ABSTRACT
Discs large (Dlg) is an essential polarity protein and a tumor suppressor originally characterized in Drosophila but also well conserved in vertebrates. Like the majority of polarity proteins, plasma membrane (PM)/cortical localization of Dlg is required for its function in polarity and tumorigenesis, but the exact mechanisms targeting Dlg to the PM remain to be fully elucidated. Here, we show that, similar to recently discovered polybasic polarity proteins such as Lgl and aPKC, Dlg also contains a positively charged polybasic domain that electrostatically binds the PM phosphoinositides PI4P and PI(4,5)P2. Electrostatic targeting by the polybasic domain contributes significantly to the PM localization of Dlg in follicular and early embryonic epithelial cells, and is crucial for Dlg to regulate both polarity and tumorigenesis. The electrostatic PM targeting of Dlg is controlled by a potential phosphorylation-dependent allosteric regulation of its polybasic domain, and is specifically enhanced by the interactions between Dlg and another basolateral polarity protein and tumor suppressor, Scrib. Our studies highlight an increasingly significant role of electrostatic PM targeting of polarity proteins in regulating cell polarity.

KEY WORDS: Dlg, Lgl, Scrib, aPKC, Par-6, Polybasic domain, Phosphoinositides, PI4P, PI(45)P2, Cell polarity, Tumorigenesis, Drosophila

INTRODUCTION
Polarity proteins play conserved and essential roles in regulating the apical-basal polarity in epithelial cells of both invertebrates and vertebrates. Among them, Discs large (Dlg), referred to here as Dlg), Scrib and Lgl [L(2)g1] also act as tumor suppressors and share the same basolateral subcellular localization in epithelial cells (Bilder et al., 2003; Tanentzapf and Tepass, 2003). Like many polarity proteins, plasma membrane (PM)/cortical localization is essential for Dlg, Lgl and Scrib for regulating apical-basal polarity and tumorigenesis (Hough et al., 1997; Ventura et al., 2020). Recent studies have demonstrated that multiple polarity proteins contain so-called polybasic (PB) domains that are typically of 20-40 aa length and are highly positively charged due to enriched Arg and Lys residues (Hammond and Hong, 2018). Polybasic (domain) polarity proteins, such as Lgl and aPKC, can specifically target to the PM by electrostatically binding negatively charged phospholipids, in particular PI4P and PI(4,5)P2 (PIP2), enrichment of which in the PM makes the inner surface of PM the most negatively charged membrane surface the cell (Hammond et al., 2012). Although the electrostatic PM targeting of Lgl is now well established (Bailey and Prehoda, 2015; Dong et al., 2015), the exact mechanisms targeting Dlg to the PM remain to be fully elucidated. Here, we report that Dlg, like recently characterized polybasic polarity proteins Lgl and aPKC (Dong et al., 2020), contains a polybasic domain that electrostatically targets Dlg to the PM and is necessary for Dlg to regulate polarity and tumorigenesis. Our results also suggest that Scrib specifically enhances the electrostatic PM targeting of Dlg by regulating a potential phosphorylation-dependent allosteric control mechanism.

RESULTS AND DISCUSSION
A polybasic domain in Dlg mediates its PM targeting
Dlg belongs to the MAGUK protein family and contains PDZ, SH3, HOOK and guanylate kinase (GUK) domains (Fig. 1A). The GUK domain is considered ‘kinase dead’ and instead acts as a protein-interacting domain that can bind the SH3 domain either intramoleurally or intermoleurally (McGee et al., 2001) (see below). By sequence analysis, we identified a well-conserved candidate polybasic domain that spans the C-terminal half of the SH3 domain and the N-terminal half of the HOOK domain (Fig. 1A), with a basic-hydrophobic index (Brzeska et al., 2010) of 0.90, comparable to polybasic domains in Lgl (1.01), aPKC (0.96) and Numb (1.07). In liposome pull-down assays, GST fusion of Dlg polybasic domain (GST-PB) bound both PI4P- and PI(45)P2-containing liposomes but not liposomes containing only phosphatidylserine (PS) (Fig. 1B). GST-PB-KR6Q or GST-PB-KR15Q, in which positive charges were either partially or completely eliminated by K/R->Q mutations, did not bind either liposome (Fig. 1B), supporting an electrostatic interaction between the positively charged polybasic domain and the negatively charged PI4P- or PI(45)P2-containing membrane.

