment in the background of Scrib and Dlg knockdown is consistent with the proteins functioning in a tripartite complex in epithelial tissues, as has been shown in the neuromuscular junctions (13). Indeed, Gukh and Dlg are both required for Scrib localization in the neuromuscular junctions, with greater Scrib mislocalization being observed in the double mutant (13). It remains to be determined whether this co-regulation of Scrib localization by Dlg and Gukh also occurs in epithelial tissues, however this function is consistent with the stronger enhancement of the Scrib knockdown phenotype by dual knock down of Dlg and Gukh.

In the wing, knockdown of Gukh together with Dlg resulted in a pronounced truncation of the 3rd wing vein. Since EGFR-Ras signalling is a key pathway involved in wing vein formation (33), and Scrib has been previously shown to repress this pathway (18,34), we firstly considered whether Gukh might also function together with Dlg and Scrib in regulating EGFR-Ras signalling. However, since knockdown of Scrib function would be expected to increase Ras signalling, and enhanced Ras signalling is associated with ectopic wing veins (35), which was not observed in dlg gukh impaired wing tissue, it is unlikely that the Ras pathway is up-regulated by Dlg knockdown in this context. Conversely, knockdown of Dpp (TGFβ/BMP) signalling results in truncated wing veins (36), and therefore the generation of a similar phenotype by dlg and gukh impairment suggests Dlg and Gukh might positively regulate the Dpp pathway in this context. Consistent with this notion, Scrib via its LRR domain, has been shown to bind to the BMP Type I Receptor, Tkv, Type II Receptor, Pnt, and the phosphorylated (active) Mad transcription factor in Drosophila wing posterior cross vein development, which is thought to facilitate BMP Receptor signalling (37,38). Additionally, the Scribble module protein, Lgl, has been implicated in the regulation of Dpp secretion in embryonic ectodermal cells (37,38). Further studies are required to determine whether Dlg, Scrib and Gukh interact to affect Dpp signalling during wing development.

**Molecular interaction of Gukh with Scrib**

To establish a molecular basis for the observed genetic interaction between Gukh and Scrib, we performed a biochemical analysis of individual PDZ domains of Drosophila Scrib with peptides encoding for the C-terminal PDZ domains of the Scrib module protein, Lgl, has been implicated in the regulation of Dpp secretion in embryonic ectodermal cells (37,38). Further studies are required to determine whether Dlg, Scrib and Gukh interact to affect Dpp signalling during wing development.

Molecular interaction of Gukh with Scrib

To establish a molecular basis for the observed genetic interaction between Gukh and Scrib, we performed a biochemical analysis of individual PDZ domains of Drosophila Scrib with peptides encoding for the C-terminal PDZ binding motif in Gukh. Our binding data demonstrated that the major site of interaction between Gukh and Scrib is the Scrib PDZ1 domain, thus for the first time establishing a definitive molecular basis for this interaction. These findings are in contrast to that of Mathew et al. (13), who in yeast two-hybrid assays showed that Scrib PDZ2 but not PDZ3-4 showed strong interaction with the Gukh-C terminal peptide, however they were unable to draw conclusions regarding PDZ1 in their experiments due to high background activity with these constructs (13). Thus, our findings have revealed a previously unexplored role for Scrib PDZ1 in binding to Gukh. However, in contrast to the Mathew report (13), our study did not reveal a role for Scrib PDZ2 in binding to Gukh C-terminal peptide, which might be due to differences in construct design, post-translational modification in the yeast system, or to the inherent propensity for false positives using the yeast two-hybrid system, due to heterologous protein expression and inappropriate cell localization. Importantly, our findings provide a rationale for the observation that the Scrib truncation mutant scrib5 (39), which results in the loss of Scrib PDZ3 and 4 domains, is functionally active and displays normal adherens junction and basolateral/septate junction (SJ) formation, whereas the scrib5 mutant, which lacks active PDZ domains results in disrupted SJ formation. These data, together with our
results, indicate that the presence of an intact and active PDZ1 domain in Scrib should be necessary and sufficient for normal adherens junction and septate junction formation. Moreover, since we have shown that Gukh interacts with Scrib PDZ1, and Gukh genetically interacts with Scrib and Dlg in epithelial tissues, this suggests that Gukh could contribute to the role of Scrib-PDZ1 in epithelial structure as well as potentially in directed epithelial cell migration (39).