To investigate whether the polybasic domain is required for targeting Dlg to PM in vivo, we generated transgenic flies expressing Dlg::GFP, DlgKR6Q::GFP and DlgKR15Q::GFP under the ubiquitin promoter (Dong et al., 2020). Although the dlo locus apparently expresses numerous isoforms, our ubi-dlg::GFP (dlg::GFP) based on isoform-G fully rescued the null mutants of dlo[4] and was expressed at levels similar to an endogenous GFP knock-in allele (dlg::GFP[4]) (Fig. S1A). Dlg::GFP showed typical basolateral PM/cortical localization in follicular and embryonic epithelial cells (Fig. 1C, Fig. S2A), whereas both DlgKR6Q and DlgKR15Q showed a strong reduction of PM localization (Fig. 1C, Fig. S2C) and failed to rescue dlo[4] (Table 1). Dlg interacts with multiple proteins on the PM through its three PDZ domains (Hough et al., 1997; Qian and Prehoda, 2006), and DlgAPDZ::GFP also showed reduced PM localization in follicular cells, albeit less severe than DlgKR6Q and DlgKR15Q (Fig. 1C). Consistent with both PDZ and
Fig. 1. A polybasic domain in Dlg mediates its electrostatic PM targeting. (A) Polybasic domains in human DLG1 (hDlg1, NP_004078.2) and Drosophila Dlg (NP_996405). Mutations in DlgKR6Q, DlgKR15Q, DlgS4A and DlgS4D, as well as the point mutation of dlgm30 ("m30") allele, are also shown. Deletions in DlgΔPDZ and DlgΔGUK are shown beneath. Bold black amino acids are conserved Ser/Thr residues; bold colored amino acids are point mutations introduced in mutants. (B) Western blot using GST antibody showing that GST-PB, but not GST or GST-PB-KR6Q or GST-PB-KR15Q, co-sedimented with PI4P- and PIP2-containing liposomes. (C) PM localization of wild-type and mutant Dlg::GFP in follicular cells. PM Index: values above 1 (dashed line) indicate predominant PM localization, whereas those below 1 indicate cytosolic localization. Sample numbers (n) are shown in parentheses. ****P<0.00001; ns, P>0.05. Error bars represent s.d. (D,E) Follicular cells expressing Dlg::GFP and Lgl::mCherry (D, Movie 1), or DlgΔPDZ::GFP and Lgl::mCherry (E, Movie 3), undergoing hypoxia followed by reoxygenation. (D′,E′) Kymographs (top) were sampled at the areas indicated by the boxes in D and E. Arrowheads in kymographs highlight the persistent residual PM localization of Dlg under hypoxia. Graphs show quantification of Dlg and Lgl PM localization (n=20). Time stamps ae shown in hh:mm:ss format. Error bars represent s.d. Scale bars: 5 µm.
polybasic domains contributing to PM targeting in follicular cells, Dlg<sup>APDZ-KR6Q</sup>:GFP and Dlg<sup>APDZ-KR15Q</sup>:GFP became virtually lost from the PM (Fig. 1C).

To investigate further the electrostatic PM targeting of Dlg<sub>vivo</sub>, we used a previously established assay that applies controlled hypoxia to acutely and reversibly deplete PIP2 and PI4P in the PM (Dong et al., 2015) (data not shown). Similar to observations for the polybasic polarity proteins Lgl (Dong et al., 2015) and aPKC (Dong et al., 2020), hypoxia also induced acute and reversible loss of Dlg::GFP<sup>KI</sup>, as well as ubi-Dlg::GFP, from the PM in follicular cells (Fig. 1D,D′, Movies 1 and 2). However, unlike Lgl, which became completely cytosolic within 30-60 min of hypoxia, residual Dlg::GFP<sup>KI</sup> persisted on the PM over 100 min since the start of hypoxia (Fig. 1D,D′, Fig. S1B). In contrast, hypoxia induced complete PM loss of Dlg<sup>APDZ</sup>:GFP that was identical to that observed for Lgl (Fig. 1D,D′, Movie 1). Such data suggest that in follicular cells a significant portion of Dlg electrostatically binds to the PM, whereas the rest were retained at the PM by PDZ domain-dependent interactions.

Dlg is also a component of the septate junction (SJ), which is composed of over 20 different proteins (Izumi and Furuse, 2014), making the SJ the primary target of PDZ-dependent PM localization. In early embryonic epithelial cells with undeveloped SJ, Dlg::GFP was readily lost from the PM under hypoxia, suggesting that its PM targeting is mostly electrostatic (Fig. S2A). In stage 14 or later embryos with mature SJs, PM localization of Dlg::GFP<sup>KI</sup> was significantly enhanced (Fig. S2C,D) and became completely resistant to hypoxia (Fig. S2A). In contrast, in stage 14 embryos mutant for core SJ proteins such as Coracle (cora) (Lamb et al., 1998) or Na+,K<sup>+</sup>-ATPase α-subunit (Altpa) (Paul et al., 2003), Dlg::GFP<sup>KI</sup> PM localization became sensitive to hypoxia (Fig. S2B). In addition, enhanced PM localization in late embryos was seen in electrostatic Dlg mutants, but not in mutants lacking PDZ domains (Fig. S2C,D), consistent with the suggestion that PDZ domains are required for Dlg localization to the SJ (Hough et al., 1997). Indeed, PM localization of Dlg<sup>APDZ</sup>:GFP in late embryos is electrostatic, based on its sensitivity to hypoxia (Fig. S2B).

In summary, with the help of hypoxia-based imaging assays we were able to specifically probe the electrostatic PM targeting of Dlg <sub>vivo</sub>. Our data revealed that in follicular and early embryonic epithelial cells full PM localization of Dlg requires both electrostatic PM targeting by the polybasic domain and protein-interactions mediated by PDZ domains, whereas in epithelial cells with mature SJs PDZ domains alone can act redundantly to retain Dlg on the PM.

### Potential phosphorylation events on the polybasic domain regulate the PM targeting of Dlg

Polybasic domains are often regulated by direct phosphorylations, which inhibit PM binding by neutralizing the positive charges (Hong, 2018), such as in the cases of Lgl, Numb and Miranda (Bailey and Prehoda, 2015; Dong et al., 2015). To investigate whether the Dlg polybasic domain is also regulated by phosphorylation events, we mutated four well-conserved S/T sites within its polybasic domain (Fig. 1A), of which the first two are the previously characterized phosphorylation sites of aPKC and PKCα (Golub et al., 2017; O’Neill et al., 2011). Both phosphomimetic Dlg<sup>S4A</sup>:GFP and non-phosphorylatable Dlg<sup>S4A</sup>:GFP were significantly reduced in the PM (Fig. 3A), suggesting that these potential phosphorylation events are unlikely to regulate the polybasic domain through charge neutralization. Interestingly, PM localization of Dlg<sup>S4A</sup>:GFP nearly abolished the residual PM localization of Dlg<sup>APDZ-S4A</sup>:GFP (Fig. 3A), suggesting that the residual PM localization of Dlg<sup>S4A</sup>:GFP is non-electrostatic but PDZ dependent.