Interestingly, our measurements of Scrib PDZ-Gukh interactions identified the PDZ1-Gukh interaction as unusually tight for PDZ domain interactions with an endogenous ligand, with tight nanomolar interactions typically found in PDZ-synthetic ligand complexes such as those of Erbin or ZO-1 interactions with peptides derived from phage display. To better understand the structural basis for this, we determined the crystal structure of Scrib PDZ1-Gukh. Superimposition of the PDZ1 domain from Drosophila bound to Gukh over the human Scrib PDZ1 domain [PDZ 2W4F] reveals no significant structural changes in the ligand binding groove upon Gukh binding (Fig. 2B), which has previously been observed for the GRIP1 PDZ6-peptide complex (40). Examination of the interface of the PDZ1-Gukh complex revealed an extensive net of hydrogen bonds and ionic interactions, which supplement the insertion of the C-terminal Gukh L1788 into a hydrophobic pocket. In particular the ionic interaction between Gukh E1785 and Scrib R765 is reminiscent of Erbin R49-p120-catenin-like peptide E4 (41). Loss of this ionic interaction in a Scrib R765A mutant leads to a ~8-fold loss of Gukh binding to Scrib, suggesting that the Arg765<sub>PDZ1</sub>Glu1785<sub>Gukh</sub> salt bridge is important. Interestingly, despite displaying nanomolar affinity, Gukh does not contain any Trp residues in its PDZ binding motif. In the case of Erbin, a Trp in the -1 position has been shown to be important for binding, with a second Trp in position -4 position being critical for the high affinity interaction by engaging the β2-3 loop (Fig. 2D). A similar key role is played by a Trp in the -6 position of a synthetic peptide in complex with ZO1 (42,43), which has been shown to contribute substantially to the binding of ZO1 by inserting into the β2-3 loop in a similar location as the -4 Trp in the Erbin complex (43). In contrast, Gukh harbors a Phe in the -4 position rather than a Trp, which nonetheless makes significant contacts with the Scrib PDZ1 domain via π-stacking with H796. Thus, the -4 position in Gukh is still able to contribute substantially to binding to PDZ1 by exploiting the opposite side of the ligand binding groove. Indeed, mutation of H796 to an Ala abrogates binding to Gukh, supporting the notion that the His796<sub>PDZ1</sub>:Phe1784<sub>Gukh</sub> π-stacking is important for Scrib:Gukh interaction. Furthermore, Gukh only forms a single hydrogen bond with the β2-3 loop via the main chain carbonyl of P1782, indicating that the engagement of the β2-3 loop is not necessary for a high affinity nanomolar interaction.

In conclusion, our study has revealed novel roles and regulatory mechanisms for Gukh in epithelial development. Our discovery of a novel role for Drosophila Scrib PDZ1 in the interaction with Gukh, and the important function of Gukh together with Scrib and Dlg in epithelial tissue morphogenesis and differentiation, increases our understanding of a previously poorly-studied protein. It will now be important to investigate whether the vertebrate Gukh orthologs also interact with Scrib and Dlg in a similar manner in epithelial tissue development.

**EXPERIMENTAL PROCEDURES**

**Scrib PDZ domain expression and purification**

Protein expression constructs encoding the PDZ domains of Drosophila Scrib (Uniprot accession number Q7KRY7: PDZ1 726-820, PDZ2 929-1019, PDZ3 1237-1328 and PDZ4 1335-1427) were obtained as synthetic cDNA codon optimized for E. coli expression and cloned into the pGex-6P3
vector (Bioneer). Mutant PDZ1 R765A and H796A were obtained as synthetic cDNA codon optimized for *E. coli* expression and cloned into the pGex-6P1 vector (Genscript). Individual PDZ expression plasmids were transformed into *E. coli* BL21 (DE3) pLysS. Bacterial cells were grown in Super Broth media (3.2% w/v tryptone, 2% w/v yeast extract, 0.5% w/v NaCl and 5 mM NaOH) supplemented with 0.2 mg/ml ampicillin. Protein overexpression was induced by the autoinduction method (44) by supplementing the growth media with 100 mM NaCl, 50x5052 (25% glycerol, 10% lactose and 2.5% glucose), 10 mM Tris pH 7.6 and 1 mM MgSO₄. Bacterial cultures were incubated at 16°C with shaking at 160 rpm for 72 hours. Bacterial cells were harvested by centrifugation at 3,724 x g for 15 min at 4°C using the Avanti J-E centrifuge (Beckman Coulter), JLA-9.1000 rotor. Bacterial pellets were resuspended in GST purification buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1mM EDTA) and lysed using TS series bench top cell disruptor (Constant systems Ltd) at 25 kPsi at 4°C. Bacterial cell lysates were clarified by centrifugation at 3,724 x g for 15 min at 4°C using the Avanti J-E centrifuge (Beckman Coulter), JA-25.50 rotor. Supernatants containing the target proteins were subjected to affinity chromatography using glutathione Sepharose 4B (GE Healthcare), and target proteins were liberated from the column overnight at 4°C by addition of HRV 3C protease (1:10 weight ratio). Flow-through containing target proteins was concentrated using centrifugal concentrators (Millipore) with a 3 kDa molecular weight cut-off. Concentrated protein was subjected to size exclusion chromatography using a HiLoad 16/600 Superdex 75 column (GE Healthcare) mounted on an AKTA Pure (GE Healthcare) equilibrated with 25 mM HEPES, pH 7.0 for DM-PDZ2, DM-PDZ1 and DM-PDZ4, HEPES, pH 8.0 for DM-PDZ3, supplemented with 150 mM NaCl.

**Isothermal titration calorimetry (ITC).** Purified *Drosophila* Scrib PDZ domains were used in titration experiments against 8-mer peptides spanning the C-terminus of *Drosophila* Gukh isoform-A (LPSFETAL, GenScript). Raw heats were measured using a Microcal NanoITC200 system (GE Healthcare) at 25°C. Due to lack of useful aromatic amino acids in PDZ domain proteins, protein concentrations were calculated using the Scope method (45) by measuring absorbance at 205 nm and 280 nm using a NanoDrop 2000 spectrophotometer (Thermo Scientific). As controls a non-binding Gukh mutant peptide (LPSFEAAA, GenScript) and a superpeptide (RSWFETWV, GenScript) engineered to harbor pan-PDZ binding activity (46) as positive control were used. Binding isotherms were analysed using Origin 7.0 E (MicroCal).

**dmPDZ1:Gukh complex crystallization and data collection**

The complex of dmPDZ1 with Gukh peptide was reconstituted by mixing protein and peptide at a 1:2 molar ratio. The dilute protein complex was then concentrated to 20mg/mL using a 3kDa molecular weight cut-off centrifugal concentrator (Millipore), flash-cooled and stored under liquid nitrogen. Crystallization trials were carried out using 96-well sitting-drop trays (Swissci) vapour diffusion at 20 °C either in-house or at the CSIRO C3 Collaborative Crystallization Centre, Melbourne Australia. 0.15 µL dmPDZ1:Gukh peptide complex was mixed with 0.15 µL of various crystallization conditions using a Phoenix nanodispenser robot (Art Robbins). Commercially available screening kits (PACT Suite and JCSG-plus Screen) were used for the initial crystallization screening, with hit optimization performed using a 96-well plate at the CSIRO C3 Centre. Crystals of dmPDZ1 in complex with Gukh peptide were obtained at 20 mg/mL in 0.2 M zinc acetate dehydrate, 0.1 M sodium cacodylate pH 6.5 and 10 % (v/v) propanol. The crystals were cryo-protected using 30 %
(v/v) ethylene glycol and flash cooled at 100 K using liquid nitrogen. Hexagonal rod crystals were obtained belonging to space group P3_212. Unfortunately, structure determination failed with those crystals, which were subsequently used in a cross-seeding experiment into the Shotgun screen at CSIRO C3. After two months crystals of dmPDZ1 in complex with Gukh peptide were obtained at 20 mg/mL in 30 % (v/v) PEG 4000, 0.2 M sodium acetate and 0.1M Tris chloride pH 8.5. The crystals were cryo-protected using 30 % (v/v) ethylene glycol and flash cooled at 100 K using liquid nitrogen. All diffraction data were collected on the MX1 beamline at the Australian Synchrotron using ADSC Quantum 315r CCD detector (Area Detector Systems Corporation, Poway, California, USA) with an oscillation range of 1.0° per frame using a wavelength of 0.9537 Å. Diffraction data were integrated using XDSme (47) and scaled using AIMLESS (48). The structure of dmPDZ1-Gukh peptide was solved by molecular replacement using Phaser (49) with the structure of hsPDZ1 (PDB: 5VWC) as a search model. The final TFZ and LLG values were 19.1 and 471, respectively. The solution produced by Phaser was manually rebuilt over multiple cycles using Coot (50) and refined using PHENIX (51). Data collection and refinement statistics details are summarized in Table 1. Molprobity scores were obtained from the Molprobity web server (52). Shape complementarity was calculated using the program SC (53). Coordinate file have been deposited in the Protein Data Bank under the accession code 5WOU.