Our recent studies also showed that the polybasic domain in aPKC, which is a pseudosubstrate region (Psr) with no characterized phosphorylation sites, is allosterically regulated (Dong et al., 2020). The Psr in aPKC binds and autoinhibits the kinase domain, which in turn prevents Psr from electrostatically binding to PM, while binding of Par-6 to aPKC induces conformation changes that expose the Psr to allow PM binding.
Given that the SH3 and GUK domains in Dlg could also bind each other, we postulate that the loss of potential phosphorylation events in DlgS4A::GFP keeps the SH3-GUK in a closed conformation that prevents the polybasic domain from binding to the PM. Removing the GUK domain, however, resulted in DlgΔGUK::GFP localizing both on the PM and in nuclei (Fig. 3A), likely due to the fact that the Arg/Lyn-rich feature of the polybasic domain is also similar to the nuclear localization signal (NLS) (Dong et al., 2015). Consistent with the observation that removing the GUK domain exposes the polybasic domain, DlgΔGUK::GFP PM localization is partially electrostatic just like Dlg::GFP (Fig. S2B, Movie 7). In addition, the moderate nuclear localization of DlgΔGUK::GFP can be overcome by its interaction with the SJ, as DlgΔGUK::GFP was localized at the SJ in larval wing disc epithelia, which develop strong SJs (Fig. S2E) (Hough et al., 1997). In contrast, DlgS4A-ΔGUK::GFP showed overwhelmingly high nuclear localization both in follicular cells (Fig. 3A) and in wing imaginal epithelial cells (Fig. S2E), suggesting that removing the GUK domain likely also exposes the polybasic domain in DlgS4A, but the lack of potential phosphorylation events strongly enhanced the nuclear localization property of the polybasic domain.

Although additional studies are needed to confirm the phosphorylation events of Dlg in vivo, our results are consistent with a model in which the polybasic domain in Dlg is regulated by phosphorylation events that allosterically relieve its blockage by the GUK domain and promote its targeting to the PM over nuclear localization (Fig. 4D).

Scrib specifically enhances the electrostatic PM targeting of Dlg
Scrib and Lgl colocalize with Dlg at the basolateral PM and play similar functions in cell polarity and tumorigenesis, but it is unknown...
Fig. 3. Scrib enhances the electrostatic PM targeting of Dlg. (A) Follicular cells expressing Dlg::GFP or mutants as indicated. (A') Quantification of the PM localization of each mutant. (B,C) Follicular cells expressing Dlg::GFP or mutants as indicated. scrib-RNAi (B) or lgl-RNAi (C) cells were labeled by RFP. (B',C') Quantification of wild-type and mutant Dlg::GFP PM localizations in wild-type (green dots) and RNAi (red dots) cells. Error bars represent s.d. Sample numbers (n) are shown in parentheses. ****P<0.001; ns, P>0.05. Asterisks in image panels indicate RNAi cells. Scale bars: 5 µm.
Fig. 4. Electrostatic PM targeting of Dlg regulates cell polarity and tumorigenesis. (A) $\text{dlg}^{-/-}$ follicular cells were marked by the loss of RFP (blue in all merged images), except for $\text{dlg}^{-/-}$ wt in which the $\text{dlg}^{-/-}$ clones were marked by the absence of Dlg (green). Samples expressed wild-type or mutant Dlg::GFP as indicated, and were stained for GFP (green) and aPKC (red). Note that $\text{dlg}^{-/-}$, $\text{dlg}^{m30-\Delta GUK}$ clones showed roughly equal frequency of rescued and non-rescued polarity defects. Asterisks highlight $\text{dlg}^{-/-}$ mutant cells. (B) Eyes from adult flies of RasV12, or $\text{dlg}$-RNAi or RasV12/dlg-RNAi combined with additional expression of wild-type and mutant Dlg as indicated. ctrl, RasV12/dlg-RNAi only. (C) Quantification of the rescue of RasV12/dlg-RNAi lethality by wild-type and mutant Dlg as indicated. (D) A hypothetical model on the potential phosphorylation-dependent allosteric control of the Dlg polybasic domain and its regulation by Scrib. Potential regulatory defects in Dlg$m30$, Dlg$^{S4A}$ and Dlg$^{\Delta GUK}$ are also shown. Kinases phosphorylating the polybasic domain of Dlg remain to be characterized but could include aPKC and PKC$\alpha$. The sequential order between Scrib binding to Dlg and its phosphorylation is postulated. Scale bars: 5 µm.
how Scrib and Lgl specifically regulate the electrostatic PM targeting of Dlg. Consistent with recent studies demonstrating that Scrib, but not Lgl, appears to interact physically with Dlg (Khoury and Bilder, 2020; Ventura et al., 2020), knocking down Scrib by RNAi induced a partial loss of Dlg from the PM (Fig. 3B). More importantly, Scrib appears to specifically enhance the electrostatic PM targeting of Dlg, as Dlg<sup>ΔPDZ</sup>, which can only bind the PM electrostatically, was dramatically reduced from the PM in scrib-RNAi cells (Fig. 3B). In contrast, the residual PM localization of the electrostatic mutant Dlg<sup>ΔGUK</sup> was not affected by scrib-RNAi (Fig. 3B). Scrib interaction depends on the SH3 domain in Dlg and a single point mutation in the SH3 domain in the ΔGUK mutant (Fig. 1A) abolishes such interaction (Khoury and Bilder, 2020). Indeed, similar to electrostatic mutants such as Dlg<sup>ΔK6Q</sup>, Dlg<sup>S4A</sup> and Dlg<sup>ΔGUK</sup>, PM localization of Dlg<sup>ΔPDZ</sup>::GFP was also reduced and became resistant to scrib-RNAi (Fig. 3B), supporting the hypothesis that interactions between Scrib and Dlg are necessary for Scrib to enhance Dlg electrostatic PM targeting. More importantly, PM localization of Dlg<sup>S4A</sup> or Dlg<sup>ΔGUK</sup>, two mutants defective in the potential phosphorylation-dependent allosteric control of the polybasic domain, was also resistant to scrib-RNAi (Fig. 3B), suggesting that Scrib may act by facilitating such a phosphorylation-allosteric mechanism (Fig. 4D).