Drosophila melanogaster stocks and genetic analysis.

w^{1118} (wild type with white eyes), g lukh\_RNAi (P[TRiP.GL01345]), ey-GAL4, dpp^{Gal4\_Gal4} (dpp-GAL4), en-GAL4, UAS-lacZ, UAS-GFP, UAS-GAL80\_C, ey-FLP, hsp70-FLP, FRT82B Ubi-GFP, FRT82B Ubi-GFP and g lukh\_BG02660 (the g lukh P element mutant) stocks were obtained from the Bloomington Drosophila stock Centre. UAS-\beta-gal\_RNAi was obtained from K. Harvey. The Scrib-GFP protein trap (CA07683, homozygous viable) was obtained from the Flytrap collection (L. Cooley). The following Vienna Drosophila Resource Centre stocks were used: UAS-scrib\_RNAi (101128, VDRC stock 105412), UAS-scrib\_RNAi (11663 C2V, gift from B. Dickson, 2nd chromosome); UAS-scrib\_RNAi (11663 C3S, gift from B. Dickson) 3rd chromosome, VDRC stock 45555), UAS-dlg\_RNAi (4689 C2V, gift from B. Dickson (VDRC stock 31134), 2nd chromosome. The UAS-gukh-C (C terminus transgene, missing the N-terminal actin binding domain) was obtained from V. Budnik (13). The efficacy of dlg 4699 C2V and scrib 11663 RNAi lines in targeting these proteins has been previously confirmed (11), and we have confirmed that the scrib RNAi 101128 efficiently knocks down Scrib protein (data not shown). We also confirmed the efficacy of the g lukh-RNAi line by testing its ability to decrease Gukh levels when expressed via engrailed-GAL4 in the larval wing epithelium, and verified the specificity of the Gukh antibody by showing that Gukh immunoreactively was decreased in g lukh\_BG02660 mutant clones in the eye epithelium (Fig. 3).

Stocks of ey-GAL4 and UAS-scrib\_RNAi 11663 C2V and UAS-scrib\_RNAi 11663 C3S, or ey-GAL4 and UAS-dlg\_RNAi 4689 C2V and UAS-scrib\_RNAi 11663 C3S were generated and balanced over CyO and TM6B. Stocks of dpp-GAL4 UAS-GFP with UAS-scrib\_RNAi 11663 C2V or UAS-dlg\_RNAi 4689 C2V were generated and maintained at 18°C (where expression of the transgenes is low).

For induction of g lukh clones in the eye epithelium, g lukh\_BG02660 FRT82B flies were crossed to ey-FLP FRT82B Ubi-GFP at 25°C, and the eye-antennal discs were dissected from third instar larvae.
Fly crosses were performed at 25°C or 29°C (as indicated) and grown on standard fly media (Molasses 93.3 g/L, Agar 5.6 g/L, Glucose 10.6 g/L, Fresh yeast 60 g/L, Semolina (Coarse) 66 mg/L, Acid Mix 0.92% v/v).

Analysis of genetic interactions in the Drosophila eye and wing tissues.

For analysis of genetic interactions in Drosophila eyes, crosses of ey>scrib\textsuperscript{RNAi} (2\textsuperscript{nd}) scrib\textsuperscript{RNAi} (3\textsuperscript{rd}); ey>dlg\textsuperscript{RNAi} (2\textsuperscript{nd}) scrib\textsuperscript{RNAi} (3\textsuperscript{rd}) to UAS-gukh-C, UAS-gukh\textsuperscript{RNAi}, UAS-dlg\textsuperscript{RNAi} or UAS-lacZ (control) were conducted at 29°C. Crosses of ey-GAL4 to each UAS-RNAi or UAS-transgene were performed as controls. Drosophila adults were collected 7 to 8 days after crossing at 29°C and at least 50 progeny were examined from each cross, and photographs obtained for at least 6 samples for each genotype. Eye size was measured using Adobe Photoshop Extended tools.

For analysis of genetic interactions in Drosophila wings, dpp>scrib\textsuperscript{RNAi} 11663 C2V (2\textsuperscript{nd}) or dpp>dlg\textsuperscript{RNAi} 4689 C2V (2\textsuperscript{nd}) were crossed to UAS-gukh-C, UAS-gukh\textsuperscript{RNAi}, UAS-dlg\textsuperscript{RNAi} or UAS-GFP (control) at 25°C. Crosses of dpp-GAL4 to each UAS-RNAi or UAS-transgene were performed as controls. Scoring was performed based on the presence or absence of the truncated wing vein phenotype. At least 50 individual Drosophila adults were scored from each cross. For imaging, 10 Adult flies wings per sample were carefully detached from the torso and mounted on glass slides using a mixture of methyl salicylate and Canada Balsam (Sigma-Aldrich) in ratio of 1:1 and were left to dry overnight.

Microscopy images of Drosophila eyes and wings were obtained at ×2.5 and ×3.2 magnification, respectively, on an Olympus SZX7 microscope equipped with an INFINITY-1 camera and images were processed using INFINITY capture software.

Immunofluorescent staining, confocal microscopy and quantification

Primary antibodies were rabbit anti-Gukh antibody (1/500 (V. Budnik) raised to the N-terminus of Gukh), mouse anti-Dlg (Developmental Studies Hybridoma Bank, 4F3). Antibody staining was performed using similar methodology as previously described (11). The secondary antibodies were goat-anti-rabbit AlexaFluor-568 (Invitrogen), and goat-anti-mouse AlexaFluor-633 (Molecular Probes). Wing and/or eye imaginal discs were mounted onto glass slides in one drop of ProLong® GOLD Antifade Mountant (Molecular Probes, Cat. #P36934) or 80% glycerol in PBS and covered with a glass coverslip. All confocal images were taken on either a Confocal Leica TCS (true confocal scanner) SP5 (Leica Microsystems, Germany) or a Confocal Zeiss ELYRA (Carl Zeiss, Germany) microscope. Staining intensity and cell migration were quantified using the Fiji (ImageJ) image analysis software. Quantification of Pixel intensity was determined for Gukh staining after RNAi-mediated knockdown (marked by the expression of GFP) or in gukh mutant clones (marked by the absence of GFP expression) versus the wild type tissue using Fiji software. Adult eye size was determined by drawing a region of interest followed by area measurement (Size = 0-infinitiy pixel units, Circularity = 0.00-0.10). Statistical analysis was conducted with Student’s t-test using Graphpad Prism, where p < 0.05 (Version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com).

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The authors have no conflicts of interest to report.

**Author Contributions:**
SC, CMM and TS designed and performed experiments, analyzed the data and wrote the manuscript. MP, KYBL, IH and BZS designed and performed experiments and analyzed the data. POH, HER, MK conceived the project, designed the experiments, analyzed the data and wrote the manuscript. All authors reviewed and commented on the final manuscript.