In agreement with the recent findings that Lgl does not appear to associate physically with Dlg and Scrib (Khoury and Bilder, 2020; Ventura et al., 2020), lgl-RNAi did not affect the PM localization of Dlg, Dlg<sup>ΔK6Q</sup>, Dlg<sup>S4A</sup>, Dlg<sup>ΔPDZ</sup> and Dlg<sup>ΔGUK</sup> (Fig. 3C, Fig. S4C). However, PM localization of Dlg<sup>ΔPDZ</sup> was mildly reduced in lgl-RNAi cells (Fig. 3C). Thus, Lgl could also play a major role in enhancing Dlg electrostatic PM targeting, through mechanisms that are presently unclear. Finally, knocking down GukH, which interacts with the GUK domain and is required for aPKC-regulation of Dlg in neuroblasts (Golub et al., 2017), did not affect the electrostatic PM targeting of Dlg<sup>ΔPDZ</sup> (Fig. S4C), consistent with the suggestion that GukH appears to be dispensable for epithelial polarity (Caria et al., 2018).

**Electrostatic PM Targeting is essential for Dlg to regulate cell polarity and tumorigenesis**

We also investigated how electrostatic PM targeting may specifically contribute to Dlg function in polarity and tumorigenesis. Follicular cells of dlg<sup>Δ−/−</sup> mutants showed dramatic disruption of cell polarity evidenced by the mislocalization of apical polarity proteins such as aPKC (Fig. 4A). This phenotype was fully rescued by ectopic expression of Dlg and Dlg<sup>ΔPDZ</sup>, but not electrostatic mutant Dlg<sup>ΔK6Q</sup> or Dlg<sup>ΔPDZ</sup>ΔK6Q (Fig. 4A), suggesting that electrostatic PM targeting is essential for Dlg to regulate apical-basal polarity in follicular cells. Surprisingly, dlg<sup>ΔK6Q</sup>/Y; dlg<sup>S4A</sup>Δ::GFP and dlg<sup>ΔK6Q</sup>/Y; dlg<sup>ΔGUK</sup>Δ::GFP males are viable, although sterile (Table 1); thus, the potential mechanisms regulating the polybasic domain may be largely dispensable for Dlg function in normal development.