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**FOOTNOTES**
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**TABLES**

**Table 1:** Summary of thermodynamic binding parameters for Scrib PDZ domain interactions with Gukh peptide measured at pH 7.5 and 25 °C.

|        | K_D (nM) | -ΔH (kcal/mol) | TΔS (cal/mol/K) | N       |
|--------|----------|----------------|-----------------|---------|
| PDZ1   | 664±129  | 12.5±0.8       | -13.8±2.5       | 0.91±0.18 |
| PDZ2   | NB       | NB             | NB              | NB      |
| PDZ3   | 27800±7400 | 2.8.3±1.0     | -12.7±3.4       | 0.88±0.05 |
| PDZ4   | NB       | NB             | NB              | NB      |

**Table 2:** Crystallographic data collection and refinement statistics.

| Data collection | PDZ1: Gukh peptide |
|-----------------|--------------------|
| Space group     | C222_              |
| No of molecules in AU | 1+1                |
| Cell dimensions | 36.43, 53.88, 94.22 |
| a, b, c (Å)     | 90.00, 90.00, 90.00 |
| Resolution (Å)* | 31.42-1.55 (1.58-1.55) |
| R_s || | 0.094 (0.580) |
| I / σI*         | 18.4 (4.3) |
| CC(1/2)         | 0.99 (0.92) |
| Completeness (%)* | 98.2 (94.6) |
| Redundancy*     | 13.8 (13.4) |
| Wilson B-factor | 11.8              |
| No. unique reflections | 13578 (617) |
| No. of observed reflect. | 187879 (8282) |
| Refinement     | Resolution (Å)     | 31.42-1.55       |
| R_work / R_free | 0.197/0.224        |
| No. non-hydrogen atoms | Protein: 766 |
|                  | Ligand/ion: 38     |
|                  | Water: 135         |
Table 3: Summary of binding parameters for wild-type and mutants of Scrib PDZ1 domain interactions with Gukh peptide measured at pH 7.5 and 25°C.

| Scribble       | $K_D$ (nM) | N       |
|----------------|-----------|---------|
| WT PDZ1        | 664±129   | 0.91±0.18 |
| PDZ1 R765A     | 5100±2220 | 0.89±0.04 |
| PDZ1 H796A     | NB        | NB      |
| PDZ1 G747W     | NB        | NB      |

FIGURE LEGENDS

FIGURE 1: Interactions between Scrib PDZ domains and the C-terminus of Gukh. (A) Raw heats of titration were measured using isothermal titration calorimetry for interactions between Scrib PDZ1, PDZ2, PDZ3 and PDZ4 domains with an 8-mer peptide encoding the C-terminal PDZ binding motif of Gukh (LPSFETAL, Gukh residues 1781-1788). All experiments were performed in triplicate, error is STD. NB denotes no binding. (B) SDS-PAGE analysis of wild type Scrib PDZ domains 1 to 4 (lanes 1-4). All experiments were performed in triplicate, error is STD. NB denotes no binding.

FIGURE 2: The crystal structures of Drosophila melanogaster Scrib PDZ1 bound to a Gukh peptide. The Gukh peptide engages the PDZ1 domain via a shallow groove located between the β2 and α2. (A) PDZ1 (light pink) is shown as a cartoon with β-PIX peptide (cyan) represented as sticks. Side-chains of the residues involved in interactions (shown as dashed black lines) are displayed as sticks and are labelled. (B) Overlay of cartoons of drosophila PDZ1 (light pink, residues 726-819, 1.65 Å resolution) bound to Gukh peptide (cyan) and human PDZ1 (green, residues 724-819, 1.91 Å resolution). (C) Simulated anneal composite omit electron density map encompassing the binding groove of Scrib PDZ1 in complex with Gukh. PDZ1 is shown as light pink sticks whereas Gukh is shown as cyan sticks. The electron density map is shown as a blue mesh contoured at 1.0 σ and was calculated by omitting the entire Gukh peptide. (D) Cartoon representation of Scrib PDZ1:Gukh complex (light pink and cyan) with the Erbin PDZ domain bound to a synthetic p120-like peptide (sky blue and orange, PDB...
Figure 3: Gukh is expressed in epithelial tissues. (A) Confocal images of immunofluorescently stained third instar larval imaginal wing discs with an anti-Gukh antibody (red). The engrailed (en)-GAL4 driver was used to drive expression of either a UAS-β-galRNAi control or UAS-gukhRNAi in the En domain indicated by the GFP marked posterior compartment of the wing disc (green). In en-driven gukhRNAi discs downregulation of Gukh staining was observed compared to the uniform staining seen in the control disc. (B) Generation of ey-FLP clones in otherwise wild type imaginal eye discs by crossing gukhBG02660 FRT82B flies to ey-FLP FRT82B Ubi-GFP at 25°C. The absence of GFP represents patches of mutant tissue and reveals downregulation of Gukh staining. (C) Quantification of the intensity ratio in the wing samples (from A), which is measured by the intensity of Gukh staining in GFP domains compared to the non-GFP domain. There is a significant decrease (by ~25%) in the intensity ratio when gukhRNAi is expressed (n = 14-18 wing discs). (D) Quantification of the intensity ratio in the eye samples (from B), showing that the intensity ratio of Gukh staining in non-GFP mutant tissue compared to GFP-marked tissue was significantly decreased by ~50% (n = 10-18 eye discs). Images were taken at 40x (A) and 20x (B) magnification. Scale bars = 50µm. Error bars represent mean ± SEM. Student’s t-test used to test for significance. **** = p ≤ 0.0001.

Figure 4: Gukh co-localizes with Scrib and Dlg in Drosophila third instar larval epithelia. Representative confocal images of third instar larval eye and wing epithelial tissue immunofluorescently stained with anti-Gukh and anti-Dlg antibodies. A Scrib-GFP protein trap (homozygous viable), where GFP is inserted in the endogenous gene, allowed visualization of Scrib protein localization. Each row shows a planar (top) and a cross sectional (bottom) image of the disc. Stainings are shown in grey scale and in merge (right panel) with Gukh shown in green and Dlg or Scrib in red. (A) In wing epithelia Scrib and Dlg localize to the cell cortex, mainly at the apical region of the cells. Gukh shows a strong cytoplasmic localization in comparison but is still highly concentrated at the cortex of cells at the apical region. (B) In eye epithelia, Gukh shows similar localization to that observed in wing epithelia, additionally showing enrichment in the apical region of photoreceptor cells. Images taken at 63x magnification. Scale bars = 10µm. Arrowheads point to the same region on each image to aid comparisons.

Figure 5: Gukh genetically interacts with Dlg and Scrib. (A-C) Representative images of male adult eyes are shown for the genotypes indicated. (A) Interactions with the Scrib knockdown phenotype using ey>scribRNAi (2nd) scribRNAi (3rd). Knockdown of Scrib using the ey-GAL4 driver results in a small rough eye phenotype, which is strongly enhanced upon knockdown of Dlg, and mildly enhanced when Gukh function is impaired, using either RNAi or the dominant-negative transgene (gukh-C). (B) Control phenotypes of crosses to ey-GAL4, showing normal eye phenotypes. (C) Knockdown of Scrib together with Dlg using the ey-GAL4 driver results in a small rough eye phenotype, which is strongly enhanced upon impairment of Gukh function, using either RNAi or the dominant-negative transgene, gukh-C. (D, E) Representative images of adult female wings are shown for the genotypes indicated. (D) Control phenotypes of gukhRNAi or gukh-C crossed to dpp-GAL4, showing normal wing phenotypes. (E) Interactions with the Dlg knockdown wing phenotype using dpp>dlgRNAi. The Dpp driver results in expression along between the 3rd and 4th wing veins. Knockdown of Dlg did not noticeably affect wing morphology, however impairment of Gukh function using gukhRNAi or the gukh-C dominant
negative transgene resulted in truncation of the 3rd wing vein (arrow) in the majority of flies (~60-70%). (F) Graph of the comparison of eye size between genotypes (as indicated) shown in A. (G) Graph of the comparison of eye size between genotypes (as indicated) shown in C. Values are the mean eye size and error bars represent mean ± SEM. Student’s t-test used to test for significance. (H) Quantitation of wing phenotypes observed in indicated fly genotypes shown in (D) and (E). Values are % of flies displaying vein truncation. Number of flies scored: UAS-GFP: 68; UAS-gukH RNAi: 60; UAS-gukH-C: 54.
A

PDZ1:Gukh
$K_D = 664 \text{ nM} \\
+/- 129$

PDZ2:Gukh
$K_D = \text{NB}$

PDZ3:Gukh
$K_D = 27.8 \text{ \mu M} \\
+/- 7.4$

PDZ4:Gukh
$K_D = \text{NB}$

Figure 1

B

[Image of protein gel]
Drosophila melanogaster Guk-holder interacts with the Scribbled PDZ1 domain and regulates epithelial development with Scribbled and Discs Large

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