We then used a well-established tumor model (Brumby et al., 2011) to investigate how electrostatic PM targeting is required for the tumor suppressor function of Dlg. Overexpression of constitutively active Ras<sup>VT12</sup> in larval eye disc produced a moderate rough eye phenotype (Fig. 4B). Although dlg-RNAi alone in the eye did not produce obvious phenotypes (Caria et al., 2018), combining Ras<sup>VT12</sup> overexpression and dlg-RNAi (Ras<sup>VT12</sup>/dlg-RNAi) produced massive eye tumors, which resulted in strong larval/pupal lethality, with very few adult escapers with reduced eye size and dark tumor tissues (Fig. 4B). We made transgenic stocks that express wild-type or mutant Dlg proteins from cDNA sequences modified to be resistant to dlg-RNAi (Fig. S4C, Table S1). Each of the ΔPDZ mutants is also resistant to dlg-RNAi as the RNAi targets the sequence near the N-terminal coding region. As expected, expression of RNAi-resistant dlg<sup>ΔPDZ</sup>::GFP (Dlg<sup>ΔPDZ</sup>−::GFP) fully rescued Ras<sup>VT12</sup>/dlg-RNAi lethality and eye morphology (Fig. 4B,C). Both Dlg<sup>ΔPDZ</sup> and the polybasic mutant Dlg<sup>ΔK6Q</sup>−::GFP rescued the lethality of Ras<sup>VT12</sup>/dlg-RNAi, but eyes in dlg<sup>ΔK6Q</sup>−::GFP flies still showed strong tissue overproliferation (Fig. 4B). In contrast, Dlg<sup>ΔPDZ</sup>ΔK6Q−::GFP failed to rescue either lethality or eye morphology and tumorigenesis (Fig. 4B). Thus, both electrostatic and PDZ-dependent PM targeting are required for the tumor suppressor function of Dlg and they act redundantly in rescuing the Ras<sup>VT12</sup>/dlg-RNAi tumor lethality, although electrostatic PM targeting appears to be more specifically required for inhibiting the overproliferation of Ras<sup>VT12</sup>/dlg-RNAi tumor cells.

Notably, Dlg<sup>ΔPDZ</sup>ΔGUK, but not Dlg<sup>ΔK6Q</sup> or Dlg<sup>ΔPDZ</sup>ΔPDZ, partially rescued the polarity defects in dlg<sup>Δ−/−</sup> cells (Fig. 4A). RNAi-resistant Dlg<sup>ΔPDZ</sup>− only moderately rescued the lethality caused by Ras<sup>VT12</sup>−/dlg-RNAi, and survivors still showed strong overproliferation in eyes (Fig. 4B). Consistent with the polarity rescue results in Fig. 4A, removing GUK but not PDZ domains in Dlg<sup>ΔPDZ</sup>− significantly enhanced the rescue of the overproliferation phenotype in Ras<sup>VT12</sup>−/dlg-RNAi eyes (Fig. 4B). Such data are consistent with the observation that eliminating the potential allosteric inhibition of the polybasic domain in Dlg partially compensated for the loss of Scrib-dependent enhancement electrostatic PM targeting. Interestingly, although both Dlg<sup>S4A</sup> and Dlg<sup>ΔK6Q</sup>− were defective in electrostatic PM targeting, Dlg<sup>S4A</sup>− showed strong rescue activity, whereas Dlg<sup>ΔK6Q</sup>− did not (Table 1), suggesting that Scrib/Dlg interaction is crucial for Dlg function besides regulating the electrostatic PM targeting of Dlg. In addition, unlike Dlg<sup>S4A</sup>− or Dlg<sup>ΔGUK</sup>−, Dlg<sup>S4AΔGUK</sup> showed no rescue activity (Table 1), probably because its strong nuclear concentration made protein levels at the PM too low to rescue (Table 1).

The electrostatic nature of Dlg PM targeting makes Dlg a new member of the polybasic polarity protein family that includes at least Lgl, aPKC, Numb and Miranda. Our results also make it increasingly clear that electrostatic PM targeting is a key molecular mechanism widely used by polarity proteins for achieving controlled subcellular localization and for regulating cell polarity. It will be of great interest for future studies to integrate the electrostatic PM targeting mechanism into the regulatory network of polarity protein and their interacting partners, by uncovering the essential molecular mechanisms regulating this simple but elegant physical interaction between polarity proteins and the PM.

**MATERIALS AND METHODS**

**Fly stocks**

Flies carrying transgenic ubi-dlg::GFP or ubi-dlg::GFP mutant (ubi-dlg**::GFP**) alleles were generated by a phiC31-mediated integration protocol (Huang et al., 2009), att<sup>P<sup>903</sup></sup> δ5 (Bloomington Drosophila Stock Center, BL#24868) stock was used to integrate ubi-dlg::GFP and ubi-dlg**::GFP constructs to the 2nd chromosome. Transgenic alleles of ubi-dlg::GFP and ubi-dlg**::GFP were further recombined with w dgl<sup>A1</sup> FRT19A/FM7 (Bloomington Drosophila Stock Center, BL#57086). A summary of the ubi-dlg::GFP and ubi-dlg**::GFP alleles is given in Table S1.

The PM localization of Dlg<sup>ΔPDZ</sup>− was further characterized by the cell-specific reporter phiC31<sup>Δpdz</sup>−/LM6<sup>Δpdz</sup>− and phiC31<sup>Δpdz</sup>−<sup>Δpdz</sup>− TM6<sup>Δpdz</sup>−<sup>Δpdz</sup>− were gifts from Dr Greg Beitel, (Northwestern University, USA). w UAS<sup>tcp</sup>−::mRFP<sup>−</sup> (‘FKBP-IPNPE’): FKBP<sup>−</sup>::mRFP<sup>−</sup> and w UAS<sup>tcp</sup>−<sup>−</sup> Lck-FRB<sup>−</sup>:CFP<sup>−</sup> were gifts from Dr Stefano De Renzi (EMBL Heidelberg, Germany) (Reversi et al., 2014). ey-Gal4, UAS-Ras<sup>VT12</sup>− stocks were a gift from Dr Helena Richardson (University of Melbourne, Australia). w P[w<sup>+]mC]=PTT-GC[dlg<sup>17C0055</sup>]<sup>−</sup>(dlg<sup>−</sup>); [GFP<sup>−</sup>], BL#50859, UAS-Pi4KIIA−
RNAs (BL#35256), UAS-scrib-RNAi (BL#29552), UAS-igl-RNAi (BL#38989), UAS-dlg-RNAi (II) (BL#39035), UAS-dlg-RNAi (III) (BL#34854) and gukh-RNAi (BL#42486) (Caria et al., 2018) were from the Bloomington Drosophila Stock Center. w; lgf::mCherry knock-in and w; lgf::GFP knock-in stocks were previously published (Dong et al., 2015). Drosophila cultures and genetic crosses were carried out at 25°C.

The genotypes of Drosophila samples shown in figures are given in supplementary Materials and Methods.

Molecular cloning
To make ubi-dlg::GFP, the ubiquitin promoter (1872 bp) was PCR amplified from plasmid pWUM6 (a gift from Dr Jeff Sekelsky, University of North Carolina at Chapel Hill, NC, USA) using primers 5′-AGTGTCC- GAATTTCCGGCACTACGGAAGGGGAGTCCGACGAGCGGTGGATTATTCTGCAGCTGCAGCGACGATCGCCGATGGGC and 5′-CTGGAGCGGGCGC- GCCTGGTGGATTATTCTGCGGG and inserted into the pGE-attB vector (Huang et al., 2009) to generate the vector pGU. DNA fragments encoding Dlg::GFP were then inserted into the pGU vector. More details about DNA constructs used in this report are given in Table S1. The sequence of the Dlg isoform used in this study can be found in the NCBI RefSeq database (BL#34854).

Liposome pull-down assays
Liposomal binding assays were carried out as described (Kim et al., 2008). A lipid mixture of 37.5% PC (840051C), 10% PS (840032C), 37.5% PE (840021C), 10% cholesterol (700000P) and 5% PI(4,5)P2 (840046X) or PI4P (840045X); all lipids were purchased from Avanti Polar Lipids) was dried and resuspended to a final concentration of 1 mg/ml of total phospholipids in HEPES buffer and subjected to 30 min sonication. Formed liposomes were harvested at 16,000 μg for 10 min and resuspended in binding buffer (20 mM HEPES, pH 7.4, 120 mM KC1, 20 mM NaCl, 1 mM EGTA, 1 mM MgCl2, 1 mg/ml bovine serum albumin). Approximately 0.1 μg of purified protein or protein complex was mixed with 50 μl of liposome suspension in each liposome-binding assay. Liposomes were pelleted at 16,000 μg for 10 min after 15 min incubation at room temperature, and were analyzed by western blot to detect co-sediment of target protein(s).

Generation of mitotic mutant clones in Drosophila follicular epithelia
Mutant follicular cell clones of dlgA were generated by standard FLP/FRT techniques (St Johnston, 2012). Young females were heat-shocked at 37°C for 1 h and their ovaries were dissected 3 days later.

Live imaging and hypoxia treatment in Drosophila epithelial cells
Embryos and dissected ovaries were imaged according to previously published protocols (Dong et al., 2015; Huang et al., 2011). Embryos were staged by timing and kept in 25°C for 2 h before imaging. Ovaries from adult females of 2 days old were dissected in halocarbon oil (#95). Follicular cells containing overexpressing or RNAi clones were generated by heat-shocking the young females of the correct genotype at 37°C for 15-30 min and their ovaries were dissected 3 days later. To ensure sufficient air exchange to samples during the imaging session, dechorionated embryos or dissected ovaries were mounted in halocarbon oil on an air-permeable membrane (YSI Membrane Model #5793, YSI) sealed by vacuum grease on a custom-made permeable slide, and imaged live as previously described (Dong et al., 2015; Huang et al., 2011).

Immunostaining and confocal imaging
Immunostaining of follicular cells and embryos was carried out as previously described (Huang et al., 2009). Primary antibodies were: chicken anti-GFP (Aves Lab, GFP-1010; 1:5000); mouse anti-Dlg (Developmental Studies Hybridoma Bank, 4F3; 1:50); rabbit anti-apkC (Santa Cruz Biotechnology, Sc-216; 1:1000). Secondary antibodies were: Cy2-, Cy3- or Cy5-conjugated goat anti-rabbit IgG, anti-mouse IgG and anti-chicken IgG (Jackson ImmunoResearch, 111-225-003, 115-165-003 and 106-175-003; all at 1:400). Images were collected on Olympus FV1000 confocal microscopes (Center for Biologic Imaging, University of Pittsburgh Medical School) and processed in Adobe Photoshop for compositions.

Image processing and quantification
Time-lapse movies were first stabilized using the HyperStackReg plug-in in ImageJ. Images or movies containing excessive noisy channels were denoised using the PureDenoise plugin in ImageJ prior to quantification. PM localization of GFP or RFP in images or movies were measured in ImageJ by custom macro scripts. In each image or the first frame of the movie, regions of interest (ROIs) approximately 20-40 μm² were drawn across selected cell junctions. In most cases, custom macros was used to automatically generate PM masks by threshold-segmentation that was based on the mean pixel value of the ROI. Custom macros were then used to automatically measure PM and cytosolic intensities of each fluorescent protein in ROIs in an image or throughout all the frames of a movie. Backgrounds were manually measured based on the minimal pixel value of the whole image or the first frame of the movie. The PM localization index for each fluorescent protein was auto-calculated by the macro as the ratio of (PM – background)/(cytosol – background). In live-imaging experiments, the ‘Normalized PM Index’ was calculated by normalizing (PM Index –1) over the period of recording against the (PM Index –1) at 0 min. Data were further processed in Excel, visualized and analyzed in Graphpad Prism.

Tumorigenesis assays
RNAi-resistance wild-type or mutant ubi-dlg::GFP (dlgA::GFP) transgenic alleles were crossed with ey-Gal UAS-Ras(32); CyO-Gal80; UAS-dlg-RNAi/TM6B in 3-4 days. F1 progeny from each cross were scored into two groups based on their genotypes: dlgA::GFP; ey-Gal UAS-Ras(32); UAS-dlg-RNAi/+ (group#1) or dlgA::GFP; ey-Gal UAS-Ras(32); TM6B/+ (group#2). The ratio between groups #1 and #2 was calculated as the ‘Rescue’ index for each dlgA::GFP allele. Representative eyes of flies from group#1 were imaged.

Statistics
In all quantifications, a two-tailed Student’s t-test was carried out to calculate the P value. Differences with P>0.05 are considered to be statistically insignificant.

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Competing interests
The authors declare no competing or financial interests.
Author contributions

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